Department of Basic Medical Sciences
Division of Molecular Cell Signaling (1)

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

1. Role of the Sho1 transmembrane protein in activation of the yeast stress-responsive Hog1 MAPK pathway

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The adaptive response to hyper-osmotic stress in yeast, termed the high osmolarity glycerol (HOG) response, is mediated by two independent upstream osmosensing pathways that converge on the Pbs2 MAP kinase kinase (MAPKK), leading to the activation of the Hog1 MAP kinase (MAPK). Hog1 is the yeast homolog of the mammalian p38 stress-responsive MAPK. One of the upstream branch involves an osmosensory histidine kinase, termed Sln1, and a multistep phosphorelay reaction that culminates in the activation of the Ssk2 MAP kinase kinase kinase (MAPKKK), which then activates the Pbs2 MAPKK. Another upstream branch is dependent on the Sho1 transmembrane protein. Sho1 is composed of four transmembrane segments (TM1-TM4) clustered in its N-terminal region, and an SH3 domain at the C-terminus. Both its N- and C-termini lie in the cytoplasm, exposing two short segments (one between TM1 and TM2, and another between TM3 and TM4) to the extracellular milieu. The physiologically relevant target of the Sho1 SH3 domain has been identified as the Pbs2 MAPKK. A proline-rich sequence (KPLPPLPVA) that is reminiscent of the consensus SH3-binding sequence was found in the N-terminal non-catalytic domain of Pbs2, and was indeed shown to be the Sho1 binding site. A mutation in the central proline residue in this sequence inhibits the binding of Pbs2 to Sho1, and causes a phenotype that is indistinguishable from the *sho1*Δ mutation, namely the inability to activate Pbs2-Hog1 when the Sln1 pathway is also mutationally inactivated. Thus, interaction between the Sho1 SH3 domain and Pbs2 is essential for Hog1 activation.

Complete deletion of the Sho1 transmembrane segments (thus its SH3 domain is no longer membrane associated) also abolishes Sho1 activity. Replacement of the Sho1 transmembrane segments with another membrane targeting sequence, however, rescued the Sho1 activity. For example, the transmembrane segments from the Ste2 pheromone receptor could functionally substitute the Sho1 transmembrane segments. Furthermore, an N-terminal myristoylation site could also functionally replace the Sho1 transmembrane segments. Thus, membrane targeting of the Sho1 SH3 domain is sufficient for its role in the osmotic stress response. In fact, if the Pbs2

MAPKK is artificially translocated to the plasma membrane by fusing Pbs2 to a transmembrane protein, the need for the Sho1 protein in osmosensing could be circumvented, at least partially. By using Sho1-GFP fusion protein, it was found that Sho1 is localized to sites of polarized growth, such as emerging buds in vegetatively growing cells and shmoo tips in mating cells. Thus, the Sho1 transmembrane segments might be important for localization of Pbs2 at proper subregions in the plasmamembrane, so that the efficiency of Hog1 activation is optimized.

2. Role of Cdc42 GTPase and Ste20 PAK-like kinase in the Sho1-dependent activation of the yeast Hog1 stress-responsive MAPK pathway

Desmond C. Raitt, Francesc Posas and Haruo Saito

In addition to the Sho1 membrane protein, the Ste11 MAPKKK and the Ste11-binding protein Ste50 were known to be required for activating the Hog1 MAPK. In order to identify additional elements that are involved in the Sho1 branch of the HOG pathway, we conducted a systematic mutant screening. As a result, we found that *ste20*Δ cells that have additional mutations in the Snl1 branch exhibited an enhanced osmosensitivity when grown in media containing galactose rather than glucose. Thus, *ssk2*Δ *ssk22*Δ *ste20*Δ mutant cells can grow on YPGlu media containing 1.5 M sorbitol but fail to grow on YPGal media containing 1.5 M sorbitol. Ste20 is a PAK-like protein kinase that binds the the small Rho-type GTPase protein Cdc42. In order to define more precisely the role of Ste20 in the osmotic stress response, we analyzed the ability of various mutant forms of *STE20* to rescue the osmosensitivity caused by a *ste20*Δ mutation. In this regard, two specific domains of Ste20 were found to be essential, namely the C-terminal kinase domain and a region of 36 amino acids between residues 334-369 which is the Cdc42-binding (CRIB) domain.

To further investigate the role of both Ste20 kinase activity and binding of Cdc42 in the osmotic stress response, we directly assayed Hog1 activation in response to osmotic stress by immunoblot analysis. Thus, it was found that activation of Hog1 in response to osmotic shock was dependent on both Ste20 kinase activity and the presence of the Cdc42 binding domain, since Ste20 mutants defective in either kinase activity or Cdc42 binding failed to induce Hog1 activation. In clear contrast, only Ste20 kinase activity, but not the Cdc42-binding, is necessary in the mating pheromone signal transduction.

From these and other results, we propose the following model for the molecular mechanism of the Sho1 osmosensing pathway. The membrane-bound Sho1, through its direct interaction with the Pbs2 MAPKK, anchors the Ste11-Pbs2-Hog1 MAP kinase module to selected regions in the plasmamembrane, namely to the bud during the G1 and S phases and to the shmoo tip in cells treated with mating factor. At the same time, Ste20 binds the small G-protein Cdc42, which is known to localize to the similar regions in the plasmamembrane when it is in the active (GTP-bound) form. Thus, both Ste20 and Ste11 are localized to the same regions in the plasmamembrane, allowing the two proteins to interact with each other. In response to osmotic stress, the membrane-localized Ste20 phosphorylates the membrane-localized Ste11, which then activates the Pbs2 MAPKK and the Hog1 MAPK in the same complex.

3. Identification of a substrate for the yeast stress-responsive Hog1 MAPK

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Osmotic activation of Hog1 MAPK results in induction of a set of osmoadaptive responses, which allow cells to survive in high osmolarity environments. Little is known about how the MAPK activation results in induction of these responses, mainly because no direct substrates for Hog1 have been identified. We conducted a two-hybrid screening using Hog1 as a bait to identify potential substrates for the MAPK, and identified the Rck2 protein kinase as an interactor for Hog1. Both two-hybrid analyses and co-precipitation assays demonstrated that Hog1 binds strongly to the C-terminal region of Rck2. Upon osmotic stress, Rck2 was phosphorylated in a Hog1 dependent manner. Furthermore, purified Hog1 was able to phosphorylate Rck2 *in vitro*. Rck2 phosphorylation occurred specifically at Ser519, a residue located within the C-terminal autoinhibitory domain, and phosphorylation at Ser519 by Hog1 resulted in an increase of Rck2 kinase activity. Overexpression of Rck2 partially suppressed the osmosensitive phenotype of *hog1*Δ cells under osmotic stress, suggesting that Rck2 is acting downstream of Hog1. Consistently, growth arrest caused by hyperactivation of the Hog1 MAPK was abolished by deletion of the *RCK2* gene. Furthermore, overexpression of a catalytically impaired (presumably dominant inhibitory) Rck2 kinase resulted in a decrease of osmotolerance in wild type cells but not in *hog1*Δ cells. Taken together, these data suggest that Rck2 acts downstream of Hog1 controling a subset of the responses induced by the MAPK upon osmotic stress.

