Department of Basic Medical Sciences

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

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Protein phosphorylation and dephosphorylation are among the most important intracellular signaling mechanisms, and are mediated respectively by protein kinases and protein phosphatases. We study various aspects of cellular signal transduction with a particular emphasis on the role and regulation of protein phosphorylation and dephosphorylation in cellular stress responses, using both mammalian and yeast cells.

1. Activation of MTK1/MEKK4 by GADD45 through induced N-C dissociation and dimerization-mediated trans-autophosphorylation of the MTK1 kinase domain.

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Mammalian cells are frequently exposed to cellular stresses, which are defined as diverse environmental conditions that are detrimental to the normal growth and survival of the cells. Typical cellular stresses include ultraviolet (UV), ionizing radiation (IR), genotoxins, hyperosmolarity, oxidative stress, low oxygen supply (hypoxia), and inhibition of protein synthesis by antibiotics and plant toxins. In coping with the barrage of these and other cellular stresses, multi-cellular eukaryotic organisms have developed a strategy as to how damaged cells will respond to stresses. In general, if the intensity of damage is moderate, the affected cell will seek to repair the damage. If, however, the damage to a cell is too severe for a complete repair, the affected cells are eliminated by apoptosis. This reduces the risk to the organism as a whole, such as development of a cancer. Such a crucial decision-making between repair or death is, at least in part, mediated by the Stress-activated MAP kinase (SAPK) pathways.

As the name implies, the SAPK pathways are homologous to and share many characteristics with the classic (ERK1/2) MAPK pathway. Eukaryotic MAPK pathways are conserved signaling modules that serve to transmit signals from the cell surface to the nucleus. The core of any MAPK pathway is composed of three tiers of sequentially activating protein kinases, namely, MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. Activation of MAPKs is achieved by phosphorylation of a threonine and a tyrosine residues within a conserved Thr-Xaa-Tyr motif in the activation loop (also called the T-loop) catalyzed by MAPKKs. MAPKKs, in turn, are activated by any of several MAPKKKs, via phosphorylation of serine and/or threonine residues within their activation loop.

All eukaryotic cells possess multiple MAPK pathways, each of which is activated by distinct sets of stimuli. In the budding yeast *Saccharomyces cerevisiae*, for example, hyperosmotic stress

activates the Hog1 MAPK pathway, whereas mating pheromones activate the Fus3/Kss1 MAPK pathway (13, 18). In mammalian cells, four different subfamilies of MAPKs are present, namely, ERK1/2, JNK1/2/3, p38α/β/γ/δ, and ERK5. The ERK1/2 MAPK pathway is preferentially activated in response to mitogenic stimuli, such as growth factors and phorbol esters, and plays a role in cell growth and cell survival. The ERK1/2 pathway is mainly regulated by the monomeric GTPase Ras, which recruits MAPKKKs of the Raf family to activate the two downstream MAPKKs: MEK1/2. These MAPKKs, in turn, activate the ERK 1/2 MAPKs. The JNK and p38 MAPKs (collectively called SAPKs), in contrast, preferentially respond to various cellular stresses, and are thus called SAPK pathways. Besides cell stresses, the SAPK pathways are also activated by cytokines such as IL-1, TNFα, and TGF-β. The JNK subfamily of MAPKs are activated mainly by the MKK4 and MKK7 MAPKKs, while the p38 subfamily MAPKs are activated by the MKK3 and MKK6 MAPKKs. In clear contrast to this limited number of MAPKKs in the SAPK pathways, there are numerous MAPKKKs that function upstream of the JNK and p38 MAPKs. These include MEKK1/2/3, MTK1 (also known as MEKK4), TAK1, ASK1/2, TAO1/2/3, MLKs, and perhaps others. This multiplicity at the level of MAPKKK reflects the vastly diverse stress stimuli that can recruit these SAPK pathways.

MTK1 is one of the human MAPKKKs belonging to the SAPK pathways, and the mouse ortholog is called MEKK4. The kinase domain of MTK1 (MEKK4) is homologous to other MAPKKKs, especially similar to mammalian MEKK1/2/3 and ASK1/2 and yeast SSK2/SSK22, but its N-terminal non-catalytic domain (regulatory domain) is unique. Analyses using MEKK4-deficient mice have shown that the MEKK4 signaling pathway integrates signals from both the T cell antigen receptor and IL-12/STAT4 in developing Th1 cells, and promotes STAT4-independent IFNγ production, and that MEKK4 is essential for normal neural and skeletal development.

In a yeast two-hybrid screening aimed to identify MTK1 activator(s), we found three Growth-Arrest and DNA Damage-inducible 45 (GADD45) family proteins to be strong binding partners of MTK1. The GADD45 gene was originally identified as a UV-inducible gene in Chinese hamster cells. The human genome encodes three GADD45-like proteins, GADD45α (the original GADD45), GADD45β, and GADD45γ. These will be referred to collectively as the GADD45 proteins. The three GADD45 genes are all inducible by cellular stresses, although optimal stimuli for each gene appear to be different. The GADD45 proteins interact with various intracellular molecules, such as proliferating cell nuclear antigen (PCNA), Cdc2-CyclinB1 complex, $p21^{Waf1/Cip1}$, and core histones, and play important roles in stress-adaptive processes including growth control, maintenance of genomic stability, DNA repair, and apoptosis. In other words, the GADD45 proteins are emergency calls in damaged cells.

Expression of transfected GADD45 genes strongly activates co-expressed MTK1 kinase and induces apoptosis in mammalian cells. TGF-β-induced GADD45β expression also activates p 38 MAPK through MTK1 activation. MEKK4-deficient mice have lost GADD45-induced IFNγ production. Activation of the SAPK pathway by MTK1 and GADD45 is temporally a slow process, because it requires induction of GADD45 gene expression prior to activation of MTK1. Thus, the activation of MTK1 by GADD45 differs from other modes of SAPK activation that occur within minutes. GADD45/MTK1 mediated SAPK activation may therefore serve as a more long-term adaptive mechanism for stressed cells.

We previously proposed that binding of GADD45 to the N-terminal region of MTK1 counters the autoinhibitory effect of the MTK1 N-terminal segment on the kinase domain, and at the same time enables the MTK1 kinase domain to bind its cognate MAPKKs (MKK3 and MKK6) via the latter's DVD docking sites. The details of MTK1 activation by GADD45, however, remained obscure. This year, we investigated the molecular mechanism by which GADD45 regulates MTK1 kinase activity.

