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. Conserved docking site is essential for activation of mammalian MAP kinase kinases by specific MAP kinase kinase kinases.

Mutsuhiro Takekawa, Kazuo Tatebayashi, and Haruo Saito

The family of the mitogen activated protein kinases (MAPKs) is a major cellular device by which eukaryotic cells adaptively respond to extracellular stimuli. In mammalian cells, there are at least four subfamilies of MAPKs, namely ERK (also known as the classical MAPK), JNK (also called SAPK), p38 (also called RK and CSBP), and ERK5. The ERK subfamily members (ERK1 and ERK2) are activated by mitogenic stimuli, such as growth factors and phorbol 12-myristate 13-acetate (PMA), and are associated with proliferative responses. In contrast, the members of two other subfamilies (JNK1, 2, and 3, and p38 α, β, γ, and δ) are more potently activated by extracellular stresses (e.g., osmotic shock, oxidative stress, UV and gamma irradiations, and translation inhibitors) and inflammatory cytokines (e.g., IL-1 and TNFα), and are associated with reparative and/or apoptotic responses. Activated MAPKs enter the nucleus, and phosphorylate nuclear substrates including transcription factors, protein kinases, and cell cycle regulators. Cytoplasmic and membrane-associated substrates are also known.

Each MAPK is activated through a cascade of three successively activating protein kinases, namely MAPK, MAPK kinase (MAPKK or MEK), and MAPKK kinase (MAPKKK or MEKK). For mammalian organisms, a relatively small number of structurally conserved MAPKKs are known to activate specific subsets of MAPKs: MEK1 and MEK2 activate the ERK family MAPKs; MKK4 (also known variously as MEK4/SEK1/JNKK1) and MKK7 (MEK7/SEK 2/JNKK2) activate mainly the JNK family MAPKs; MKK3 and MKK6 (also known as MEK 3 and MEK6, respectively) activate only the p38 family MAPKs; and MEK5 activates ERK5. There are significantly more (at least 17) MAPKKKs belonging to several structurally diverse families. The MAPKKKs of the Raf and Mos families phosphorylate and activate MEK1/2, whereas other MAPKKKs such as MEKK1/2/3, MTK1 (MEKK4), TAK1, ASK1 (MAPKKK5), TAO, and mixed lineage kinases (MLKs) prefer-

entially activate some or all of the stress-activated MAPKKs, namely MKK3, 4, 6, and 7.

Several mechanisms are likely to be involved in ensuring the fidelity and efficiency of signal flow through these kinase cascades. Naturally, the specific interaction between the kinase catalytic center and the substrate phospho-acceptor site should be important. Recently, however, equally important contributions of scaffold proteins and docking interactions are being appreciated. A scaffold protein is a third party molecule that tethers two or more proteins together. A docking interaction, in contrast, is the formation of a binary complex through docking sites that are distinct from their catalytic center and the phospho-acceptor site. The docking interactions mediated by the CD domain in MAPK and the D domain in MAPKKs or in MAPK substrates have been extensively characterized. In contrast, no convincing evidence existed for specific docking interactions between mammalian MAPKKKs and MAPKKs, although various pairs have been shown to form stable complexes.

In yeast, we recently documented the first case of MAPKKK-MAPKK docking interaction between Ssk2/Ssk22 and Pbs2. A docking site in the N-terminal "regulatory" region of the Pbs2 MAPKK is required for the specific interaction with Ssk2 or Ssk22 MAPKKK. This year, we identified a conserved docking site, termed DVD, in the mammalian MAP kinase kinases (MAPKKs) belonging to the three major subfamilies, namely MEK1, MKK4/7, and MKK3/6. The DVD sites bind to their specific upstream MAP kinase kinase kinases (MAPKKKs), including MTK1 (MEKK4), ASK1, TAK1, TAO2, MEKK1, and Raf-1. DVD site is a stretch of about 20 amino acids immediately on the C-terminal side of the MAPKK catalytic domain. Mutations in the DVD site strongly inhibited MAPKKs from binding to, and being activated by, their specific MAPKKKs, both in vitro and in vivo. DVD mutants could not be activated by various external stimuli in vivo. Synthetic DVD oligopeptides inhibited specific MAPKK activation, both in vitro and in vivo, demonstrating the critical importance of the DVD docking in MAPK signaling.

2. Roles of Sho1 and Ste50 as dynamic docking proteins in the SHO1 branch of the yeast osmoregulatory HOG signal pathway.

Kazuo Tatebayashi, Katsuyoshi Yamamoto, Takashi Maruoka, Kei-ichiro Tanaka, Taichiro Tomida, Eri Kasukawa, and Haruo Saito

When exposed to hyperosmotic extracellular environments, the budding yeast (*Saccharomyces cerevisiae*) activates the High Osmolarity Glycerol (HOG) signaling pathway, which culminates in phosphorylation and activation of the Hog1 MAPK. Activated Hog1 initiates an adaptive program that includes adjustments in cell cycle progression, regulation of protein translation, induction or repression of various genes, and synthesis and intracellular retention of the compatible osmolyte glycerol. Yeast has two putative osmosensors, Sln1 and Sho1, which are structurally distinct and functionally independent of each other. Signals emanating from these sensors converge at a common MAPKK homolog, Pbs2, which is the specific activator of the Hog1 MAPK. The entire signal pathway from the cell surface sensors to the Hog1 MAPK will be referred to as the HOG pathway, while the upper two branches are called the SLN1 and SHO1 branches, respectively.

Sln1 is a homolog of prokaryotic two-component sensor histidine kinases, and, through the Sln1-Ypd1-Ssk1 multi-step phosphorelay reaction, controls the phosphorylation state of the response regulator Ssk1. Un-phosphorylated Ssk 1 binds and activates the functionally redundant and structurally homologous Ssk2 and Ssk22 MAPKKKs. Both Ssk2 and Ssk22 are constitutively bound to their substrate, the Pbs2 MAPKK, through a specific docking interaction. When activated by un-phosphorylated Ssk1, either Ssk2 or Ssk22 can phosphorylate and activate Pbs2.