4. Regulation of the mammalian stress-responsive MTK1 MAPKKK by GADD45-like proteins

Mutsuhiro Takekawa and Haruo Saito

The mammalian stress-responsive MAPKs (p38 and JNK) are activated by diverse environmental stresses (osmotic stress, UV, X-ray and DNA-damaging reagents). These MAPK pathways play pivotal roles in cellular stress responses such as cell cycle arrest and apoptotic cell death. In addition, recent studies have revealed that the stress-responsive MAPK pathways are involved in inflammatory responses as well as the responses of cancer cells to cytotoxic therapies. We have been studying the regulatory mechanisms of the human stress-responsive MAPK cascades essential for cellular responses to environmental stresses and cytokines.

The mechanism by which the p38 and JNK pathways are activated by environmental stresses is only vaguely defined. Previously, we demonstrated that the mammalian homolog of the yeast Ssk2 MAPKKK, namely MTK1, is involved in stress-induced activation of the p38 and JNK MAPKs. In order to study the regulation of the MTK1 MAPKKK, we also cloned molecules that specifically bind to and activate MTK1. These were identified as three human GADD45-like proteins (GADD45α/β/γ). The GADD45-like genes are induced by environmental stresses, including MMS, UV, and γ-irradiation. More recently, we found that expression of GADD45β was specifically and efficiently induced by TGFβ, and that the timing of the TGFβ-induced p38 activation was almost identical to that of GADD45β induction.

Expression of the constitutively active TGFβ receptor type I activated the p38 pathway, and this activation was further enhanced by coexpression of Smad proteins. More importantly, overexpression of Smad proteins alone was capable of activating the p38 pathway. In addition, the p38 activation induced by the constitutively active TGFβRI was strongly inhibited by expression of the dominant-negative Smad4 mutant. Thus, these findings suggest that TGFβ activates the p38 pathway, at least in part, through Smad-dependent transcription of GADD45β.

5. Roles of protein phosphatase type 2C (PP2C) in down regulation of the stress-responsive MAPK pathways

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We previously revealed that PP2Cα, a member of the serine/threonine specific protein phosphatase type 2C family, has a role in down regulation of the mammalian stress-responsive p38 and JNK MAPK pathways. We believe that the major function of PP2Cα is to maintain the MAPK activities low in the absence of any external stimuli, because the PP2Cα is expressed constitutively and is evenly localized to both the cytoplasm and the nucleus. In contrast, expression of another member of the PP2C family, Wip1, is induced following γ or UV radiation, and is predominantly localized in the nucleus. Expression of Wip1 is dependent on the wild-type p53 gene function. The roles of the Wip1 phosphatase in DNA damage-induced stress responses, however, remained obscure.

In order to elucidate the function of Wip1, we initially carried out Northern blot analyses of *Wip1* gene expression under various stress conditions. Expression of Wip1 mRNA was highly inducible not only by γ or UV radiation, but also by other environmental stresses such as anisomycin, H_2O_2, and methyl methane sulfonate. We also found that UV-induction of Wip1 required p38 activity in addition to the wild-type p53 activity. *In vivo* and *in vitro* experiments showed that ectopic expression of Wip1 inhibits activation of the p38 MAPK pathway, but not the JNK and ERK MAPK pathways, through specific dephosphorylation of a conserved threonine residue in the p38 MAPK. Moreover, Wip1 was found to form a stable complex with p38 *in vivo*.

Recent studies have shown that p38, when activated by genotoxic stresses, enhances p53 activity by phosphorylation and eventually leads to cell cycle arrest or apoptosis. We thus examined whether Wip1 attenuates UV-induced p53 phosphorylation at Ser33 and Ser46, residues previously reported to be phosphorylated by p38. Wip1 expression strongly suppressed UV-induced phosphorylation of p53 on Ser33 and Ser46, but not ser392. Wip1 expression also suppressed both p53-mediated transcription and apoptosis in response to UV radiation. However, purified GST-Wip1 failed to dephosphorylate p53 on Ser33 and Ser46 *in vitro*, suggesting that Wip1 reduces p53 phosphorylation indirectly, possibly by inactivating p38. Thus we propose that p53-dependent expression of Wip1 mediates a negative feedback regulation of p38-p53 signaling and contributes to suppression of the UV-induced apoptosis.

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Department of Basic Medical Sciences
Division of Molecular Cell Signaling (2)

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

1. Proteolysis and cell death

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

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

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

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

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

cal role of calpain in apoptosis are under investigation.

b. Reduced expression of procaspase-8 in Jurkat cells desensitized with cytotoxic anti-Fas antibody

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Human T-leukemic Jurkat cells express Fas antigen and are sensitive to Fas ligand. Agonistic anti-Fas monoclonal antibodies kill Jurkat cells by apoptosis where caspase-8-dependent pathway should be activated by clustering of Fas bound with antibodies. When cultured in the presence of anti-Fas antibody Jurkat cells became resistant to the antibody in two weeks. Cell-suface expression of Fas was not changed as analyzed by flow chtometry. The Fas-desensitized cells was sensitive to staurosporine suggesting that apoptotic machinery via mitochondorial pathway retains. Among apoptosis-related proteins examined expression of procaspase-8 was reduced in the Fas-desensitized cells. Moreover, proteolytic activation of procaspase-8 was not seen upon stimulation of Fas. Quantitative expression of procaspase-8 may be critical for execution of receptor-mediated apoptosis.

c. Limited proteolysis of actin in neutrophils

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

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

2. Phagocytic differentiation and apoptosis

Yuichi Niikura, Ryoko Iizuka, Takashi Nonaka and Shinobu Imajoh-Ohmi

Among phagocytes macrophages are long-lived to play important roles in the defense system of the host after settlement in various tissues. On the other hand, polymorphonuclear leukocytes spontaneously undergo apoptosis. Using a human monoblast U937 cell line we have investigated relationship between differentiation and cell death. The U937 cells differentiate to superoxide anion-producible cells after cultured with interferon γ (IFNγ), 1α, 25 dihydroxy vitamin D3 (VD) or retinoic acid (RA). RA- and VD-differentiated U937 cells seem to be more closely related to mature macrophages than IFNγ-differentiated cells, since RA and VD induce cell surface expression of CD11b. Although RA and VD do not change the expression of Fas antigen and tumor necrosis factor receptor, they desensitize these death receptors in U937 cells after differentiation.

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

When RA-differentiated U937 cells were treated with inhibitors for proteasomes or protein kinases, apoptotic cell death could be induced as well as in undifferentiated and IFNγ-differentiated cells. In such cells proteolytic activity of caspase-3/7 increased and cleavage of PARP was observed. But activation of procaspase-8 remained suppressed in RA-differentiated cells, indicating that activity of this caspase is particularly inhibited in RA-differen-

tiated cells. Interestingly, activation of procaspase-8 was seen in undifferentiated or IFNγ-differentiated U937 cells treated with proteasome inhibitors. Apoptotic signal downstream of caspase-3/7 may come back near the receptor and presumably enhances the death signal. Fas ligand and other death factors are induced by some apoptotic stimuli. On the other hand, procaspase-8 is reported to activated independently of interaction between Fas and Fas ligand. Such positive feedback pathways should function in apoptotic cells.