The data from this study lead to the following model of the GADD45 mediated activation of the MTK1 MAPKKK. Activation of MTK1 by GADD45 occurs through a series of molecular steps (I through VI). In brief, each step is as follows. (I) In unstimulated cells, MTK1 is in a closed (inhibited) conformation in which the N-terminal autoinhibitory domain (AID) blocks the C-terminal kinase catalytic domain (KD). (II) Extracellular stimuli, such as MMS exposure, induce the expression of stress-inducible GADD45 proteins, which bind to the MTK1 N-terminal GADD45-binding domain (BD). (III) GADD45-binding to MTK1 dissociates the latter's AID from the C-terminal kinase catalytic domain. (IV) At the same time, GADD45-binding unmasks the MTK1 dimerization domain, inducing homo-dimer formation. At this stage, the MTK1 kinase domain is in open conformation (i.e., not actively inhibited), but not yet fully active as a kinase. (V) Dimerized MTK1 becomes fully activated when Thr-1493 is trans-autophosphory-

lated. (VI) GADD45 binding also unmasks a site in the MTK1 kinase domain that interacts with the MAPKK DVD docking sites, allowing MTK1 to interact with, and phosphorylate, the cognate MAPKKs, namely MKK3 and MKK6.

Thus, full activation of MTK1 by GADD45 entails four different molecular mechanisms: removal of the autoinhibitory domain; dimerization; phosphorylation of the activation loop; and unmasking of the docking site for MAPKKs. Individually, these mechanisms are used by other MAPKKKs. However, the details are different for each MAPKKK, reflecting their different physiological roles. In this study, we revealed how the binding of one protein (GADD45) orchestrated these mechanisms, thereby converting an inert enzyme (MTK1) to a fully active one.

2. Adaptor functions of Cdc42, Ste50, and Sho1 in the yeast osmoregulatory HOG MAPK pathway.

Kazuo Tatebayashi, Katsuyoshi Yamamoto, 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 *pbs*2 or *hog*1 mutant cellss, however, the cross-talk barrier breaks down, and osmostress induces the mating-specitic reporter *FUS1-lacZ*.

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, Sho1 and Ste50 act as adaptive docking proteins that restrict the osmostress signal to flow from Ste20/Cla4 to Pbs2, via Ste11.

Publications

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Department of Basic Medical Sciences

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

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

1. NMDA receptor phosphorylation and synaptic plasticity

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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 generated mice with a knockin mutation of the Tyr-1472 site to phenylalanine (Y1472F) and show that Tyr-1472 phosphorylation is essential for functions of the amygdala. The knockin mice show impaired fear-related learning and reduced amygdaloid LTP. NMDA receptor-mediated calcium/calmodulin-dependent protein kinase II (CaMKII) signaling is impaired in YF/YF mice. Electron-microscopic analyses reveal that the Y1472F mutant of the NR2B subunit shows improper localization at synapses. We thus identify Tyr-1472 phosphorylation as a key mediator of synaptic plasticity and fear-related learning in the amygdala.

2. Inhibitory modulation of synaptic plasticity is stronger in the dentate gyrus than in the CA1 region of the hippocampus.

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Long-term potentiation (LTP) is a phenomenon that the efficacy of synaptic transmission is enhanced after high-frequency activation of the synapse. It was first discovered in the hippocampus, and it has been widely accepted as a cellular basis of certain forms of memory. In the medial perforant pathway-dentate gyrus granule cell synapse and in the CA3-CA1 pyramidal cell synapse, LTP is induced by a similar mechanism (postsynaptic N-methyl-D-aspartate receptor dependent), while several reports suggested that the modulation of LTP by γ-aminobutyric acid type A ($GABA_A$) receptor-mediated inhibitory inputs is stronger in the medial perforant pathway-dentate gyrus granule cell synapse. To explore the underlying molecular mechanism that makes the difference between the two regions, we compared LTP in the presence of the $GABA_A$ receptor antagonist picrotoxin with LTP in its absence in the CA1 region and in the dentate gyrus using acute slices of the rat hippocampus. We then compared the inhibitory monosynaptic responses with excitatory monosynaptic responses, and also compared their summation during an LTP-inducing stimulus between the two regions. Our results suggest that the stronger inhibitory modulation of LTP in the dentate gyrus may be due to the balance biased towards inhibition between the summated inhibitory and excitatory postsynaptic currents during conditioning in the dentate gyrus. Besides these examinations of synaptic inhibitory inputs, several reports suggested that continuous activation of extrasynaptic $GABA_A$ receptors by ambient GABA is different in several aspects between the two regions, which could also contribute to the finding about LTP modulation as well. For example, it is reported that continuous activation of extrasynaptic $GABA_A$ receptors is mediated by the receptor with different subunit compositions between the two regions, which may result in different properties of the inhibition. Thus, we are currently examining whether this kind of inhibition is associated with the stronger inhibitory modulation of LTP in the dentate gyrus using the whole-cell patch-clamp technique.

3. Functional properties of the NMDA receptor in the lateral amygdala: a comparison with those in the hippocampal CA1 region

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The amygdala is a crucial brain structure for the acquisition and expression of fear memory. The N-methyl-D-aspartate (NMDA)-type glutamate receptor channel, composed of the NR1 (GluRζ) and NR2 (GluRε) subunits, plays a key role in synaptic plasticity in the central nervous system. NR2 subunits (NR2A-NR2D) are differentially expressed, depending on developmental stages and brain regions, but their functional roles in the amygdala are still largely unknown. Here, we have investigated the properties of synaptic NMDA receptors in the lateral nucleus of the amygdala (LA), comparing them with those in the hippocampal CA1 region. We find that the biophysical properties of NMDA receptors and NR2A/NR2B ratio in the LA are distinct from those in the CA1 region and that the NR2B subunit contributes to synaptic transmission and LTP induction to a greater extent in LA than in the CA1 region. Our data suggest that these properties of NMDA receptors in the LA are responsible for unique properties of amygdaloid synaptic function and plasticity.

4. Analysis of muscarinic acetylcholine receptor functions using knockout mice

Minoru Matsui, Shun Hamada, Shinji Kusakawa, Yuji Kiyama, 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 stra-

ins, 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 smooth muscle contraction /relaxation, salivary secretion, regulation of GABAergic neuron activity in the dorsal horn, and regulation of the endocannabinoid signaling in the striatum (see the publication list for details).

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 Ramón 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. Stability and mechanical property of actin filaments are generally regulated by their side-binding proteins. This project aims to elucidate a role of reorganization of the spinous actin cytoskeleton in synaptic functions.

Drebrin, one of the actin side-binding proteins, is highly enriched in dendritic spines of the mature brain. 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 20 % 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 in progress. 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 be-

yond 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 trisynaptic circuit classically proposed in the lamellar hypothesis. We found that an adenosine A_1 receptor antagonist, 8-cyclopentyltheophylline (8-CPT), produced population spikes in CA 1 neurons in the transverse slices. These data indicate that endogenous activity of adenosine A_1 receptors is involved in the inhibition of signal propagation from the CA3 to CA1 region beyond lamellar organization. We have started to analyze spatial and temporal patterns of the signal propagation from the CA3 to CA1 region 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 recordings 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 supramammillary nucleus (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 papers on these results.