Sho1 contains four transmembrane segments and a cytoplasmic SH3 domain that binds a proline-rich motif in the N-terminal region of Pbs2. Sho1 predominantly localizes to the cytoplasmic membrane at the polarized growth, such as emerging bud and bud neck. Sho1 is absolute required for activation of Hog1 via the SHO1 branch. Nonetheless, it is unclear whether Sho1 is an osmosensor in strict sense, because a Sho1 construct in which the four transmembrane segments were replaced by a membrane-targeting myristoylation site (Myr-Sho1) could efficiently complement *sho1*Δ mutation.

In addition to Sho1 itself, Cdc42, Ste20, Ste50, and Ste11 have been implicated in the SHO1 branch. Cdc42 is a small G-protein of the RHO family, and is known to bind and activate Ste20, a prototype of the PAK family protein kinases. Activated Ste20 phosphorylates Ste11 on Ser-302, Ser-306, and Thr-307, in the N-terminal regulatory domain. Phosphorylation at these sites dissociates the Ste11 N-terminal inhibitory domain from its C-terminal catalytic domain, thereby allowing autophosphorylation and activation of Ste11. Ste50 and Ste11 contain a SAM

domain, and they bind each other constitutively via an SAM-SAM interaction. Ste50 is essential for the SHO1 branch signaling, but its role has been unknown.

The same set of proteins, namely Sho1, Cdc42, Ste20, Ste50, and Ste11, are also involved in the filamentous growth (FG) pathway, and at least Cdc42, Ste20, and Ste11 are also essential in the mating pheromone responsive MAPK pathway. In spite of this highly overlapped involvements of the same set of molecules, non-physiological cross-talk activation is severely limited. For example, pheromone stimulation activates only the Fus3/Kss1 MAPKs; cross-talk activation of the Hog1 MAPK does not occur. This is mainly due to the scaffold function of Ste5. Similarly, osmostress activates only the HOG pathway. In *pbs2* or *hog1* mutant cellss, however, the cross-talk barrier breaks down, and osmostress induces the mating-specitic reporter *FUS1-lacZ*.

We have shown, for both mammalian and yeast cells, that a direct docking interaction between MAPKKKs and their congnate MAPKKs is essential for signaling through the MAPK cascades. Mammalian MAPKKs, including MEK1, MKK3/MEK3, MKK4/SEK1/JNKK1, MKK6/MEK6, and MKK7/SEK2/JNKK2, have a conserved docking site termed DVD at or near their C-terminus. Without the DVD site, activated MAPKKKs cannot phosphorylate their specific MAPKKs. Similarly, the yeast Ssk2 and Ssk22 MAPKKKs bind to the Pbs2 MAPKK by direct docking interaction. Thus, specific docking of an activated upstream MAPKKK to a downstream MAPKK seems a universal mechanims for MAP kinase signaling.

Direct docking interaction, however, is unsuitable for the yeast Ste11 MAPKKK, because this kinase must interact with at least two differnt MAPKKs (Pbs2 and Ste7) depending on the context of the upstream stimulation. In this paper, we investigated how Ste11 interact with upstream and downstream kinases in the SHO1 branch of the HOG pathway. We isolated gain-of-function mutants as well as defective mutants from the *SHO1, CDC42, STE50*, and *STE11* genes. Through their characterization, a common theme has arisen: both Ste50 and Sho1 function as adaptive docking sites by bringing an activated upstream kinase and a downstream target kinase together, to effect specific activation of the HOG pathway.

This year, to investigate how osmostress activates only the Hog1 MAP kinase module, we isolated and characterized constitutively activated alleles in three key genes involved in the pathway, namely *STE11, STE50*, and *SHO1*. Binding of Cdc42 activates the upstream kinases in the HOG pathway, Ste20 and Cla4. Binding of Cdc42 to the Ste11-bounding protein, Ste50, brings activated Ste20/Cla4 and their substrate, the Ste11 MAPKKK, together. Activated Ste11 and its HOG-specific substrate, the Pbs2 MAPKK, are then brought together by binding of the Ste50-Ste11 complex to the cytoplasmic domain of Sho1, to which Pbs2 is also bound. Thus, Sho 1 and Ste50 act as adaptive docking proteins that restrict the osmostress signal to flow from Ste20/Cla4 to Pbs2, via Ste11.

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Department of Basic Medical Sciences

Division of Neuronal Network
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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

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In a variety of brain regions, excitatory synaptic transmission is regulated dynamically depending on the pattern of synaptic activation: high-frequency activation induces long-lasting enhancement of the 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 the 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, 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 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. The achievement of this year includes elucidation of mAChR functions in intestinal ACh release, smooth muscle contraction/relaxation, gastric acid secretion and hippocampal synaptic plasticity (see the publication list for details).

3. Neuromodulators and synaptic plasticity

Shizuka Kobayashi, Ayako M. Watabe, Norikazu Katayama, Hiroyuki Kato, Michiko Nakamura, Takayuki Morimoto, Saknan Bongsebandhu-Phubhakdi, Fumiko Goto, Noriko Kumazawa, Itone Nishizaki-Ogawa, Masataka Umitsu, and Toshiya Manabe

Neuronal leucine-rich repeat proteins (NLRRs) are type I transmembrane proteins and expressed in neuronal tissues, but their function remains unknown. We identified and characterized a new member of the NLRR family, NLRR 4. In order to elucidate its roles in the central nervous system, we generated NLRR4-deficient (NLRR4(−/−)) mice and found that they showed impaired memory retention. In hippocampus-dependent learning tasks, NLRR4(−/−) mice were able to learn and maintain the memories for one day but unable to retain the memories for four days after learning. In contrast, in a hippocampus-independent task, NLRR4 (−/−) mice were able to retain the memory normally for at least seven days. These results suggest that NLRR4 plays a key role in hippocampus-dependent long-lasting memory.