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

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Upon invasion into tissues bacterial phathogens are phagocytosed by resident macrophages to be killed and digested. Some bacteria can escape into the cytosol and induce cell death of the host cell. *Shigella flexneri* is also reported to induce apoptosis in murine macrophages where a bacterial invasion plasmid antigen B (IpaB) activates a cellular protease triggering apoptotic cell death. However, other investigators observed necrotic cell death in *Shigella*-infected human macrophages derived from peripheral monocytes. Cell death of macrophages caused by bacterial invasion remains to be characterized on the basis of molecular interaction.

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

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

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

a. Cleavage-site-directed antibodies

Ryoko Iizuka, Hiroyuki Mori, Reiji Aihara, Hidehiko Kikuchi, Masahiko Kato, Takashi Nonaka, and Shinobu Imajoh-Ohmi

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

Similar antibodies are now available for *in situ* analysis and detection of apoptosis. Poly(ADP-ribose) polymerase (PARP), an nuclear enzyme catalyzing poly(ADP-ribosyl)ation of nuclear proteins and involved in DNA repair, is known to be

cleaved by caspases during apoptosis in many cells. Cleavage of PARP occurs at a specific site between amino-terminal DNA binding domain and carboxyl-terminal catalytic domain. Although proteolysis of PARP is thought to be not essential for execution of apoptosis, such a specific cleavage may be a hallmark for apoptotic cell death. *In situ* confocal microscopic analysis by means of cleavage-site-directed antibodies for PARP revealed that proteolysis of PARP preceded DNA fragmentation detected by the TUNEL method. Moreover, mobilization of PARP-derived fragments could be seen in individual cells. The DNA binding fragment was left in the nucleus after cleavage but the catalytic fragment was released into the cytosol, suggesting that nuclear proteins did not interact with cleaved PARP.

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.

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Department of Basic Medical Sciences
Division of Neuronal Network

Regulation of gene expression is a main interest in this Division. Over the past decade, molecular and cellular studies of living organisms carried out in many laboratories resulted in the identification of genes, factors and signals involved in the processing, modification, splicing, translation, transport or editing of mRNAs, and uncovered numerous novel mechanisms of gene expression. These accomplishments clearly emphasized the biological importance and interest of the regulatory role of RNA and the mechanisms underlying the post-transcriptional control of gene expression. We aim to clarify these molecular basis from novel aspects in translational control as well as the fate of RNA.

1. Molecular Mimicry between Translational Release Factor and tRNA

Koichi Ito, Makiko Uno, Kuniyasu Yoshimura, Fumiyoshi Sei, 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. Discovery of tripeptide 'anticodon' for stop codon recognition

Bacterial RF1 and RF2 catalyze termination of polypeptide synthesis at UAG/UAA and UGA/UAA stop codons, respectively. How these polypeptide release factors read both non-identical and identical stop codons is puzzling. We have discovered the basis of this recognition. Swaps of each of the seven conserved domains between RF1 and RF2 in an RF1/RF2 hybrid led to the identification of a domain which could switch recognition specificity. A genetic selection among clones encoding random variants of this domain showed that the tripeptides Pro-Ala-Thr in RF1 and Ser-Pro-Phe in RF2 determine release factor specificity *in vivo*. An *in vitro* release study of the tripeptide variants indicated that the first and third amino acids independently discriminate the second and third purine bases, respectively. Further analysis using stop codons containing base analogs suggested that the C-2 amino group of purine is a primary target of discrimination of G from A. These findings demonstrate that the discriminator tripeptide of bacterial release factors is functionally equivalent to that of the anticodon of tRNA, irrespective of the difference between protein and RNA. This finding implies that a tripeptide as well as a trinucleotide can contribute equivalent codon recognition specificity. Our discovery of the discriminator tripeptide, or "peptide anticodon", in bacterial release factors clearly solves the long standing coding problem of how a RF reads a stop codon. Although the peptide anticodon functionally mimics the tRNA anticodon, the structure must be significantly different. Nevertheless, it is important to point out that our experimentally de-

fined discriminator tripeptide agrees with the predictions of our RF-tRNA mimicry hypothesis.

b. The position of release factor 1 in the ribosome determined by directed hydroxyl radical probing

We have made *E. coli* RF1 derivatives in which single cysteine residues are introduced into 12 different (perhaps surface) positions. These cysteines were covalently attached to Fe-mediated hydroxyl radical generator (Fe-BABE) and these tethered proteins were bound to the ribosome in the presence of the stop codon for mapping the position of RF1 in the ribosome by directed hydroxyl radical probing methods. These experiments identify two conserved domains of RF1: one that interacts with functionally important rRNA elements of the small ribosomal subunit surrounding the decoding site, and the other that interacts with rRNA elements of the large subunit involved in interactions with tRNA and other translational factors. The interaction of RF1 with the ribosome is closely similar to that of elongation factor EF-G, which catalyzes translocation, suggesting that the two factors interact with the ribosome in a similar manner while catalyzing distinct mechanisms. These results provide a basis for understanding the mechanism of stop codon recognition coupled to hydrolysis of peptidyl-tRNA, mediated by a protein release factor. These experiments were done in collaboration with Drs. Kevin S. Wilson and Harry F. Noller (University of California, Santa Cruz).

2. Regulation of Ribosome Recycling

a. Functional mapping of ribosome-contact sites in the ribosome recycling factor: a structural view from a tRNA mimic

Toshinobu Fujiwara, Koichi Ito and Yoshikazu Nakamura

After release of nascent polypeptides by the release factor in translation termination, the post-termination complex composed of the ribosome, deacylated tRNA and mRNA needs to be dissociated for the next round of protein synthesis. In bacteria, the decomposition is catalyzed by a ribosome recycling factor (RRF, originally called ribosome releasing factor) probably in concert with the elongation factor EF-G. However, the mechanism of the RRF function is still very poorly understood.