7. Regulation of adenosine A_1 receptor expression

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Pertussis toxin functionally uncouples adenosine A_1 receptor (A_1AR) from its effectors. We hypothesized that this loss in the 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-G_i 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 G_i protein inactivation. Inhibition of NF-κB attenuated the increase in A_1AR induced by pertussis toxin. We conclude that pertussis toxin promotes de novo A_1 AR synthesis by activating NF-κB through an ADP ribosylation-independent mechanism involving intracellular Ca^{2+} release and PKC activation.

Further, we evaluated the role of NO in the regulation of A_1AR expression, a G protein-coupled receptor involved in cytoprotection in the central nervous system, as the expression of the A_1AR is regulated by oxidative stress. Administration of the NO donor, S-nitrosylpenicillamine (SNAP), to pheochromocytoma 12 (PC 12) cells increased A_1AR protein in a time- and dose-dependent manner. The response to SNAP was attenuated by the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5- tetramethylimidazoline -1-oxyl-3 oxide (C-PTIO), and by the inhibition of nuclear factor-jB (NF-jB), implicating this transcription factor in the regulatory process. In addition, SNAP also increased the degradation of Inhibitory jB-a (IjB-a), a marker of NF-jB activation. Furthermore, the induction of inducible nitric oxide synthase (iNOS) by lipopolysaccharide increased A_1AR in PC12 cells and in mice,

whereas the inhibition of NOS activity suppressed this response. We conclude that NO, via the activation of NF-jB, serves as an endogenous regulator of A_1AR, and speculate that the induction of the A_1AR could counteract the cytotoxicity of NO.

8. GABAergic interneurons facilitate mossy fiber excitability in the developing hippocampus.

Michiko Nakamura, Yuko Sekino, and Toshiya Manabe

Profound activity-dependent synaptic facilitation at hippocampal mossy fiber synapses is a unique and functionally important property. Although presynaptic ionotropic receptors, such as kainate receptors, contribute partially to the facilitation in the hippocampus, the precise mechanisms of presynaptic regulation by endogenous neurotransmitters remain unclear. In this study, we report that axonal $GABA_A$ receptors on mossy fibers are involved in the activity-dependent facilitation during development. In immature mouse hippocampal slices, short-train stimulation (5 pulses at 25 Hz) caused frequency -dependent facilitation of not only postsynaptic responses but also presynaptic fiber volleys that represent presynaptic activities. This fiber volley facilitation was inhibited by selective $GABA_A$ receptor antagonists, or by enkephalin that selectively suppresses excitability of interneurons. Furthermore, we directly demonstrated that this facilitation resulted from depolarization of mossy fibers in imaging experiments using a voltage-sensitive dye. This increased mossy fiber excitability caused by depolarizing action of GABA gradually decreased with development and eventually disappeared at around postnatal day 30. These results suggested that GABA released from interneurons acted on axonal $GABA_A$ receptors on mossy fibers and contributed at least partially to the activity- and age-dependent facilitation in the hippocampus.

Publications

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Department of Basic Medical Sciences

Division of Fine Morphology
分子構造解析分野

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

教 授 医学博士 片 山 榮 作

Electron microscopy provides a unique means to investigate the structure of biological materials including cells/tissues and macromolecular complexes. Instantaneous structure of functioning molecules not only in solution but also in live cells can be visualized with high contrast after adequate processing. Though the spatial resolution remains less than crystallography, the real superior feature of the method is its almost unlimited applicability to the materials whose structure cannot otherwise be pursued. Since last year, we have been involved in a new project to develop several innovative techniques to characterize three-dimensional structure of individual macromolecules under functional states in solution or in live cells in situ". Our research activities are directed to two ways; one, development of new experimental tools and the other, their application, both to realize "Structural Biology of Single Molecules".

Visualization of structural features of functioning protein molecules during various molecular events related to cell motility 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 renewed the conventional knowledge on the intrinsic properties of various motor proteins and their interactions. The most important message from this new concept is that the behavior of individual protein molecule is stochastic and should be different from the others, and that the important information could be revealed by observation and separately analysing unaveraged behavior of each single molecule. Various experimental techniques to deal single molecules have already been established and used as the most powerful and indispensable tools in current biophysical sciences. On the other hand, conventional means of structural biology; *i.e*. X-ray crystallography or multi-dimensional NMR analysis, collects the data from a vast number of particles to be averaged both in time and space, and apparently is not compatible to "single molecule" matter. Electron microscopy is unique in terms that it has a potential to visualize the instantaneous structure of individual macromolecules. Structural determination of motor-proteins under dynamic functional state is difficult to approach by other means and thus, is one of the most adequate and challenging applications to evaluate the power of novel methodologies we have developed. In order to obtain structural information of sliding actomyosin motor comparable to the results of single molecule physiology, we have been utilizing quick-freeze deep-etch replica electron microscopy to capture

transient configuration of myosin crossbridges supporting actin movement *in vitro*. In this technique, various molecular events extensively studied under fluorescence microscope can be instantaneously arrested within one millisecond and the structure of individual protein molecules under well-characterized experimental conditions might be clearly visualized with a resolution that enables us to recognize subdomain arrangement within "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 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, formation of phantom images due to this problem has been the biggest obstacle that had hampered high-resolution 3-D electron microscopy of biological material by now. Several years ago, we introduced 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 of replica specimens. Recently, we further improved the reconstruction process (patent pending) based on completely different principle from the previous ones, and now are able to reconstruct even more precisely the internal structure of various materials (whether biological or not) from much less number of tilt-series images, unaffected by phantom problem. As a complementary approach to characterize the 3-D structure of the target particles utilizing single but very high-resolution images, we devised a computer program to automatically extract the outline shape and characteristic feature pattern of the protein surface from replica images. We are able to compare it quantitatively with a comprehensive set of artificial images of the atomic-model in various configurations viewed from all 3-D angles (patent pending). The robustness of new pattern-recognition program was confirmed by examination of gallery of particles in ADP/vanadate-form (Vi-form), whose structure is well-characterized by X-ray crystallography. By that 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 including actin-sliding ones. The question to be answered here is whether putative "swinging of myosin lever-arm" actually occurs during actomyosin sliding and how the intra- and/or intermolecular structural change is reflected to the sliding of actin filament. We previously showed that the structure of actin-attached myosin during sliding is unlike kinked Vi-form as believed, but rather close to oppositely--kinked configuration (pPDM-form) which we found for the first time, in myosin head whose two reactive thiols were chemically cross-linked by bifunctional reagents. More objective analysis was carried out by modular-fitting of each protein part in replica images to artificial model-images; i.e. crystal structure of myosin head was subdivided into a motor domain and a lever-arm, and the artificial images generated from individual parts were separately compared with the counterparts in the replica images. We could determine precise orientation of lever-arm moiety respective to the main body of the motor-domain in this putative intermediate configuration. We also analyzed the images of myosin crossbridges supporting actin movement, by comparison with atomic models of various configurations including above crosslinked ones. The results confirmed our previous view that the lever-arm moiety of myosin head during sliding is not kinked as postulated in conventional "tilting lever-arm hypothesis" but actually, is oppositely-kinked as we found in SH-crosslinked myosin. Last year, we reported the presence of actin-attached myosin heads taking pPDM-form as above but in a different orientation, so that the attachment angle of the motor-domain in is almost the same as in rigor. That is probably the true primed structure immediately before the power-stroke, and the crossbridge in that configuration would eventually stretch to form strong rigor-binding, possibly by a successive release of inorganic phosphate and ADP. Now it is almost certain that structural change including lever-arm swinging would take place during sliding, but in quite a different manner expected from the conventional hypothetical movement. 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 primed configuration to extended rigor-form. Considering that actin filaments move smoothly and continuously on myosin-coated substrate, we might assume the contribution of 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. 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 Pi and ADP release. 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. There are more supporting evidences for our revised mechanisms, though we do not have space to describe them.