We are currently examining many other neuromodulators that localize in central synapses, including intracellular signal transduction molecules and adhesion molecules.

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

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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 protein tyrosine phosphatase receptor type Z (Ptprz), which is 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 Rho-associated kinase (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.

5. Dynamics of the actin cytoskeleton in dendritic spines: roles in morphological regulation and synaptic plasticity

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Dendritic spines of pyramidal cells in the mature brain receive excitatory inputs. Each spine provides a postsynaptic biochemical compartment. Since Santiago Ramon y Cajal discovered dendritic spines of neurons more than 100 years ago, it has been a long-lasting question whether shapes of spines are related to their function. Recent advanced techniques of imaging GFP-tagged proteins reveal that spine shapes are unexpectedly dynamic, responding to glutamate stimulation. The actin cytoskeleton predominates in spines, and regulates their morphological plasticity and the anchoring of certain postsynaptic molecules. Numerous studies suggest that actin remodeling is a key to understand the molecular mechanism underlying activity-dependent morphological changes. This project aims to elucidate a role of reorganization of the spinous actin cytoskeleton in synaptic functions.

Stability and mechanical property of actin filaments are generally regulated by their side-binding proteins. Drebrin, one of the actin side-binding proteins, is highly enriched in dendritic spines of mature brains. Using immunoelectron microscopy and a newly developed antibody against drebrin A, we have shown that drebrin A, a neuron-specific isoform of drebrin, localizes in sites of prospective excitatory synapses in the immature brain. We have also found that 25% of dendritic spines contain no drebrin. Since Alzheimer's disease shows major loss of drebrin in the dendritic spine and since down-regulation of the drebrin-A isoform caused by antisense oligonucleotides induces cognitive deficits, we hypothesize that the drebrin content in a dendritic spine is closely related to its synaptic function. It has been immunohistochemically shown that down-regulation of the drebrin-A isoform caused by antisense oligonucleotides in developing cultured hippocampal neurons prevents spine formation and PSD-95 accumulation in dendritic spines. We are now interested in a role of drebrin in trafficking of glutamate receptors during synaptogenesis.

We have reported that intense stimulation with glutamate induces the translocation of drebrin from dendritic spines to their parent dendrites. The translocation of drebrin might be a cause of actin reorganization associated with synaptic activity. Further immunohistochemical and DiI-labeling studies on the effects of glutamate on spine shapes are now progressing. The ionic mechanisms underlying the drebrin translocation have also been examined using glutamate receptor antagonists and Ca^{2+} channel blockers. We are now investigating the ATPase-dependent mechanism of the drebrin translocation. We have just started to examine the effects of ATPase inhibition on synaptic plasticity such as LTP at excitatory synapses in the CA1 region of the rat hippocampus.

6. Spatial and temporal patterns of the signal propagation in hippocampal neuronal circuits: gating mechanisms in the dentate gyrus and the CA2 region in the hippocampal network

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The lamellar hypothesis in the hippocampus is based on physiological data showing that stimulation of the entorhinal cortex activates only a limited number of CA1 cells arranged in a direction along the alvear fibers of the hippocampus. A simple tri-synaptic circuit (DG-CA 3-CA1), which is based on classical anatomical observations with Golgi staining, is consistent with the lamellar hypothesis. However, this hypothesis has been criticized because recent anatomical work has revealed that there is wider distribution of axons along the longitudinal axis of the hippocampus than expected in the simple tri-synaptic concept, and that there are much richer connections among hippocampal subfields (DG, CA3, CA2, and CA1). The discrepancy between results of physiological and anatomical experiments may be due to the inhibitory mechanisms that suppress signal propagation beyond lamellar organization. To examine whether such an inhibitory mechanism is present between lamellae in the rat hippocampus, hippocampal slices were prepared transversely (at a right angle to the long axis), and obliquely (along the alvear fibers). The mossy fiber stimulation evoked population spikes of CA1 neurons in the oblique slices, but not in the transverse slices. These data are consistent with the tri-synaptic circuit classically proposed in the lamellar hypothesis. We found that an adenosine A_1 receptor antagonist, 8-cyclopentyltheo-phylline (8-CPT), produced population spikes in CA1 neurons in the transverse slices. These data indicate that endogenous activity of adenosine A_1 receptors is involved in the inhibition of signal propa-

gation from CA3 to CA1 beyond lamellar organization. We have started to analyze spatial and temporal patterns of the signal propagation from CA3 to CA1 evoked by the mossy fiber stimulation in oblique and transverse slices using a newly developed low-noise CMOS sensor. We have immunohistochemically shown that adenosine A_1 receptors are highly expressed in the CA2 region. Optical recording using a voltage-sensitive dye would enclose whether CA2 neurons are activated by the application of 8-CPT and whether the activation of CA2 neurons is the source of the CA1 activity.

We are currently interested in a role of the supramammillary nucleus (SuM) of the hypothalamic nucleus in the hippocampal function, because the SuM neurons send dense fibers directly to the dentate gyrus and the CA2 region. We have previously shown that intrasupramammillary injection of the $GABA_A$ receptor agonist muscimol prevents the generation of seizure discharges in the rat hippocampus of a kainic acid-induced epileptic model. Our findings suggest that inputs from the SuM to the hippocampus gate the signal flow from the entorhinal cortex to the hippocampus. We have started a new project on signal propagation from the entorhinal cortex to the dentate gyrus using the horizontal slice preparation in which the connection between the two brain regions is preserved.

We hypothesize that the SuM controls the hippocampal memory function. We have tried to trace the fiber tracts from the SuM to the hippocampus with a tracer injection. Since the CA2 region is in the position which controls longitudinal signal propagation in the hippocampal formation, it is important to assess when and how CA2 neurons are activated in vivo. We have analyzed the number of Fos-immunopositive neurons (FN) in the SuM and the hippocampus of the rats that had been placed in an open field. Further, we have analyzed effects of SuM lesions on the increase of FN in the CA2 region. The CA2 region was identified by the absence of the mossy fibers. We are preparing a paper on these results.