The crystal structure of RRF resembles a tRNA shape (see below), with an architecturally different flexibility compared with tRNA, but its structure-and-function relationships are unknown. We here found that an RRF variant defective in ribosome binding regains the binding capacity through 20 independent secondary changes occurring in three topologically distinct regions of RRF. Since two of these regions are equivalent to the tip of the anticodon stem and the upper surface of the acceptor stem of tRNA, RRF may interact with the ribosome in a way similar to tRNA, spanning 30S and 50S subunits, to exert its action for splitting the ribosome.

b. Interaction of ribosome recycling factor and elongation factor EF-G with *E. coli* ribosomes studied by the surface plasmon resonance technique

Tetsuya Ishino[1], Tohru Yamami[1], Hiroto Hara[1], Kazuteru Yokose[1], and Yoshikazu Nakamura: [1]RRF Research Inc., Tsukuba, Japan

The formation of ribosome complexes on the surface-coupled RRF and elongation factor EF-G of *Escherichia coli* was monitored in real time with a BIACORE 2000 instrument based on the surface plasmon resonance technique. RRF interacted with 70S ribosomes as well as 50S and 30S subunits, although it interacted preferentially with 50S subunits, which was clearly seen under high but physiological ionic conditions. This 50S interaction was diminished by single amino acid substitutions for Arg132 of RRF, which did not affect appreciably the protein folding but nullified the activity *in vivo* and *in vitro*. Moreover, a set of antibiotics that inhibited the RRF-50S interaction were also inhibitory to the polysome breakdown activity of RRF *in vitro*. The BIACORE technique also worked very well in demonstrating the action of antibiotics thiostrepton and fusidic acid, which are inhibitory to the RRF function by freezing the pre- and post-translocation intermediates catalyzed by EF-G. These results suggest that the preferential interplay of RRF with the 50S subunit may be of biological significance, probably reflecting the mode of RRF action. The BIACORE technique proved useful for real-time monitoring of the interaction between the ribosome and translation factors as well as for screening of potential inhibitors for ribosome recycling factor.

3. Yeast [PSI$^+$] 'Prions' Cross-Transmissible and Susceptible beyond a Species Barrier through a Quasi-Prion State

Toru Nakayashiki, Colin Crist, and Yoshikazu Nakamura

The yeast [*PSI*$^+$] element represents an aggregated form of release factor Sup35p and is inherited by a prion mechanism. A 'species barrier' prevents cross-transmission of the [*PSI*$^+$] state between heterotypic Sup35p 'prions'. *Kluyveromyces lactis* and *Yarrowia lipolytica* Sup35 proteins, however, show interspecies [*PSI*$^+$] transmissibility, susceptibility and a high spontaneous propagation rate. Cross seeding was visualized by coaggregation of differential fluorescence probes fused to heterotypic Sup35 pro-

teins. This coaggregation state, referred to as a 'quasi-prion' state, can be stably maintained as a heritable [*PSI*$^+$] element composed of heterologous Sup35 proteins. *K. lactis* Sup35p was capable of forming [*PSI*$^+$] elements not only in *S. cerevisiae* but in *K. lactis*. These two Sup35 proteins contain unique multiple imperfect oligopeptide repeats responsible for cross-transmission and high spontaneous propagation of novel [*PSI*$^+$] elements, serving good models to unravel the molecular basis of interspecies transmission and susceptibility of prion diseases in mammals.

4. Protein Crystallography

Tomohiko Toyoda, Koichi Ito, Masayo Urata, Maria B. Garber[2], Ouliana F. Tin[2], and Yoshikazu Nakamura:[2]Protein Research Institute, Pushchino, Russia

a. Crystal structure of *Thermus thermophilus* RRF

The crystal structure of *T. thermophilus* RRF was determined at 2.6 angstrom resolution. It is a tRNA-like L shape consisting of two domains with a long three-helix bundle (domain 1) and a three-layer β/α/β sandwich (domain 2). Although their domain structures are similar to those of *Thermotoga maritima* RRF (Selmer et al., Science 286: 2349-2352, 1999), the hinge angle between two domains differs by 33° in two species. Altering the hinge angle by alanine substitution for amino acids, including proline, at the hinge region generated both dead and hyper-activated RRF variants, suggesting that the hinge angle or its variability is vital for the action of RRF. Although a tRNA mimic is speculated, the variable axis between domains 1 and 2 of RRF is 90° different from that between the anticodon stem and the acceptor stem of tRNA, and surface electrostatic potentials of two RRF proteins do not mimic the surface potential of tRNA or a tRNA mimic EF-G protein.

b. Crystallography of *Thermus thermophilus* RF-1

Polypeptide release factor one from *T. thermophilus*, ttRF1, was purified and subjected to crystallization. Thin crystalline needles were obtained but their quality was not satisfactory for X-ray diffraction. Stable fragments of ttRF1 suitable for crystallization were screened by limited proteolysis. Three major fragments were produced by thermolysinolysis and analyzed by N-terminal sequencing and electrospray mass spectrometry (in collaboration with Tsutomu Suzuki & Kimitsuna Watanabe - Dept of Chemistry and Biotechnology, University of Tokyo). They were N-terminal fragments generated by proteolysis at amino acid positions 211, 231 and 292. The corresponding recombinant polypeptides, ttRF1^{211}, ttRF1^{231} and ttRF1^{292}, were overproduced and subjected to crystallization. Of these polypeptides, ttRF1^{292} gave rise to crystals that belong to $P3_1$ (or $P3_2$) space group with unit cell parameters a = b = 64.5 Å, c = 86.6 Å and diffract up to 7 Å resolution.

5. RNA Aptamer Selection by SELEX

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

The systematic evolution of ligands by exponential enrichment (SELEX) method is based on the *in vitro* selection of oligo-nucleotide ligands from large random-sequence libraries by repeated reactions of DNA transcription, RNA selection and RT-PCR amplification. The selected oligo-nucleotide ligands are called 'aptamer' which has high affinity and specificity to the target molecule. We have initiated SELEX experiments using mammalian translation initiation factors including eIF4G and eIF4A provided by Dr. Nahum Sonenberg (McGill University, Canada). Several RNA aptamers which acquired the strong and specific binding capacity for these proteins were successfully raised. One class of eIF4A aptamers severely inhibit the ATPase activity, suggesting that they probably dock the catalytic pocket. eIF4G and eIF4A proteins are known to be crucial for catalyzing the initiation of protein synthesis by playing as a multipurpose ribosome adapter bridging eIF4E (cap-binding protein), eIF3 (40S subunit binding protein), eIF4A and Pab1p (poly-A binding protein), and an RNA unwinding helicase, respectively. Importantly, the abnormality in the protein level or the activity of either initiation factor is known to cause cell proliferation. Hence, from the therapeutic point of view, it is of great interest whether or not these RNA aptamers can suppress these malignant cells.

6. Genetic Control of Antigenic Variation of *Pneumocystis carinii*

Miki Wada, and Yoshikazu Nakamura

a. Major surface glycoprotein genes with a novel expression element

Pneumocystis carinii is a fungus which augments in the lungs of immunocompromised patients and causes severe pneumonia. *P. carinii* are coated by abundant surface proteins, named MSGs for major surface glycoproteins, that undergo antigenic variation and play important roles in host-*P. carinii* interaction. There are many MSG genes on the chromosomes but the MSG expression site is restricted to a unique site called type-I UCS and silent MSG genes can be translocated to the expression site by DNA recombination. We have reported previously the existence of alternative UCS element named type-II UCS by MSG transcript analyses (J Infect Dis, 171,

1563-1568 [1995]). In this study we characterized the feature of type-II UCS and MSG genes that are under the control of type-II UCS (hence designated the type-II MSG family) by cDNA and genomic analyses. Contrary to type-I UCS, type-II UCS elements are located at most of the *P. carinii* chromosomes and directly attached to type-II MSG genes. Type-II MSG genes consist of two subtypes in coding sizes of 2.3 kb and 3.3 kb. Antibody raised against the synthetic type-II UCS polypeptide detected products in molecular masses ranging between 90 and 100 kDa. Although the cloned type-II MSG sequences are significantly diverse, cysteine residues that are rich and highly conservative in type-I MSGs are also strictly conserved in type-II MSG proteins, showing the importance of multiple cysteines in the structural integrity of MSGs in the cell surface of *P. carinii*.