Next, we focused our attention on the behavior of the processive movement of unconventional myosin-V, that plays an important role in intracellular vesicular transport. Burgess et al. (2003) examined the structure of "walking" myosin-V along actin filament, by a combination of negative staining and single-particle analysis. They averaged negatively stained images of lead and trail 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 respectively, assuming that lever-arm tilts in the analogous manner to myosin-II's swinging, 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 the same 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 interpretation. The processive behavior of unconventional myosin might thus be accounted for as to correspond to the latter process of conventional myosin-II including crossbridge stretching.

Development and application of new marker probes for electron and fluorescence microscopy

Tominaga, M.

We have been developing a rod-shaped protein module that enables us high-resolution protein surface mapping by putting landmarks in electron microscopic images of the target, together with fluorescence detection of the same molecules within cells or solutions (Katayama; Pat. Pend., 2005). The marker probe was designed to include Green Fluorescent Protein (GFP) on one end and fusible on the other end to any position along the amino-acid sequence of the given protein. Myosin-V has a pair of heavy chains, each with six calmodulin (CaM) as light chains, possibly providing the rigidity of the neck domain necessary to act as a lever-arm and to regulate the processivity through Ca^{2+}-dependent reversible dissociation. A novel probe was fused to two components of myosin-V, heavy chain and CaM, both to the N-termninus, aiming to examine their mode of binding to the other components. Electron microscopy of probe-tagged CaM showed dumbbell-like morphology reflecting two globular structures of GFP and CaM moieties. Characteristic Ca^{2+}-dependent activities of original CaM such as mobility change in SDS gel, binding to myosin light-chain kinase and activation of calcineurin remained almost normal after fusion, suggesting the advantageous feature of the probe module. Probe-tagged myosin-V which was transiently expressed in HeLa cell showed filamentous appearance and/or punctate distribution at the extreme edge of lamellipodia. Simultaneous visualization of actin filaments revealed the accumulation of mutant myosin V at the end of actin bundles, in a similar manner to wild-type molecules, indicating at least its motor activity was little affected by the fusion of the large probe. Further analyses are in progress on the structure and function of probe-tagged proteins.

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片山栄作，木森義隆，馬場則男：回転式モルフォロジーフィルター及びそれを用いた画像処理法（出願番号：2006–280441　2006年10月13日）

片山栄作，村山　尚，樫山　拓，小林琢也：分子モジュール（出願番号：2006–332530　2006年12月８日）

Department of Basic Medical Sciences

Division of Molecular Neurobiology (1)
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 spines 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 b-trefoil domain and a carboxy-terminal a-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 b -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.

A) Inositol 1,4,5-trisphoaphate receptor type 1 in granule cells, not in Purkinje cells, regulates the dendritic morphology of Purkinje cells through BDNF production.

Hisatsune, C., Kuroda, Y., Akagi, T., Torashima, T., Hirai, H., Hashikawa, T.,

Inoue, T. & Mikoshiba, K.

Here, we show that cultured Purkinje cells from inositol 1,4,5-trisphosphate receptor type 1 knock-out (IP_3R1KO) mice exhibited abnormal dendritic morphology. Interestingly, despite the huge amount of IP_3R1 expression in Purkinje cells, IP_3R1 in granule cells, not in the Purkinje cells, was responsible for the shape of Purkinje cell dendrites. We also found that BDNF application rescued the dendritic abnormality of IP_3R 1KO Purkinje cells, and that the increase in BDNF expression in response to activation of AMPA receptor (AMPAR) and metabotropic glutamate receptor (mGluR) was impaired in IP_3 R1KO cerebellar granule cells. In addition, we observed abnormalities in the dendritic morphology of Purkinje cells and in the ultrastructure of parallel fiber-Purkinje cell (PF-PC) synapses in IP_3R1KO mice *in vivo*. We concluded that activation of AMPAR and mGluR increases BDNF expression through IP_3R1-mediated signaling in cerebellar granule cells, which contributes to the dendritic outgrowth of Purkinje cells intercellularly, possibly by modifying PF-PC synaptic efficacy.

B) Cytosolic inositol 1,4,5-trisphosphate dynamics during intracellular calcium oscillations in living cells.

Matsu-ura, T., Michikawa, T., Inoue, T., Miyawaki, A., Yoshida, M. & Mikoshiba, K.

We developed genetically encoded fluorescent inositol 1,4,5-trisphosphate (IP_3) sensors that do not severely interfere with intracellular Ca^{2+} dynamics and used them to monitor the spatiotemporal dynamics of both cytosolic IP_3 and Ca^{2+} in single HeLa cells after stimulation of exogenously expressed metabotropic glutamate receptor 5a or endogenous histamine receptors. IP_3 started to increase at a relatively constant rate before the pacemaker Ca^{2+} rise, and the subsequent abrupt Ca^{2+} rise was not accompanied by any acceleration in the rate of increase in IP_3. Cytosolic [IP_3] did not return to its basal level during the intervals between Ca^{2+} spikes, and IP_3 gradually accumulated in the cytosol with a little or no fluctuations during cytosolic Ca^{2+} oscillations. These results indicate that the Ca^{2+}-induced regenerative IP_3 production is not a driving force of the upstroke of Ca^{2+} spikes and that the apparent IP_3 sensitivity for Ca^{2+} spike generation progressively decreases during Ca^{2+} oscillations.

C) IRBIT specifically binds to and activates pancreas-type Na^+/HCO_3^- cotransporter 1, pNBC1.

Shirakabe, K., Priori, G., Yamada, H., Ando, H., Horita, S., Fujita, T., Fujimoto, I., Mizutani, A., Seki, A. & Mikoshiba, K.

Inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3 Rs) are IP_3-gated Ca^{2+} channels that are located on intracellular Ca^{2+} stores. We previously identified an IP_3R binding protein, termed IP_3R binding protein released with IP_3 (IRBIT). Because IRBIT is released from IP_3R by physiological concentrations of IP_3, we hypothesized that IRBIT is a signaling molecule that is released from IP_3R and regulates downstream target molecules in response to the production of IP_3. Therefore, in this study, we attempted to identify the target molecules of IRBIT, and we succeeded in identifying Na HCO_3 cotransporter 1 (NBC1) as an IRBIT binding protein. Of the two major splicing variants of NBC1, pancreas-type NBC1 (pNBC1) and kidney-type NBC1 (kNBC1), IRBIT was found to bind specifically to pNBC1 and not to bind to kNBC1. IRBIT binds to the N-terminal pNBC1-specific domain, and its binding depends on the phosphorylation of multiple serine residues of IRBIT. Also, an electrophysiological analysis in *Xenopus* oocytes revealed that pNBC 1 requires coexpression of IRBIT to manifest substantial activity comparable with that of kNBC1, which displays substantial activity independently of IRBIT. These results strongly suggest that pNBC1 is the target molecule of IRBIT and that IRBIT has an important role in pH regulation through pNBC1. Also, our findings raise the possibility that the regulation through IRBIT enables NBC1 variants to have different physiological roles.

D) IRBIT suppresses IP_3 receptor activity by competing with IP_3 for the common binding site on IP_3 receptor in a phosphorylation-dependent manner.

Ando, H., Mizutani, A., Kiefer, H., Tsuzurugi, D., Michikawa,T. & Mikoshiba, K.

The inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) are IP_3-gated intracellular Ca^{2+} channels. We previously identified an IP_3R binding protein, IRBIT, which binds to the IP_3 binding domain of IP_3R and is dissociated from IP_3R in the presence of IP_3. In the present study, we showed that IRBIT suppresses the activation of IP_3R by competing with IP_3 by [3H]IP_3 binding assays, in vitro Ca^{2+} release assays, and Ca^{2+} imaging of intact cells. Multiserine phosphorylation of IRBIT was essential for the binding, and 10 of the 12 keyamino acids in IP_3R for IP_3 recognition

participated in binding to IRBIT. We propose a unique mode of IP_3R regulation in which IP_3 sensitivity is regulated by IRBIT acting as an endogenous "pseudoligand" whose inhibitory activity can be modulated by its phosphorylation status.

E) Distinct role of the N-terminal tail of the Na, K-ATPase Catalytic subunit as a signal transducer.

Zhang, S., Malmersjo, S., Li, J., Ando, H., Aizman, O., Uhlen, P., Mikoshiba, K. & Aperia, A.

Mounting evidence suggests that the ion pump, Na, K-ATPase, can, in the presence of ouabain, act as a signal transducer. A prominent binding-motif, linking the Na, K-ATPase to intracellular signaling effectors, has however not yet been identified. Here we report that the Nterminal tail of the Na, K-ATPase catalytic α-subunit (αNT-t) binds directly to the Nterminus of the inositol 1,4,5-trisphosphate receptor ($InsP_3$ R). Three amino acid residues, LKK, conserved in most species and most α-isoforms, are essential for the binding to occur. In wild-type cells, low concentrations of ouabain trigger low frequency calcium oscillations that activate NF-κB and protect from apoptosis. All these effects are suppressed in cells overexpressing a peptide corresponding to αNTt but not in cells overexpressing a peptide corresponding to αNT-tΔLKK. Thus we have identified a well-conserved Na, KATPase motif that binds to the $InsP_3R$ and can trigger an anti-apoptotic calcium signal.

F) Short-term potentiation at the parallel fiber -Purkinje cell synapse.

Goto, J., Inoue, T., Kuruma, A. & Mikoshiba, K.

Changes in synaptic efficacy at the parallel fiber (PF)-Purkinje cell (PC) synapse are postulated to be a cellular basis for motor learning. Although long-term efficacy changes lasting more than an hour at this synapse, i.e., long-term potentiation and depression, have been extensively studied, relatively short lasting synaptic efficacy changes, namely short-term potentiation (STP) lasting for tens of minutes, have not been discussed to date. Here we report that this synapse shows an apparent STP reliably by a periodic burst pattern of homosynaptic stimulation. This STP is presynaptically expressed, since it accompanies with a reduced paired-pulse facilitation and is resistant to postsynaptic Ca^{2+} reduction by BAPTA injection or in P/Q-type Ca channel knockout cerebella. This novel type of synaptic plasticity at the PF-PC synapse would be a clue for understanding the presynaptic mechanisms of plasticity at this synapse.

G) 4.1N binding regions of inositol 1,4,5-trisphosphate receptor type 1.

Fukatsu, K., Bannai, H., Inoue, T. & Mikoshiba, K.

Zhang et al. and Maximov et al. [S. Zhang, A. Mizutani, C. Hisatsune, T. Higo, H. Bannai, T. Nakayama, M. Hattori, and K. Mikoshiba, Protein 4.1N is required for translocation of inositol 1,4,5-trisphosphate receptor type 1 to the basolateral membrane domain in polarized Madin-Darby canine kidney cells, J. Biol. Chem. 278 (2003) 4048-4056; A. Maximov, T.S. Tang, and I. Bezprozvanny, Association of the type 1 inositol (1,4,5)-trisphosphate receptor with 4.1N protein in neurons, Mol. Cell. Neurosci. 22 (2003) 271-283.] reported that 4.1N is a binding partner of inositol 1,4,5-trisphosphate receptor type 1 (IP_3R 1), however the binding site of IP_3R1 differed: the former determined the C-terminal 14 amino acids of the cytoplasmic tail (CTT14aa) as the binding site, while the latter assigned another segment, cytoplasmic tail middle 1 (CTM1). To solve this discrepancy, we performed immunoprecipitation and found that both the segments had binding activity to 4.1N. Both segments also interfered the 4.1N-regulated IP_3R1 diffusion in neuronal dendrites. However, IP_3R1 lacking the CTT14aa does not bind to 4.1N [S. Zhang, A. Mizutani, C. Hisatsune, T. Higo, H. Bannai, T. Nakayama, M. Hattori, and K. Mikoshiba, Protein 4.1N is required for translocation of inositol 1,4,5-trisphosphate receptor type 1 to the basolateral membrane domain in polarized Madin-Darby canine kidney cells, J. Biol. Chem. 278 (2003) 4048-4056.] and its diffusion constant is larger than that of IP_3R1 full-length in neuronal dendrites. We conclude that both the CTT14aa and CTM1 sequences can bind to 4.1N in peptide fragment forms. However, we propose that the responsible binding site for 4.1N binding in full-length tetramer form of IP_3R1 is CTT14aa.

H) ATP autocrine/paracrine signaling induces calcium oscillations and NFAT activation in human mesenchymal stem cells.

Kawano, S., Otsu, K., Kuruma, A., Shoji, S., Yanagida, E., Muto, Y., Yoshikawa, F., Hirayama, Y., Mikoshiba, K. & Furuichi, T.