7. Regulation of Adenosine A_1 Receptor Expression

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Pertussis toxin ADP-ribosylates G_i- and G_o-transducing proteins and functionally uncouples adenosine A_1 receptor (A_1AR) from its effectors. We hypothesized that this loss in receptor coupling could lead to de novo A_1AR synthesis by the cell in a futile attempt to re-establish normal receptor function. To test this hypothesis, we used hamster ductus deferens tumor (DDT1 MF-2) cells, a cell culture model for studying A_1AR, and showed that pertussis toxin (100 ng/ml) produced a time-dependent loss in A_1AR-Gi interaction and abolished A_1AR activation of extracellular signal regulated kinase (ERK)1/2. Interestingly, pertussis toxin increased the expression of A_1AR, as measured by real time PCR, immunocytochemistry and [^{3}H]-cyclopentyl-1,3-dipropylxanthine (DPCPX) binding, suggesting a compensatory response to Gi protein inactivation. DDT1 MF-2 cells exposed to pertussis toxin demonstrated activation of nuclear factor (NF)-κB within 30 min of exposure, a time point which preceded the loss of function of the A_1 AR. Inhibition of NF-κB attenuated the increase in A_1AR induced by pertussis toxin. Cells exposed to B-oligomer subunit of pertussis toxin, devoid of significant ADP ribosyltransferase activity, showed increased A_1AR protein expression, preceded by activation of NF-κB. B-oligomer increased intracellular Ca^{2+} in DDT1 MF-2 cells. Chelation of intracellular Ca^{2+} with 1,2-bis (2-aminophenoxy) ethane-N,N,N′, N′-tetraacetic acid tetra(acetoxymethyl)ester (BAPTA) or inhibition of protein kinase C (PKC) with bisindolylmaleimide hydrochloride (BIM), reduced the activation of NF-κB and [^{3}H] DPCPX binding. We conclude that pertussis toxin promotes de novo A_1AR synthesis by activating NF-κB through an ADP ribosylation-independent mechanism involving intracellular Ca^{2+} release and PKC activation.

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Department of Basic Medical Sciences

Division of Biomolecular Imaging
分子構造解析分野

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Electron microscopy provides a 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 three-dimensional 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

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

We have been investigating the three-dimensional (3-D) architecture of various macromolecular assemblies that might play crucial roles in a number of cell motility and intracellular signal-transduction systems. 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 molecule is stochastic and should be different from the others, and that the 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 tilt-series 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 had 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 from heavy metal shadowing, virtual model with the shape of protein particle was placed in cyber-space, and its artificial 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). Previously, the best-matched face of the model had been roughly selected by observer's visual inspection using interactive model-viewer, and the simulated image was prepared for that view-angle. We now devised a computer program to automatically extract the outline shape and characteristic feature pattern of the protein surface from real replica image and comprehensive model-images prepared for all 3-D view-angles, then to compare two kinds of images by cross-correlation, and finally recognize the best-matched face from several ten-thousands of model-images in various configurations and viewed from various directions. By that automated procedure, we could objectively and reliably determine the orientation of given particles picked up from the replica images. Hence, we applied such strategy 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 current issue 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. The validity of the new pattern-recognition program was confirmed by 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 in myosin head in putative intermediate state, whose two reactive thiols 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 structures as above and reconfirmed the previous conclusion that the lever-arm moiety of myosin head is not kinked as postulated in conventional "tilting lever-arm hypothesis" but actually, is kinked 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. After extensive search for the image to link above two structures, we could pick up several particles still in pPDM-form; whose lever-arm moiety was kinked to the opposite direction of Vi-form, but its motor-domain attached to actin with the angle like that in rigor-complex. The particles were tethered to actin filament only through the tip of the upper-50K subdomain. Now, it is almost certain that lever-arm swinging takes place during sliding, but in quite a different manner from the conventional hypothetical movement. The crossbridge in that configuration would eventually stretch to form strong rigor-binding, probably by a successive release of inorganic phosphate and ADP. From purely structural aspect, there are at least two 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. Considering that actin filaments move smoothly and continuously on myosin-coated substrate, we might assume the presence of the former actin-translocating process that does not involve active crossbridge tilting. According to conventional tilting mechanism, subdomain-1 of actin would work as a major myosin binding site during crossbridge

cycle. Surprisingly, however, Siddique et al. (2005) showed that chimeric acto-S1 in which total actin sequence was inserted into the actin-binding loop of S1, still can slide quite smoothly on myosin-coated glass surface upon addition of ATP. During chimeric acto-(glass-adsorbed) myosin sliding, that site cannot experience normal strong binding to myosin on the glass, because of closely tethered S1 in the chimera. Yasunaga et al. also reported a mysterious phenomenon under in vitro motility condition, where actin filaments keep moving under extremely low, nanomolar range ATP concentration, if ADP and inorganic phosphate are present. This might reflect the presence of yet unaccounted process other than mechanical lever-arm tilting. Thus, we speculate the presence of two separate mechanisms of actin movement that might take place during sliding; one, continuous rocking movement that translocates actin without developing much force (weakly-bound state), and the other, a real "power-stroke" that occasionally operates accompanying the release of Pi and ADP. The other possibility is the change on the rate of conversion from weakly-bound to primed state, adapting on the load to actin-bound myosin head. Yanagida's team claims that the travel distance of actin-filament by single ATP hydrolysis flexibly changes according to the applied load. Such chemo-mechanical "loose coupling" might be reasonably incorporated to the revised operation mechanism, if the sliding consists of the mixture of two separate processes as above.