b. Immunological characterization of surface subtilisin-like protease

P. carinii express cell surface molecules, MSG (major surface glycoproteins) and SSP (surface subtilisin-like protease). They seem to be functionally related because of their close genomic organization and similar localization on the cell surface. SSP genes are polymorphic and encode proteins homologous to members of the eukaryotic subtilisin-like protease family which cleave prohormones for maturation. We expressed catalytic domain(s) of SSP gene as histidine-tagged protein in *E. coli* and purified by affinity chromatography using Ni-NTA resin. Anti-SSP antibody was raised against the recombinant SSP, and used to examine *P. carinii* proteins by Western blot. As predicted from the SSP cDNA size (i.e., 2.7 kb), the antibody detected approximately 100-110 kDa band, which is close in size to MSG. Although the protein mass of detected SSP was close to that of abundant MSG, they were clearly distinct by 2-dimensional gel electrophoresis analyses.

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Department of Basic Medical Sciences
Division of Fine Morphology

Electron microscopy provides a useful means to investigate the structure of biological macromolecules. If the specimens are properly prepared, It can capture the instantaneous structure of functioning molecules not only in solution but also in live cells, and visualize their details with high contrast. Though the spatial resolution is next to X-ray diffraction, its real unique feature exists in its almost limitless applicability to those whose structure cannot otherwise be pursued.

I. Laboratory of Biomolecular Imaging

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

E. Katayama, N. Ichise and T. Shiraishi

We have been investigating the three-dimensional (3-D) architecture of various kinds of macromolecular machinery which might play crucial roles in a number of cell motility and intracellular signal transduction systems. Some projects are in collaboration with other laboratories, mostly utilizing our expertise technique, the quick-freeze deep-etch replica electron microscopy. With this cryo-EM methodology, all the biological events are instantaneously arrested within one millisecond and the fine structure of individual macromolecules not only in solution but also in cells or tissues can be clearly visualized with high contrast. In the former case, combined use of mica-flakes as protein adsorbent helps greatly to improve the performance of this methodology, so that even the subdomain arrangement in a protein molecule might be recognized. As one of the applications of such techniques, we have been studying the molecular mechanism of muscle contraction by an attempt to capture transient three-dimensional configuration of myosin crossbridges supporting actin movement. Acto-heavy meromyosin (HMM) complex in *in vitro* motility assay systems is a material suitable for such purpose, since a variety of molecular events which were extensively studied under fluorescence microscope can be instantaneously arrested and fine structural features of individual protein molecules under well-characterized experimental conditions can be clearly visualized by metal shadowing (Katayama, 1998). We noticed that the images obtained in such way seem to reveal the detailed surface features of the objects which might enable us to discriminate subdomain arrangement in "individual" protein molecules, a hard subject to attain by any other means. Since replica specimens are extremely tolerant to high-dose electron beam irradiation, it should be possible to take many micrographs of the same field, a mandatory procedure to reconstruct the 3-D structure by a conventional back-projection method. As a preliminary test for such methodology, we reconstructed the 3-D image of single HMM molecule complexed with ADP/inorganic vanadate (Vi) (Katayama et al., 1998) and compared it with the atomic model of S1 obtained by X-ray crystallography (Rayment et al. 1993) or that of smooth muscle S1 motor-domain plus essential light-chain complexed with ADP/AlF_4 to which regulatory chain structure was artificially added (Dominguez et al., 1998). The fundamental subdomain arrangement of HMM-ADP/Vi in our reconstructed image was similar to the second model, as expected, confirming the feasibility of our novel approach. In order to complement the weakness of back–projection method; i.e. impaired resolution along Z-axis due to the “missing-wedge problem”, we employed precise parallax measurement of many reference points on the replica surface to determine

their actual heights (Katayama et al., 1996), and combined the data form both methods for the final processing. Hence, we applied such strategy to examine the structural features of crossbridges that slide actin filaments [in collaboration with Dr. N. Baba's team; Kogakuin University]. In reconstructed 3-D images of actin-associated HMM, the cleft dividing upper and lower 50K domain was evident and the N-terminal β-barrel worked as a good landmark to assign the subdomain arrangement in a molecule. The structure of functioning crossbridges was classified roughly into three categories by the mode of actin-attachment; two weakly-bound configurations and one (probably) strongly-bound. In the first type of weakly-bound configuration, upper 50K-domain was split into two and its tip portion (myopathy domain), seemingly be pulled apart from its main body, was the only connection between the motor-domain and actin filament. In the second weakly-bound form, actomyosin contact was through lower 50K-domain, and the upper 50K appeared as a hook. Here, myopathy domain was directly continued to the main body of the upper 50K-domain. In strongly-bound configuration, crossbridges were linked to actin through above two (possibly more) sites, though the "lever-arm" was often oriented opposite from the putative rigor structure. On the other hand, true rigor structure without nucleotide appeared almost identical to the published docking-model. (Milligan, 1996). It is notable that the rotation angle around the long axis of myosin head differed by about 70 degrees in between true rigor and weakly-bound configurations.

As a complementary approach for the structural study as above, the appearance of the molecule in the replica specimen was simulated using the atomic coordinates of the given molecule, by light-rendering computer program. Though we are still searching for the optimal rendering parameters, tentative results with actin filament and acto-myosin rigor complex show excellent matching with real replica images, suggestive for the feasibility and usefulness of such strategy. Since the atomic model can be substituted by a structure reconstructed by ourselves, we may apply such means to examine the fidelity of our own reconstruction.

Many research groups interpret their experimental results of acto-myosin sliding, along a line of "tilting lever-arm mechanism", though nobody directly observed such scene. Reconstructed 3-D images of myosin crossbridges supporting free and unloaded actin-sliding as observed here may not necessarily be compatible with those expected from that popular hypothesis.

Several studies were carried out in collaboration with Drs. K. Yamane and T. Tsuruo, (Cancer Chemotherapy Center) on the structural aspect of DNA-binding proteins related to tumor-suppression.

The other collaborative studies are proceeding mostly on the structural change accompanied with the function of various motility-related protein stystems. These projects include the characterization of dynein molecules (cytoplasmic and axonemal) in search for the origin of force to slide microtubules (with Dr. C. Shingyoji's team; Dept of Biology, Univ. of Tokyo and Dr. K. Oiwa's team, Kansai Adv. Res. Ctr,), detailed structures of myosin/metallo-fluoronucleotide complex with stabilized intermediate structure (with Dr. S. Maruta's team, Soka Univ.), mutant myosin (with Dr. T.Q.P. Uyeda's team, Nat'l Inst. for Adv. Interdiscip. Res.), structural change of myosin-V (with Dr. T. Yanagida's team, Osaka Unv., and Dr. M. Ikebe's team, Univ. of Massachusetts, and separately with Dr. T. Ando's team), the srtructure of bacterial flagella and needles (with Dr. C. Sasakawa's team, Div. of Bacterial Infection in this Institute and Dr. S.-I. Aizawa's team, Teikyo Univ.).