Human bone marrow-derived mesenchymal

stem cells (hMSCs) have the potential to differentiate into several types of cells. Calcium ions (Ca^{2+}) play an important role in the differentiation and proliferation of hMSCs. We have demonstrated that spontaneous $[Ca^{2+}]i$ oscillations occur without agonist stimulation in hMSCs. However, the precise mechanism of its generation remains unclear. In this study, we investigated the mechanism and role of spontaneous $[Ca^{2+}]i$ oscillations in hMSCs and found that IP_3-induced Ca^{2+} release is essential for spontaneous $[Ca^{2+}]i$ oscillations. We also found that an ATP autocrine/paracrine signaling pathway is involved in the oscillations. In this pathway, an ATP is secreted via a hemi-gap-junction channel; it stimulates the P2Y1 receptors, resulting in the activation of PLC-beta to produce IP_3. We were able to pharmacologically block this pathway, and thereby to completely halt the $[Ca^{2+}]i$ oscillations. Furthermore, we found that $[Ca^{2+}]i$ oscillations were associated with NFAT translocation into the nucleus in undifferentiated hMSCs. Once the ATP autocrine/paracrine signaling pathway was blocked, it was not possible to detect the nuclear translocation of NFAT, indicating that the activation of NFAT is closely linked to $[Ca^{2+}]i$ oscillations. As the hMSCs differentiated to adipocytes, the $[Ca^{2+}]i$ oscillations disappeared and the translocation of NFAT ceased. These results provide new insight into the molecular and physiological mechanism of $[Ca^{2+}]i$ oscillations in undifferentiated hMSCs.

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Department of Basic Medical Sciences

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

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

Eukaryotic pathogens represent some of the most important and complex disease causing agents on a global scale. Apicomplexan protozoa are the most successful parasites in terms of morbidity and mortality; malaria kills over a million people every year and toxoplasma parasite infects one third of the human population. The genetic complexity of these species, once a major hurdle for detailed elucidation, can now be exploited with the advent of genome sequencing. The generation and assemblage of genome sequence provides a plethora of new information for biological investigation. During the past ten years we have been studying genomics of these organisms using full-length cDNA libraries and produced two databases: Full-Parasites that contain the numerous 5'end-one-pass sequences of full-length cDNA libraies of various parasites and Comparasite that contains the homologous Reffull sequences of apicomplexa protozoa. We have also extended our interest to arthropod vectors, including mosquitoes, mites and ticks where co-evolution and complex ecology are to be addressed.

Full-Parasites

Junichi Watanabe, Hiroyuki Wakaguri, Masahide Sasaki, Yutaka Suzuki and Sumio Sugano

Full-Parasites is compilation of full-length cDNA database describing the 5′end-one-pass sequences from various parasites mapped onto their genome sequences and found at http://fullmal.ims.u-tokyo.ac.jp.

Full-malaria *P. vivax* library

Josef Tuda and Mihoko Imada: Sam Ratulangi University, Manado, Indonesia, Keio University

We have produced a full-length cDNA library from the intraerythrocytic-stage parasites and gametocytes of *Plasmodium vivax* using the blood collected from the Indonesian patients. 9,633 5′-end-one-pass sequences were mapped onto the contig genome sequences of *P. vivax*. This is our first full-length cDNA library produced from a clinical sample.

Full-Cryptosporidium

Isao Kimata and Xuenan Xuan: Osaka City University, Obihiro University of Agriculture and Veterinary Medicine

A full-length cDNA library was produced from the hatched sporozoites of *Cryptosporidium parvum* propagated in SCID mice. 5,921 5′end-one-pass sequences were mapped onto the genome sequences and approximately 700 independent genes were represented.

Full-Toxoplasma

James Ajioka, Matt Berriman: Cambridge University, Wellcome Trust Sanger Institute

Toxoplasma gondii is a globally distributed pro-

tozoan parasite that can infect virtually all warm-blooded animals and humans. Despite the existence of a sexual phase in the life cycle, *T. gondii* has an unusual population structure dominated by three clonal lineages that predominate in North America and Europe, (Types I, II and III). These lineages were founded by common ancestors approximately 10,000 years ago. The recent origin and widespread distribution of the clonal lineages is attributed to the circumvention of the sexual cycle by a new mode of transmission: asexual transmission between intermediate hosts. Asexual transmission appears to be multigenic, and although the specific genes mediating this trait are unknown, it is predicted that all members of the clonal lineages should share the same alleles. Genetic mapping studies suggested that Chromosome Ia was unusually monomorphic compared to the rest of the genome. To investigate this further, we sequenced Chromosome Ia and Chromosome Ib in the Type I strain, RH, and the Type II strain, ME49. Comparative genome analyses of the two chromosomal sequences revealed that the same copy of Chromosome Ia was inherited in each lineage, whereas Chromosome Ib maintained the same high frequency of between strain polymorphism as the rest of the genome. Sampling of Chromosome Ia sequence in seven additional representative strains from the three clonal lineages supports a monomorphic inheritance, which is unique within the genome. Taken together our observations implicate a specific combination of alleles on Chromosome Ia in the recent origin and widespread success of the clonal lineages of *T. gondii.*

Full-Eimeria

Damer Blake, Fiona Tomley, Martin Shirley: Institute for Animal Health

A full-length cDNA library was produced from the hatched sporozoites of *Eimeria tenella* propagated in chickens.

Full-Echinococcus

Yuzaburo Oku, Nariaki Nonaka, Jun Matsumoto, Masao Kamiya, Atsushi Toyoda, Yoshiyuki Sakaki: Hokkaido University, Rakuno Gakuen University, Riken

The full-length cDNA library was produced using the Vector-trapper method from hydatid cysts developed in cotton rats that were infected with *Echinococcus multilocularis*. 10,966 5′end-one -pass sequences were compared with the non-redundant database of DDBJ/Genbank/EMBL using BLAST and TBLASTX programs.

Comparasite

Junichi Watanabe, Hiroyuki Wakaguri, Masahide Sasaki, Yutaka Suzuki and Sumio Sugano

Comparasite, an integrated database of our original full-length cDNA sequence data, consists of seven sub-databases of apicomplexa protozoa, *Plasmodium falciparum* (12,484 cDNA sequences), *Plasmodium yoelii* (11,262 sequences), *Plasmodium vivax* (9,633 sequences), *Plasmodium berghei* (1,518 sequences), *Toxoplasma gondii* (7,400 sequences), *Cryptosporidium parvum* (5,921 sequences) and a tapeworm, *Echinococcus multilocularis* (10,966 sequences). Gene families are clustered and comparative analysis of any combination of these seven species is implemented, such as interspecific comparisons for cellular localization, motifs or transmembrane regions and so on. For submitted keywords and other search conditions, Comparasite retrieves orthologous gene groups containing a given protein motif/ GO term etc in common or in a species-specific manner. By enabling multi-faceted comparative analyses of genes of the phylum Apicomplexa, Comparasite should help elucidate the diversity of parasitic mechanisms that have evolved to facilitate the adaptation to various hosts and resulting complexity of life cycles.