Next, we focused our attention on the behavior of the processive movement of unconventional myosin-V. Burgess et al. (2003) examined the structure of "walking" myosin-V along actin filament, by a combination of negative staining and single-particle analysis. Since processive myosin always stays on actin by attachment of at least one head, it is natural to postulate lead and trail heads to represent initial and terminal configurations of lever-arm tilting, respectively. Thus, they averaged negatively stained images of both kinds of heads separately, and compared them with artificial projected images of atomic models of scallop myosin-II in various nucleotide-bound states. They finally assigned lead and trail heads to Vi-form and near-rigor-form assuming that lever-arm tilts in the analogous manner to myosin-II, though the matching of the former structure was not as good as the latter. Since we assigned actin-attached primed structure of myosin-II to new and yet unpublished configuration; i.e. pPDM-form, we examined if that structure could give better fitting to the former averaged image. The result was exactly the case, conversely confirming the validity of our idea on the primed configuration for myosin-II tilting. We prepared quick-freeze deep-etch replica specimens of actin-bound myosin-V in the presence of extremely low concentration of ATP, to realize two-head binding of myosin-V to actin filament. Preliminary observation and the examination of the surface profile of lead and trail heads seem to be compatible with above tentative conclusion. The processive behavior of unconventional myosin might be thus accounted for as to correspond to the latter process of conventional myosin-II including crossbridge stretching. In collaboration with Prof. Yanagida's team (Osaka Unv.), we are under way to characterize the properties of each elementary step that is responsible for 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 collaboration 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 and actin filaments *in vitro* and *in situ* (with Drs T.Q.P. Uyeda, AIST), 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.).

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Department of Basic Medical Science

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脳神経発生・分化分野(1)

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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

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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 voltage-gated Na^+ channels.

2. Functional difference among three IP_3R types

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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) IP_3R clustering in the ER membrane

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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_3R2 $SI_{m2}{}^-$ 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

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We found a novel protein, termed IRBIT (IP_3R binding protein released with inositol 1,4,5-trisphosphate), which interacts with IP3R1 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 IP_3R1 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_3R, raising the possibility that IRBIT acts as a signaling molecule downstream from IP_3R.

B) ERp44

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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 requires a low ER Ca^{2+} level and the presence of lumenal cysteine residues in their reduced form. 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. Also, ERp44 overexpression protected the cells against apoptosis, showing the significance of Ca^{2+} release via IP_3R1 in regulating apoptosis. Thus, ERp44 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.

5. Physiological Roles of Type2 and Type3 IP_3 Receptors

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To evaluate the physiological roles of IP_3 receptors Type2 (IP_3R2) and Type3 (IP_3R3), we generated mice lacking genes for these intracellular calcium release channels. Mice with single gene disruption did not show any distinct phenotype at least for several months after birth, whereas double gene knockout mice died of starvation within one week following the end of the weaning period. The double mutants failed to switch to a dry food diet, and a diet of wet mashed food could rescue these mice from starvation. In accordance with this phenotype, both pilocarpine-induced saliva secretion and acetylcholine-activated calcium signaling in submandibular acinar cells, the signaling cascade responsible for fluid secretion, were severely impaired in the double knockout mice. These results demonstrate an indispensable role of IP_3R2 and IP_3R3 in saliva secretion to tolerate solid food.

Another finding came from histological analysis of the knockout mice. In double mutants, zymogen granules accumulated in the cytosol of pancreatic acinar cells, and the *in vitro* experiment using dissociated acinar cells demonstrated that secretion of digestive enzymes (such as amylase and lipase) in response to muscarinic acetylcholine receptor stimulation, was abolished. These results show that IP_3R2 and IP_3R3, co-localized in the extreme apical regions of acinar cells, play crucial roles in exocrine function of the pancreas. Despite approximately equal caloric intake, double mutants had reduced body weights and lower blood glucose levels than their wild type littermates. It is hypothesized that defects in digestion could account for the malnourishments seen in these mice.

Besides, we found that IP_3R2 and IP_3R3 were involved also in brain development. During the specific period (after postnatal day 12) in the postnatal development of the cerebellum, more cells were found to be present in the external granular layer in double mutants than littermate control animals. Taken together, these results show that IP_3R2 and IP_3R3, having some degree of functional redundancy, play critical roles in physiologically important phenomena such as

exocrine function of the salivary gland and the pancreas, as well as brain development.

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

Takafumi Inoue, Akinori Kuruma[1], Jun-ichi Goto, and Katsuhiko Mikoshiba

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 (50 Hz, 350-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 ~100 μM, 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 (AMPA) 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. Amplification of Ca^{2+} Signaling by Diacylglycerol-Mediated IP_3 Production

Chihiro Hisatsune[1], Kyoko Nakamura[2], Yukiko Kuroda, Takeshi Nakamura[2], Katsuhiko Mikoshiba

Stimulation of various cell surface receptors leads to production of inositol-l, 4, 5-trisphospha te (IP_3) and diacylglycerol (DAG) through phospholipase C (PLC) activation, and the IP_3 and DAG in turn trigger Ca^{2+} release through IP_3 receptors (IP_3Rs) and protein kinase C (PKC) activation, respectively. The amount of IP_3 produced is particularly critical to determining the spatio-temporally coordinated Ca^{2+} signaling patterns. We report a novel signal crosstalk between DAG and the IP_3-mediated Ca^{2+} signaling pathway. We found that a DAG derivative, l-oleoyl-2-acyl-*sn*-glycerol (OAG), induces Ca^{2+} oscillation in various types of cells, independentl y of PKC activity and extracellular Ca^{2+}. The O AG-induced Ca^{2+} oscillation was completely abolished by depletion of Ca^{2+} stores or inhibitio n of PLC and IP_3 receptors (IP_3Rs), indicating th at OAG stimulates IP_3 production through PLC activation and thereby induces IP_3-induced Ca^{2+} release. Furthermore, intracellular accumulation of endogenous DAG by a DAG-lipase inhibitor greatly increased the number of cells responding to agonist stimulation at low does of ATP in COS-7 cells. These results suggest a novel physiological function of DAG, that is, amplification of Ca^{2+} signaling by enhancing IP_3 production via its positive feedback effect on PLC activity.