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II. Laboratory of Fine Morphology

Hirosi Sagara and Emiko Suzuki

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

1. Genetic and molecular anatomy of the Drosophila nervous system

Emiko Suzuki

Genetic analyses of the Drosophila nervous system have demonstrated that the structure of neurons and their connections develop under the control of genetically determined programs. We are interested in how such elaborated programs are carried out at the subcellular level. We are taking three ways of approach to this issue. First, we are searching for the genes which are involved in the development of the nervous system by screening the P-element insertion mutants whose behavioral phenotype is decreased motor activity of adults. This project has been carried out in collaboration with Dr. Chihiro Hama (NCNP) supported by CREST JST. Through this screening, we have luckily found the genes *hig* and *sif*, and recently we cloned the homologue of *sif*, *trio*. HIG protein is a member of Ig super family with complement binding domains. It is secreted into the synaptic cleft and required for the functional synapse formation. SIF and TRIO proteins are GDP-GTP exchange factors which activate Rho-family small G-proteins. SIF-protein induces the formation of the ruffling membranes through Rac-GTPase. Immunoelectron microscopy showed that SIF protein co-localizes with FAS2 to the peri-active zone of the synaptic terminals. These results and the analysis of genetic interaction indicated that SIF functions in synapse morphogenesis in collaboration with FAS2 by reorganization of cytoskeletal elements in the peri-active zones. On the other hand, TRIO protein localized to both axon and neurite terminals. The *trio* loss-of-function mutation severely affects the directional extension of CNS axons, suggesting that TRIO regulates the direction of neurite extension. The function of TRIO protein in the neuronal terminals are under investigation.

The second approach is to visualize the dynamic interaction of pre- and postsynaptic cells by single cell labeling methods. This project was done in collaboration with Dr. Akira Chiba (University of Illinois). The time lapse recording of the live muscle cells visualized by the genetic labeling of membrane targeted GFP revealed the random extension and retraction of the muscle filopodia, myopodia, prior to the arrival of projecting axons. When the axonal growth cone reached the target muscles, the myopodia and the neuro-filopodia clustered at the sites of contacts and changed their shape into lamellipodia so as to make tight adhesion. The electron microscopy of the labeled neurons revealed the partner-specific adhesion of the myopodia and growth cones. To analyze the regulatory mechanism of such cell-specific interaction, we have done the ectopic expression experiments of the target recognition molecules. When Fas 3, a "positive" target recognition molecule for RP3 neuron, was ectopically expressed in neighboring muscles, the RP3 growth cone developed tight adhesion to the Fas III misexpressing non-target muscles. In contrast, when Toll, a "negative" target recognition molecule normally expressed by neighboring non-target muscles, is misexpressed on target muscles, 6 and 7, the selective association of the myopodia from these muscles with RP3 neuron was blocked. From these observations, we propose that postsynaptic cells are not merely passive acceptors and that, by expressing specific target recognition molecules, they dynamically regulate both their own and the presynaptic cells' structures as well as their selective adhesion.

The third approach is to study how the signaling molecules which ensure the proper networking and the morphology of synaptic connections are assembled at the proper subcellular sites. We have chosen photoreceptor cells as a model system for studying these aspects. Drosophila photo-transduction is a phosphoinositide(PI)-mediated and calcium-regulated signaling cascade. We have revealed that many of the proteins involved in the PI metabolism, ex. eye-diacylglycerol kinase (eye-DGK), phosphatidylinositol transfer protein (PITP), CDP- diacylglycerol synthase (CDS), are concentrated on the subrhabdomeric cisternae (SRC), the intracellular calcium pool adjacent to photoreceptive rhabdomeres. These findings strongly suggest that PI-metabolism and calcium signalling are topologically coupled on the membrane organella. In addition to the topological coupling of signaling cascade on SRC, we have found association of the principal components of phototransduction on a PDZ-domain protein, INAD, in the photoreceptive microvilli. This work was done in collaboration with Dr.Charles S. Zuker (University of California). We have shown that INAD binds TRP (the major membrane ion channel), INAC (eye-protein kinase C that is required for the termination of phototransduction), and NORPA (PLCβ which initiates the PI cascade of phototransduction) by specific PDZ domains. The binding of these proteins to INAD protein was necessary for their proper subcellular localization. These topological couplings of signaling molecules were essential for the phototransduction, indicating that many cellular events require the assembly of the proper molecules at the proper subcellular sites. Then how such spatial regu-

lation is achieved genetically? To answer this question, we are analyzing the mechanism of assembly and anchoring of these signaling molecules by the expression studies using germ-line transformation. The studies on mutated RDGA (eye DGK) proteins revealed the involvement of the cystein-rich zinc-finger domains in the protein targeting and the enzyme activity of RDGA protein. And the studies on INAD, showed the independent anchoring and assembly mechanisms of INAD complexes. These studies proved that the *in vivo* expression studies are very powerful for the analysis of protein targeting in the nervous system.

2. Vitamin A metabolism in vertebrate and invertebrate visual organs

Hirosi Sagara and Emiko Suzuki

In spite of the same photoreceptive function, structures of the photoreceptive organs of vertebrates and invertebrates are quite different. However, use of the same vitamin A derivative, 11-cis retinal, as a chromophore of the visual pigment, rhodopsin, and the induction of the eye under the control of the same homeobox gene Pax/eyeless in both vertebrates and invertebrates indicates that the same basic mechanisms are involved in the formation and maintenance of the photoreceptive organ.

In the vertebrate eye, the retinal pigment epithelium (RPE), a single layer of cells adjacent to the photoreceptor cells, has the vital role in visual acuity. Its main functions include the synthesis of 11-cis retinal, which are then transported to the photoreceptor cells to produce rhodopsin. In the course of clarifying the functions of RPE cells, we have prepared several monoclonal antibodies (MAbs) against RPE cells. Among these, three (S5D8, S5H8 and Y3H) were proved to recognize a same molecule specifically expressed on the membrane of endoplasmic reticulum of the RPE cells. Phylogenetic analysis of the reactivity of these MAbs to the eye of various vertebrates species indicated that the antigenic protein, RPE65, is highly conserved among vertebrates and that it is very likely to have the function related to the retinoid cycle. In favor of this notion, the genetic knock out of RPE65 carried out by other group showed that the RPE65 deficient retina accumulates all-trans retinyl ester (Redmond et al., 1998). In spite of the growing circumstantial evidence, no one has demonstrated the precise function of RPE65. To solve this problem, we have decided to clone a homologue of RPE65 in *Drosophila melanogaster* which has great advantage in genetic manipulation. The striking similarity of the amino acid sequence of RPE65 from fish to humans also encouraged us to search for the invertebrate homologues of RPE65. Indeed we have found a gene homologous to vertebrate RPE65 by screening of cDNA library from adult Drosophila heads. The putative Drosophila RPE65 (DRPE65) had 35% -37% homology to vertebrate RPE65s and was specifically expressed in the eyes. The protein sequence alignment revealed several regions highly conserved among Drosophila, putative gene of *C.elegans*, vertebrate RPE65s, β-carotene dioxygenase, and plant 9-cis-epoxylcarotenoid dioxygenase. In parallel to our investigation, von Linting and Vogt (2000) also cloned DRPE65 and found that it has β-carotene dioxygenase activity. From these results, we would like to propose that RPE65-related proteins constitute a carotenoid- and retinoid-metabolizing proteins family throughout the animal and plant kingdom.