Full-Bugs

Sadao Nogami, Shinichi Noda, Chihiro Sugimoto, Masahira Hattori, Aksoy Serap: Nihon University, Kagoshima University, Hokkaido University, The University of Tokyo, Yale University

We have produced full-length cDNA libraries using the Vector-trapper method form various arthropods, including dust mite, *Dermatophagoides farinae* that were propagated in the lab, *Leptotrombidium scutellare* (tate tsutsugamushi) that were collected from the fields, and *Glossina morsitans* (tsetse flies) that were propagated in the lab. 5′end-one-pass sequences and the entire sequences were being determined for further analysis.

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Common Inheritance of Chromosome Ia Associated with Clonal Expansion of Toxoplasma gondii Running head: Common Inheritance of T. gondii Chromosome Ia. Asis Khan 1*, Ulrike Bo _hme 2*, Krystyna A. Kelly 3,4* Ellen Adlem 2, Karen Brooks 2, Mark Simmonds 2, Karen Mun-

gall 2, Michael A. Quail 2, Claire Arrowsmith 2, Tracey Chillingworth 2, Carol Churcher 2, David Harris 2, Matthew Collins 2, Nigel Fosker 2, Audrey Fraser 2, Zahra Hance 2, Kay Jagels 2, Sharon Moule 2, Lee Murphy 2, Susan O'Neil 2, MarieAdele Rajandream 2, David Saunders 2, Kathy Seeger 2, Sally Whitehead 2, Thomas Mayr 3, Xuenan Xuan 5, Junichi Watanabe 6, Yutaka Suzuki 7, Hiroyuki Wakaguri 7, Sumio Sugano 7, Chihiro Sugimoto 5, Ian Paulsen 8, Aaron J. Mackey 9, David S. Roos 9, Neil Hall 2,8, Matthew Berriman 2, Bart Barrell 2, L. David Sibley 1, James W. Ajioka 3† 1 Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO, 63110, USA 2 The Wellcome Trust Sanger Institute, The Wellcome Trust Genome Campus, Hinxton, CB10 1SA, UK 3 Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, UK 4 MRC Biostatistics Unit, Institute of Public Health, Robinson Way, Cambridge, CB2 2SR, UK 5 National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido, 080-8555, Japan 6 Department of Parasitology, Institute of Medical Science, The University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo, 108-8639, Japan 7 Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Japan 8 The Institute for Genomic Research, 9712 Medical Center Drive, Rockville, MD, 20850, USA 9 Department of Biology, University of Pennsylvania, Philadelphia, PA, 19104, USA Genome Res. 2006 Sep; 16(9): 1119-25. Epub 2006 Aug 10.

Comparasite: a database for comparative study of transcriptomes of parasites defined by full-length cDNAs

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RNA no longer stands behind DNA or protein but stands in front of DNA and protein. Recent achievements and discovery in biological science clearly emphasized the importance of RNA in life; the discovery of RNA interference, molecular mimicry between protein and RNA, and ribosome structure at atomic resolution. Moreover, the completed human genome project revealed, to our great surprise, the existence of a large amount of protein-noncoding RNAs (ncRNAs). These ncRNAs can be classified into two types: one, like antisense and microRNA, those function with the sequence complementarity to the target mRNA or DNA, while the other, like aptamer, those function independent of the sequence complementarity. In our laboratory, we aim to: 1) uncover the natural aptamers encoded in human genome; and 2) create artificial aptamers to target proteins of therapeutic interest. By studying these natural and artificial RNA aptamers, we hope to clarify superior potential of RNA, which would be highly beneficial to the development of RNA medicine and the comprehensive understanding of human genome RNA function. In addition to these RNA oriented study, two lines of translation orientated studies are in progress: 1) the molecular mechanism of translation termination and the molecular basis of mimicry between translation factors and tRNA; and 2) the 'prion' nature associated with yeast translation factor Sup35.

1. RNA Aptamers and Therapeutic Applications

Shoji P Ohuchi, Yuki Fujimoto, Toshiro Iwagawa, Yusuke Mori, Akiko Oide, Ritsuko Tanaka-Fujita, Toshie Kawai, Yasuko Yamamura, Shizue Kasanuki, Akihiro Oguro, Kei Endo, Daisuke Ito, Hironori Adachi, Shin Miyakawa[1], Masatoshi Fujiwara[1], Ling Jin[1], Emire Inomata[1], Hisayo Yasumoto[1], Kei Hodoyama[1], Taichi Sakamoto[2], Gota Kawai[2], 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, eIF4AI and eIF4AIII in collaboration with Dr. Nahum Sonenberg (McGill University, Canada) and Dr. John Hershey (UC-Davis, USA). eIF4G and eIF4 AI 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 protein level or the activity of either initiation factor is known to cause cell proliferation. We aim to test the possibility of developing anti-eIF RNA aptamers for novel diagnostic and therapeutic tools.

a. Selection of RNA aptamers against recombinant cell surface receptors

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. Moreover, aptamers raised against purified extracellular portions of target receptors are not always reactive to the native targets in the native conditions. To overcome these problems, 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 are isolating and characterizing RNA aptamers against several human cell-surface receptors or surface antigens that are useful for therapeutic applications. These include transforming growth factor-beta receptors, RANK for the receptor activator of NF-kB, ascular endothelial growth factor (VEGF) receptor.

b. 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 eIF 4G, 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.

2. Translation Termination

Koichi Ito, Miki Wada, Hiroyuki Kodama, Yuya Watanabe, 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. The role of N-terminal domain of translational release factor eRF3 for the control of functionality and stability in *S. cerevisiae*

Eukaryotic release factor eRF1 recognizes all

three stop codons and induces polypeptide release, while eRF3 binds to eRF1 and participates in translation termination. However, the regulatory role of eRF3 is still unknown. Importantly, eRF3 interacts with various proteins of distinct biological functions. Here, we investigated the effect of these binding factors on functionality and stability of eRF3 using a temperature-sensitive mutant eRF3ts, which is susceptible to factor binding to change the growth phenotype or cellular protein level. Of factors tested, Itt1 over-expression and Sla1 knockout severely impaired viability of eRF3ts cell and its protein abundance in permissive and semi-permissive conditions. Sla1 over-expression reversed the phenotype. Itt1 and Sla1 bind to the N-terminal extension domain (NED) of eRF3, unlike the other no-effect factors that bind to the C-terminal domain. Although NED itself is dispensable, NED-less eRF3 altered in the stability and functionality. Moreover, Itt1-induced eRF3ts lethality was significantly restored by *pep4*, *prb1*, and *prc1* knockouts that are defective in vacuolar proteolysis. These findings suggest that NED functions to switch the functional mode of eRF3 depending on the nature of binding factors.