8. An RNA-interacting Protein, Syncrip, and mRNA transport

Hiroko Bannai[10], Kazumi Fukatsu, Akihiro Mizutani, Tohru Natsume[11], Shun-ichiro Iemura[11], Toru Ikegami, Takafumi Inoue and Katsuhiko Mikoshiba

mRNA transport and local translation in the neuronal dendrite is implicated in the induction of synaptic plasticity. Recently, we cloned an RNA-interacting protein, SYNCRIP (heterogeneous nuclear ribonucmRNA transport and local translation in the neuronal dendrite is implicated in the induction of sylear protein Q1/NSAP1), that is suggested to be important for the stabilization of mRNA. We report here 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 bidirectionally within the dendrite at approximately 0.05 microm/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 alpha. SYNCRIP was also found to be co-transported with green fluorescent protein-tagged human staufen1 and the 3′-untranslated region of inositol 1,4,5-trisphosphate receptor type 1 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

9. Dynamic visualization of IP_3R1 receptor by atomic force microscopy

Wakako Suhara[14], Mime Kobayashi[15], Hiroshi Sagara[16], Kozo Hamada[2], Touichiro Goto, Ichiro Fujimoto[14], Katsuhiko Mikoshiba and Keiichi Torimitsu[15]: [14]The Division of Neural Signal Information NTT-IMSUT, [15]Materials Science Laboratory, NTT Basic Research Laboratories, NTT Corporation, [16]Fine Morphology Laboratory, IMSUT

Inositol 1,4,5-trisphosphate receptor (IP_3R) mediates neuronal signals by releasing calcium ions from the endoplasmic reticulum (ER). The functional IP_3R in the ER membrane consists of four subunits. Analyzing negatively stained particles of detergent-solubilized IP_3R by electron microscope (EM), it has been suggested that the receptor changes its conformational state depending on Ca^{2+} concentrations. However, the EM images were obtained from static specimens in a vacuum. Dynamic aspects of the conformational change have never visualized in a natural environment. In this study, we attempted to observe the structure of the receptor on a biological membrane by atomic force microscopy (AFM). AFM can be used to study samples in solution, which is expected to represent a natural environment. AFM revealed a globular structure on the purified ER membrane, which was identified to be cytosolic domain of type 1 IP_3R (IP_3R1) by monoclonal antibody and EM. The authentic IP_3 R1, which was immunopurified from mouse cerebella had about the same dimensions with those of the protrusion found on the membrane. Various shapes of the purified IP_3R1, including a square-shaped structure in the Ca^{2+}-free buffer and a flattened structure resembling the windmill-like state in the presence of Ca^{2+}, provided an indication of dynamic conformations in solution. Establishing an AFM observation technique for biological samples will add a new dimension to the analysis of the structure-functional relationships of membrane proteins.

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Department of Basic Medical Sciences

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

Research Associate Junichi Watanabe, M.D., Ph.D. 助 手 医学博士 渡 邉 純 一

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. Furthermore, we have started to apply full-length cDNA analysis to Echinococcus, an important helminth in Hokkaido.

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. 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 causing 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 is 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.

5. Full-length cDNA library of *Echinococcus multilocularis*

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Echinococcus is a helminth indigenous in Japan, and expansion of infected areas and increasing prevalence in the final host, the glacier fox, is a great threat to public health. Contaminated feces of the final host cause infection. We have made a full-length cDNA library from parasites propagated in cotton rats and performed the 5′ end-one-pass-sequencing of random clones.

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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. RNA Design

Shoji P Ohuchi, Takashi Ohtsu, Akihiro Oguro, Ritsuko Tanaka-Fujita, Toshie Kawai, Shin Miyakawa[1], Masatoshi Fujiwara[1], Ling Jin[1], Yukiko Iwata[1], Taichi Sakamoto[2], Gota Kawai[2], Kiyotaka Mochizuki, Kei Endo, Daisuke Ito, Hironori Adachi, Eiko Futami-Takada, Katsushi Koda, Nahum Sonenberg[3], Hirotaka Imataka[4] and Yoshikazu Nakamura: [1]Ribomic Inc., [2]Department of Industrial Chemistry, Faculty of Engineering, Chiba Institute of Technology, [3]Department of Biochemistry and McGill Cancer Center, McGill University, [4]RIKEN, Genomic Science Center, Yokohama.

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 eIF4AI and eIF4AIII provided by Dr. Nahum Sonenberg (McGill University, Canada) and Dr. John Hershey (UC-Davis, USA). eIF4G and eIF4AI 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), eIF4AI and Pab1p (poly-A binding protein), and an RNA unwinding helicase, respectively. Importantly, the abnormality in the pro-

tein 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. Selection of RNA aptamers against recombinant transforming growth factor-beta type III receptor displayed on cell surface

In most cases, anti-protein aptamers are selected by SELEX using purified recombinant protein targets. Cell surface proteins, however, are not easy targets for SELEX due to the difficulties associated with their purification. Here, we developed a novel SELEX procedure (referred to as TECS-SELEX) in which cell-surface displayed recombinant protein is directly used as the selection target. Using this method, we isolated RNA aptamers against transforming growth factor-beta type III receptor expressed on Chinese hamster ovary (CHO) cells. TbRIII plays some key roles in signal transduction through functional cooperation with the TGF-beta type II receptor or the activin receptor. One of the RNA aptamers has a dissociation constant in the 1 nM range and competed with transforming growth factor-beta to bind to the cell surface receptor *in vitro*. Structural and functional analysis of selected and optimized anti-TbRIII RNA aptamer have been studied. The development of TECS-SELEX provides a useful, novel method to isolate aptamers to any cell surface protein of interest that cannot be easily obtained to homogeneity.

b. High affinity RNA against eIF1A as a new research tool for translation initiation