Another project concerning the zebrafish eye development is being carried as a collaboration with Dr.Sumiko Watanabe at Division of Molecular and Developmental Biology.

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

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

Takayuki Michikawa, Mitsuharu Hattori, Junji Hirota[1], Maki Yamada[2], Miki Yamamoto-Hino[2], Kozo Hamada[2], Kazushi Yamauchi[2], Keiko Ohata[2], Chihiro Hisatsune[2], Akira Futatsugi[2], Akinori Kuruma[2], Hiroko Bannai[2], Toshifumi Morimura[2], Hirohide Iwasaki, Tsuyoshi Uchiyama, Hiroshi Miyauchi, Wei-Hua Cai, Rei Yokoyama, Keiko Uchida, Shuhei Tomita, Tomohiro Nakayama, Hideaki Ando, Zhang Songbai, Zhou Hong, Takafumi Inoue, and Katsuhiko Mikoshiba:[1]Mikoshiba Calciosignal Net Project (JST) and [2]Laboratory for Developmental Neurobiology, Brain Science Institute, The Institute of Physical and Chemical Research (RIKEN)

Inositol 1,4,5 trisphosphate (IP3) is a second messenger produced through the phosphoinositide turnover in response to many extracellular stimuli (hormones, growth factors, neurotransmitters, neurotrophins, odorants, lights, etc.), and controls a variety of Ca^{2+}-dependent cell functions (cell proliferation, differentiation, fertilization, embryonic development, secretion, muscular contraction, immune responses, brain functions, chemosensory, light transduction, etc.) by inducing Ca^{2+} release (IP3-induced calcium release; IICR) from intracellular store sites such as endoplasmic reticulum (ER) to cytoplasm. IP3 binds to its specific receptor (IP3R) on the Ca^{2+} store sites. IP3R is an IP3-gated Ca^{2+} release channel, and could be considered as a signal converter that exchanges the IP3 signal to the Ca^{2+} signal that physiologically acts on a wide variety of targets. Our goal in this research is to elucidate the structure-function relationships of the IP3R, and the physiological roles of IP3R-mediated Ca^{2+} signaling in various cell-types.

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

Ca^{2+} signaling via IICR often exhibits dynamic changes in time and space inside a cell (known as Ca^{2+} waves and oscillations). These complex spatiotemporal patterns are not produced by simple diffusion of cytoplasmic Ca^{2+}. The essential ingredients to generate repetitive Ca^{2+} spikes are positive feedback, cooperativity, deactivation (including negative feedback) and reactivation. Recently, we found that the positive feedback regulation by cytoplasmic Ca^{2+} is an intrinsic property of the cerebellar IP3R1, whereas the negative feedback regulation by Ca^{2+} is

mediated by calmodulin, a ubiquitous and multifunctional Ca^{2+}-dependent regulator protein. In nonneuronal cells such as *Xenopus* oocytes, IP3 is a global messenger that liberates Ca^{2+} throughout cytoplasm, but in cerebellar Purkinje cells, repetitive activation of parallel fiber-Purkinje cell synapses causes Ca^{2+} release that is restricted to individual postsynaptic local domains. The spatially restricted IICR might contribute the input specificity of the synaptic plasticity observed in the parallel fiber-Purkinje cell synapses. Our data suggests that the negative feedback regulation of Ca^{2+} mediated by calmodulin determine the spatial and temporal patterns of Ca^{2+} signaling in cerebellar Purkinje cells by limiting the amount of Ca^{2+} being released.

The coupling mechanism between ER Ca^{2+} stores and plasma membrane (PM) store-operated channels (SOCs) is crucial to Ca^{2+} signaling but has eluded detection. SOCs may be functionally related to the TRP family of receptor-operated channels. Direct comparison of endogenous SOCs with stably expressed TRP3 channels in human embryonic kidney (HEK293) cells revealed that TRP3 channels differ in being store independent. However, condensed cortical F-actin prevented activation of both SOC and TRP3 channels, which suggests that ER-PM interactions underlie coupling of both channels. A cell-permeant inhibitor of IP3R function, 2-aminoethoxydiphenyl borate (2-APB), prevented both receptor- induced TRP3 activation and store-induced SOC activation. It was concluded that IP3Rs mediate both SOC and TRP channel opening and that the IP3R is essential for maintaining coupling between store emptying and physiological activation of SOCs.

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

Takafumi Inoue, Kunio Kato[1] Akira Futatsugi[2], Akinori Kuruma[2] and Katsuhiko Mikoshiba

Analysis of the functional roles of the Ca^{2+} signaling in mammalian brain is one of the most focused topics in our research. We have shown that a neuronal IP3R1-deficient mouse strain generated by gene-targeting technique exhibit significant reduction of birthrate and abnormal behavior (ataxia and seizure). We found that cerebellar slices prepared from IP3R1-deficient mice completely lack long-term depression (LTD), a model of synaptic plasticity in the cerebellum. Moreover, a specific antibody against IP3R1, when introduced into wild-type Purkinje cells through patch pipettes, blocked the induction of LTD. These data indicate that, in addition to Ca^{2+} influx through Ca^{2+} channels on the plasma membrane, Ca^{2+} release through IP3R plays an essential role in the induction of LTD in Purkinje cells. In CA1 area of hippocampus, long-term potentiation (LTP) and LTD are well established models of synaptic plasticity. Although the IP3R1-deficient mice showed normal LTP and LTD induced by standard tetanus (100Hz, 1 sec) and low-frequency stimuli (1000 pulses at 1Hz), respectively, they showed facilitated LTP induced by a short tetanus (100 Hz, 100 ms) stimulation. We found that reduction of postsynaptic calcium influx by partial blockade of NMDA (N-methyl-D-aspartate) receptors results in a conversion of LTP to LTD and a loss of input specificity normally associated with LTP, with LTD appearing at heterosynaptic inputs. The induction of LTD at homo- and heterosynaptic sites required functional ryanodine receptors and IP3R1, respectively. In the IP3R1-deficient mice we found a conversion of LTD to LTP and elimination of heterosynaptic LTD, whereas blocking ryanodine receptors eliminated only homosynaptic LTD. Thus, postsynaptic Ca^{2+}, deriving from Ca^{2+} influx and differential release of Ca^{2+} from internal stores through ryanodine receptors and IP3R1, regulates both the polarity and input specificity of activity-induced synaptic modification.