3. Yeast Prion

Hiroshi Kurahashi, Hideyuki Hara, 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. Channel mutations in Hsp104 hexamer distinctively affect thermotolerance and prion-specific propagation

The yeast prion [PSI^+] represents an aggregated state of the translation termination factor Sup35 resulting in the tendency of ribosomes to readthrough stop codons. In this study, we constructed an auxotrophic chromosomal marker, *ura3-197* (nonsense allele), applicable to selection for loss of [PSI^+] to [psi^-]. Unlike [psi^-] yeast strains, [PSI^+] yeast strains exhibit nonsense suppression of the *ura3-197* allele and are not viable in the presence of 5-fluoroorotic acid (5-FOA) that is converted to a toxic material by the readthrough product of Ura3. We selected twenty 5-FOA resistant, loss-of-[PSI^+], mutants spontaneously or by transposon-mediated mutagenesis from *ura3-197* [PSI^+] cells. All of the 20 [psi^-] isolates were affected in Hsp104, a protein-remodeling factor. Although most of them were disabled in a normal Hsp104 function for thermotolerance, three single mutants, L 462R, P557L and D704N, remained thermotolerant. Importantly, L462R and D704N also eliminate other yeast prions [*URE3*] and [PIN^+], while P557L does not, suggesting that Hsp104 harbors a unique activity to prion propagation independent of its function in thermotolerance. The mutations that are specific to prion propagation are clustered around the lateral channel of the Hsp104 hexamer, suggesting a crucial and specific role of this channel for prion propagation.

b. A systematic evaluation by comprehensive chromosomal mutations in the protein-remodeling factor Hsp104 for [*PSI*$^+$] prion propagation in S. *cerevisiae*

Protein-remodeling factor Hsp104 is involved in thermotolerance and [PSI^+] propagation, however the structure-and-function relationship of Hsp104 for [PSI^+] remains unclear. In this study, we engineered 58 chromosomal *hsp104* mutants that affect residues considered structurally or functionally relevant to Hsp104 remodeling activity, yet most remain to be examined for their significance to [PSI^+] in the same genetic background. Many of these *hsp104* mutants were affected both in thermotolerance and [PSI^+] propagation. However, nine mutants were impaired exclusively for [PSI^+], while two mutants were impaired exclusively for thermotolerance. Mutations exclusively affecting [PSI^+] are clustered around the lateral channel of the Hsp104 hexamer. These findings suggest that Hsp104 possesses shared as well as distinct remodeling activities for stress-induced protein aggregates and [PSI^+] prion aggregates and that the lateral channel plays a role specific to [PSI^+] prion propagation.

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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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We have developed novel antibodies that discriminate post-translational modification of proteins such as phosphorylation and limited proteolysis. The powerful immunocytochemical probes visualize emzymatic reactions in situ and enable us to perform biochemical analysis of growing, differentiating and dying cells without any cell sorting.

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

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

a. Establishment of an evaluation method for cleavage-site directed antibodies

Tsuyoshi Katagiri, Chidzuko Takamura and Shinobu Imajoh-Ohmi

Phage display liblaries were used for evaluation of antigenic specificity of cleavage-site directed antibodies. Randomized sequences of synthetic oligonucleotide were introduced into phage DNA in order that a fusion protein with randomized sequences of amino- or carboxyl-terminal region. A library was applied to immobilized antibodies, and phages bound were subjected to sequence analysis for terminal regions.

b. A novel method for hunting substrates of limited proteolysis

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

During the course of study on calpain/calpastatin system in apoptosis we have found that a cleavage-site-directed antibody recognizes a novel molecule unrelated to the expected target protein. To analyze intracellular mobilization of calpastatin antibodies were raised against peptidyl haptens mimicing terminal regions of calpastatin polypeptides generated by caspases. A cleavage-site-directed antibody stained the amino-terminal 30-kDa fragment of recombinant human calpastatin cleaved in vitro by caspase-7. However, calpastatin was not detected by the same antibody in apoptotic cells, suggesting that the calpastatin fragment underwent further degradation. Instead, a 95-kDa polypepted was recognized by immunoblotting with this antibody during apoptosis. The 95-kDa band was seen specifically in apoptotic cells, and diminished in

the presence of caspase inhibitors. Under less stringent conditions a 110-kDa polypeptide was also observed in non-apoptotic cells, but decreased in apoptotic cells in parallel with appearance of the 95-kDa band, suggesting that the 110 kDa protein was cleaved to 95K by caspases during apoptosis. By further structural analysis of the two antibody-stained polypeptides by Edman degradation and mass spectrometry, however, the 110 kDa and 95-kDa polypeptides were identified as APG-2, a member of heat shock protein, and a caspase-cleaved heavy chain of myosin II-A, a non-muscle type myosin, respectively. Furthermore, we found several targets for caspases, some of which remain to be identified, by another type of cleavage-site-directed antibodies.

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

Jin Ling, Hiroyuki Fukuda and Shinobu Imajoh-Ohmi

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

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

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

2. Proteolysis and apoptotic cell death

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

a. 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 pro-

teases, 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.

b. 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.

3. Polo-like kinase 1 is required for mid-body localization of BRCA2 in cytokinesis.

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The breast cancer susceptibility protein, BRCA 2, preserves chromosomal stability through roles in the repair of DNA double-strand breaks, and possibly, cell division. BRCA2 is shown to be phosphorylated by the mitotic Polo-like kinase, Plk1 at the Ser193 residue. It is, however, unknown whether this phosphorylation influences the localization and the function of BRCA2. To address the problem, we raised antibody against phospho-Ser 193, which has the consensus motif for a Plk1 phosphorylation site (Glu/Asp-X-Ser/Thr, where X is any amino acid). Anti-phospho-Ser 193 antibody recognized a single band of the predicted size for BRCA2 on immunoblots of whole cell lysates. The antibodies specifically recognized BRCA2 prepared from mid-body and were phospho-specific, as they did not recognise BRCA2 treated with phosphatase (PP2A). Furthermore, this immunoreactive signal was completely abolished when the antibody was preincubated with the antigen phosphopeptide. These results suggest that the antibody specifically reacts with BRCA2 phosphorylated at S 193. To examine the localization of the phosphorylated form of BRCA2, HeLa cells were transfected with wild type BRCA2 and mutated BRCA2 at Ser193 to Ala, and stained with the anti-pS193 BRCA2 antibody. This antibody reacted with BRCA2 wild type in the cell mid-body, while it did not react with mutated BRCA 2. Therefore, it is likely that the antibody detects the Plk1-dependent phosphorylation of BRCA2 at S193 in the mid-body. In addition, cytokinesis is delayed or prevented when BRCA2 is depleted by siRNA. The length of mid-body is significantly extended in HeLa cells treated with BRCA2 siRNA when compared with controls. This phenotype is rescued by the exogenous expression of BRCA2 wild type, but not BRCA2 mutated at Ser 193 to Ala. We propose that BRCA2 phosphorylation by Plk1 is necessary for the recruitment of BRCA2 to the mid-body, and that the complex formation of Plk1 on BRCA2 may play crucial roles in the regulation of cytokinesis dynamics.

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