Eukaryotic translation initiation factor 1A (eIF-1A) is a highly conserved, small, acidic protein that has pleiotropic functions in protein synthesis. It has been shown that eIF1A directs the eIF2-GTP-Met-tRNAi ternary complex (TC) to the 40S ribosomal subunit with the aid of eIF3 and subsequently promotes scanning of the 5′UTR for the AUG initiation codon. The prokaryotic homolog of eIF1A, IF1, binds in the A site of 30S ribosomal subunit, and interacts with IF2. eIF1A also interacts with eIF5B (prokaryotic IF2 homolog) conserving the IF1-IF2 interaction. However, the precise function of eIF1A remains unclear. Here, we generated an RNA aptamer that bound to eIF1A with a dissociation constant of 10 nM. This RNA aptamer severely inhibits *in vitro* cap-dependent and HCV IRES-dependent translation in rabbit reticulocyte lysate. In order to reveal the inhibitory effects of this aptamer on translation, we used a pull-down assay with His-tagged eIF1A designed to reveal eIF1A binding partners in HeLa cell extracts that were inhibited in the presence of the aptamer. Western blotting of the precipitated His-tagged eIF1A-complexes using antibodies against various translation factors indicated that this RNA aptamer inhibits the interaction between eIF1A and eIF3. These results suggest that TC recruitment to the 40S ribosomal subunit was inhibited by the aptamer. We are at present determining the precise working model for this aptamer and establishing its utility as a research tool.

c. 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 (eIF4AI, eIF4G and eIF4E) and is responsible for recruiting ribosomes to mRNA. eIF4E recognizes the mRNA 5′ cap structure (m^7GpppN) 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 affinity 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.

d. RNA aptamers to mammalian initiation factor 4G inhibit cap-dependent translation by blocking the formation of initiation factor complexes

eIF4G plays a crucial multi-modulatory role in mRNA translation and decay by interacting with other translation factors and mRNA-associated proteins. We isolated eight different RNA aptamers with high affinity to mammalian eIF4G by *in vitro* RNA selection-amplification. Of these, three aptamers (apt3, apt4 and apt5) inhibited the cap-dependent translation of two independent mRNAs in a rabbit reticulocyte lysate system. The cap-independent translation directed by an HCV internal ribosome entry site was not affected. Addition of exogenous eIF4G reversed the aptamer mediated inhibition of translation. Even though apt3 and apt4 were selected independently, they differ only by two nucleotides. The use of truncated eIF4G variants in binding experiments indicated that apt4 (and probably apt3) bind to the middle domain of eIF4G, while apt5 appears to bind to the C-terminal domain of eIF4G. Corresponding to the difference in the binding sites in eIF4G, apt4, but not apt5, hindered eIF4G from binding to eIF4A and eIF3, in a purified protein solution system as well as in a crude lysate system. Therefore, the inhibition of translation by apt4 (and apt3) is due to the inhibition of formation of initiation factor complexes involving eIF4A and eIF3. The mechanism of apt5 inhibition of translation remains unknown. Apt5 had a much weaker affinity to eIF4G than apt4, but inhibited translation much more efficiently, providing evidence for a crucial role of the C-terminal region of eIF4G in translation initiation. The five additional aptamers have sequences and predicted secondary structures that are largely different from each other and from apt3 through apt5. Therefore, we speculate that these seven sets of aptamers may bind to different regions in eIF4G in different fashions.

e. NMR structures of double loops of an RNA aptamer against mammalian initiation factor 4AI

A high affinity RNA aptamer (APT58, 58 nucleotides long) against mammalian eIF4AI requires its nearly entire nucleotide sequence for efficient binding. Since splitting either APT58 or eIF4AI 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 eIF4AI, 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 eIF4AI, 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 eIF4AI.

2. Translational Control and Protein-tRNA Molecular Mimicry

Koichi Ito, Miki Wada, Hanae Sato, Yuya Watanabe, Hiroyuki Kodama, Saori Murakami, Kazuki Saito, and Yoshikazu Nakamura

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

a. Ribosomal protein L11 mutations in two functional domains equally affect release factor 1 and 2 activity

Bacterial release factors 1 and 2 catalyze translation termination at UAG/UAA and UGA/UAA stop codons, respectively. It has been shown that limiting the amount of ribosomal protein L11 affects translation termination at UAG and UGA differently. To understand the functional interplay between L11 and RF1/RF2, we isolated 21 distinct mutations in L11 as suppressors of either temperature-sensitive (ts) RF1 /RF2 strains or read-through mutants of *lacZ*

nonsense (UAG or UGA) strains. 10 of 21 mutants restored ts lethal growth of RF1 and/or RF 2 strains. All the selected L11 mutants, including the RF1ts- and RF2ts-specific suppressors, had the same effect, either enhancing or reducing, on UAG and UGA termination efficiency *in vivo*. The specific properties of the selected L11 mutations remained unchanged in an RF3 deletion strain. Moreover, ribosomes absent of L11 had equally reduced activity for both RF1- and RF2-mediated peptide release *in vitro*. These results suggest that, unlike the previous notion, L11 has a common, cooperative role with RF1 and RF2. These L11 mutations were located on the surface of two domains of L11, and interpreted to affect the interaction between L11 and rRNA or the release factors thereby leading to the altered translation termination.

3. Molecular Biology of Yeast Prions

Colin G. Crist, Hideyuki Hara, Hiroshi Kurahashi, Aiko Takahashi, Masao Ishiwata and Yoshikazu Nakamura

The Sup35 protein of the budding yeast *Saccharomyces cerevisiae* is a subunit of the eukaryotic polypeptide-release factor (eRF3) and is essential for terminating protein synthesis at stop codons. Sup35p also exists as a stable amyloid fibril, termed [PSI^+], that propagates its aberrant fold in the cytoplasm in a manner analogous to the "protein only" transmission of mammalian prion protein (PrP). [PSI^+] cells are marked by an altered protein conformation of Sup35p whereby the protein is converted from a soluble, active state to an aggregated inactive state. In the aggregated state, ribosomes often fail to release polypeptides at stop codons, causing a non-Mendelian trait easily detected by the suppression of nonsense mutations. Thus, the conversion of soluble Sup35p [psi^-] to the aggregated form [PSI^+] serves as a useful model for studying the formation of amyloid deposits and the prion-like transmission of an altered protein conformation.

a. 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.

b. A novel genetic selection of 'prion-to-nonprion' conversion

There are several genetic systems to select for [PSI^+] cells that adopt prion form of Sup35 in *S. cerevisiae*. However, there is no genetic system developed for the selection of [psi^-] cells appeared from [PSI^+] cells. Here, we constructed the *ura 3* nonsense mutant, in which readthrough translation can occur in [PSI^+] but not in [psi^-]. In [PSI^+] state, the *ura3* nonsense strain was lethal in the presence of 5-FOA since 5-FOA was metabolized to a toxic material by the readthrough product of Ura3. On the other hand, the strain allowed [psi^-] cells to grow on 5-FOA, providing us with a novel genetic selection for 'prion-to-nonprion' conversion. Using this positive selection of [psi^-] cells, numerous mutations in the *HSP104* chaperon have been isolated. These *hsp104* mutants are under investigation from the viewpoint of structure-function relationships.

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Department of Basic Medical Sciences

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

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

1. 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 nonmuscle 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. Release of a dipeptide from the carboxyl-terminus of Ku80 during apoptosis in human Jurkat T cells.

Masahiko Kato, Takashi Nonaka and Shinobu Imajoh-Ohmi

Apg-2, an Hsp110 family protein, was found to decrease during apoptosis in Jurkat T cells. Since we hypothesized that Apg-2 would be cleaved by caspase-3 or a related enzyme, a cleavage-site-directed antibody was raised against the carboxyl-terminus of the Apg-2 fragment that appears after the cleavage. This antibody, however, could not detect the Apg-2 fragment in apoptotic cells. Instead, three additional fragments were unexpectedly detected. Based on the results of microsequencing, one of these fragments was identified as Ku80, a component of DNA-dependent protein kinase. In this study, we observed that Ku80 is cleaved at Asp-730 during apoptosis, methionylisoleucine is released, and this cleavage occurs in the nucleus in the early apoptotic phase. Furthermore, Ku80 is distributed in the cytoplasm of nuclear fragmented apoptotic cells, although the cleaved fragment contains the nuclear-localization signal. Our study clearly shows that Ku80 is cleaved in thenucleus, and distributes intha cytoplasm during apoptosis.

c. 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.

2. Functional analyses of a BRCA2 protein

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Individuals with germ-line mutations in the breast cancer susceptibility gene *BRCA2* are at risk of developing breast and ovarian cancer. Consistent with a critical function for *BRCA2* in tumor suppression, tumors that develop in carriers of heterozygous *BRCA2* mutations are frequently associated with loss of heterozygosity at the *BRCA2* locus. However, mutation of the *BRCA2* gene is rarely found in sporadic breast cancer, and the role of the *BRCA2* gene in the development of sporadic breast cancer is unclear. BRCA2 plays important roles in both transcriptional regulation and DNA damage repair mediated by homologous recombination. Furthermore, it has been reported that embryonic fibroblasts with a $BRCA2^{Tr2014/Tr2014}$ mutation have an abnormal number of centrosomes. Recent studies indicate that BRCA2 inactivation in murine embryo fibroblasts and HeLa cells by targeted gene disruption or RNA interference delays and prevents cell division. Both G1 arrest and mitotic errors at cytokinesis occur in cells from which centrosomes have been removed by microsurgery. Centrosome defects are characteristic of many solid tumors and may be responsible for the origin of the mitotic spindle abnormalities and DNA aneuploidy found in cancer.

BRCA2 has been reported to localize to discrete nuclear foci during S-phase. When we examined the subcellular localization of BRCA2 at

each phase of cell cycle by immunofluorescence microscopy in HeLa cells, we observed a strong focus of BRCA2 fluorescence that corresponded to the position of the centrosome, together with the usual dot pattern of BRCA2 fluorescence in the nucleus throughout G1 and S phase. Based on this finding, we hypothesized that BRCA2 might play a vital role in the function of the centrosome checkpoint.

Here, we show that BRCA2 has a modular centrosomal localization signal (CLS), localizes to centrosomes during S and early M phases, and regulates duplication and separation of the centrosomes. Green Fluorescent Protein (GFP) fused to the CLS from BRCA2 (GFP-CLS) localizes to centrosomes and prevents endogenous BRCA2 from localizing to centrosomes. In addition, expression of GFP-CLS in cells leads to the abnormal duplication and positioning of centrosomes, resulting in the generation of tetraploid cells. Depletion of BRCA2 by RNAi has effects on cells that are similar to those observed following expression of by GFP-CLS. These results thus implicate BRCA2 in the regulation of centrosome cycle, and provide new insight into the aneuploid nature of many breast cancers.

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

We have succeeded in making an antibody to E64c. First, we tried to establish an antibody against E64c-bound calpain. A peptide corresponding to the active center of calpain was synthesized by using the multiple-antigen peptide system. E64c was chemically introduced into the SH group of active center cysteine under reducing conditions. Rabbits were immunized with the E64c-conjugated calpain-derived peptide without further conjugation with a carrier protein. Unexpectedly, an antibody thus

prepared reacted not only with E64c-inactivated calpain but also with E64c-bound other cysteine proteases such as papain and cathepsins. Low antigenicity of peptide region in the immunogen may result in such broad specificity of the antibody. Our antibody is expected to be used for identification of E64c-targeted novel proteases. When cells were treated with E64d, cell growth was suppressed and several proteins were labeled by E64c that is visualized with this antibody on immunoblotting. Structural analysis of these proteins may lead identification of novel cysteine proteases.

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

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