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

Takeo Saneyoshi, Taro Okamoto, Shoen Kume[1], Akira Muto[1], Takafumi Inoue, and Katsuhiko Mikoshiba

The phosphatidylinositol (PI) cycle has been postulated to function in dorso-ventral (D-V) axis formation of many species, as indicated by the action of lithium. Lithium is assumed to block the recycling of IP3 into inositol by inhibiting the hydrolysis of intermediate inositol phosphates. Application of lithium to cleavage stage embryos of *Xenopus laevis* induced dorsalization by conversion of ventral mesoderm to dorsal mesoderm, with a concomitant reduction in posterior structures. To determine the role of the PI cycle in patterning the body plan, we isolated functional blocking monoclonal antibodies (mAbs) against the *Xenopus* IP3R (XIP3R). Ventral injection of these monoclonal antibodies at 4-cell stage induced the formation of a secondary dorsal axis, whereas dorsal injection of the mAbs or normal mouse IgG showed no obvious effects. This implies that blockage of IICR in the ventral part of *Xenopus* embryos converted ventral mesoderm to dorsal mesoderm, thereby generating an ectopic dorsal axis. These results indicate that an active IP3-Ca^{2+} signal is required for ventral differentiation. To ask which upstream signaling pathways play roles in the ventral differentiation, we introduced a constitutively active Gαq mutant construct (GαqQ-L) in *Xenopus* early embryos. Prolonged stimulation of IP3- Ca^{2+} signaling by overexpression of GαqQ-L led to desensitization of IICR. This desensitization of IICR on the ventral side specifically induced an ectopic dorsal axis due to the conversion of ventral marginal meso-

derm to adopt a dorsal fate. Then, we examined effects of a panel of inhibitory antibodies against Gαq/11, Gαs/olf, or Gαi/o/t/z. While all these antibodies showed direct inhibition of their targets, their effects on redirection of the ventral mesoderm to a dorsal fate varied. Anti-Gαs/olf antibody showed strong induction of dorsal fate, anti-Gαi/o/t/z antibody did so weakly, and anti-Gαq/11 antibody was without effect. Injection of βARK, a Gβγ inhibitor, mimicked the dorsalizing effect of the anti-Gαs/olf antibody, whereas injection of adenylyl cyclase inhibitors did not do so. The activation of Gαs-coupled receptor gave rise to Ca^{2+} transients. All these results suggest that activation of the Gαs-coupled receptor relays dorsoventral signal to Gβγ, which then stimulates PLCβ and then the IP3- Ca^{2+} system. This signaling pathway may play a crucial role in transducing ventral signals.

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

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

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Review

Birnbaumer L., Boulay G, Brown D, Jiang M, Dietrich A, Mikoshiba K, Zhu X and Qin N: Mechanism of capacitative Ca^{2+} entry (CCE): Interaction between IP3 receptor and TRP links the internal calcium storage compartment to plasma membrane CCE channels. Recent Progress in Hormone Research

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

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

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

a. Transposition of a restriction modification gene complex

Seishi Ohashi, Yoko Ui, and Ichizo Kobayashi

Comparison of bacterial genomes supports the hypothesis that restriction modification (RM) gene complexes are mobile elements involved in various genome rearrangements and genome evolution. We now demonstrated transposition of a type II RM gene complex carried by a plasmid to the recipient *E. coli* chromosome under a condition of post-segregational host killing. Sequence analysis of the RM-*E. coli* genome junctions revealed several types of transposition events.

b. Evolution of sequence recognition by restriction modification systems

Akito Chinen, Yasuhiro Naito, Naofumi Handa, Ichizo Kobayashi

Several type II restriction-modification (RM) gene complexes kill host bacterial cells that have lost them, through attack on their chromosomal recognition sites. Two RM gene complexes recognizing the same sequence cannot simultaneously enjoy such stabilization through post-segregational host killing, because one will defend chromosomal sites from attack by the other. We analyzed intra-host competition between two RM gene complexes when the recognition sequence of one is included in that of the other. When the *Eco*RII gene complex, recognizing 5′CCWGG (W = A, T) is lost from the host, the *Sso*II gene complex, which recognizes 5′CCNGG (N = A, T, G, C) will prevent host death by protecting CCWGG sites on the chromosome. However, when the *Sso*II (CCNGG) gene complex is lost, the *Eco*RII (CCWGG) gene complex will be unable to prevent host death through attack by *Sso*II on 5′CCSGG (S = C, G) sites. These predictions were verified in our experiments in which we analyzed plasmid maintenance, cell growth, cell shape and chromosomal DNA. Our results demonstrate the presence of selective pressure for decrease in the specificity of sequence recognition by RM systems in the absence of any invading DNA.

c. C5 DNA methylase defends the genome from attack by a restriction modification gene complex

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

Some bacteria carry "orphan" DNA methyltransferase that is not paired with a restriction enzyme. Dcm in some strains of *E. coli* K-12 recognizing CC(A/T)GG provides one example. Its role has not been clear. We demonstrated that Dcm serves as molecular vaccine that protects the genome from post-segregational killing programmed by *Eco*RII, an RM system recognizing the same sequence. Strong cell killing after loss of *Eco*RII RM was moderated by *dcm* in the chromosome and completely eliminated by *dcm* overexpressed from a plasmid.

d. Genome rearrangements driven by selfish behavior of a restriction modification gene complex on the *E. coli* chromosome

Naofumi Handa, Youichi Nakayama, Naomi Saita and Ichizo Kobayashi

We examined whether an attempt of replacement of a chromosomal RM gene complex by a homologous stretch of DNA --- its competitor --- encounters resistance through chromosome restriction breakage and cell killing. We tried to replace *Pae*R7I RM gene complex inserted in *E. coli* chromosome by a homologous stretch of *Pae*R7I-modified DNA in general transduction. The efficiency of apparent replacement was decreased by R^+. To our surprise, many of these recombinant clones retained the recipient RM gene complex as well as the donor allele but slowly lost the donor allele in the absence of selection. Further analysis suggested that unequal homologous recombination between IS3 copies at distant locations has resulted in duplication of a region encompassing the RM region, and that only one of the RM copies was replaced by the donor DNA. Multiple rounds of IS-IS recombination in each of these clones had resulted in large-scale genome rearrangements.

e. Resistance of a restriction modification gene complex and a linked gene on *Bacillus subtilis* chromosome to replacement

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We obtained evidence for selfish maintenance of *Bam*HI gene complex and a linked gene on *Bacillus subtilis* chromosome. A pBR322 derivative carrying *Bam*HI gene complex ($m^+c^+r^+$) and neomycin phosphotransferase gene were inserted into a *met* locus of *B. subtilis* chromosome. We then tried to replace them by a linear homologous stretch of pBR322 carrying spectinomycin resistance gene by natural transformation. The efficiency of apparent replacement was lower, and the resulting transformant colonies were smaller in the r^+ cells than in the r^- cells. Moreover, some of these clones retained the recipient RM gene complex and neomycin phosphotransferase gene as well as the donor spectinomycin resistant gene. They have rearranged their genome in a specific way.

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

Asao Ichige and Ichizo Kobayashi

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

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

Noriko Kobayashi-Takahashi, Ichizo Kobayashi, Nanae Kotake[3], Hiroko Funaki[3], Masanori Watahiki[3]: [3]Nippongene

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

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

a. Genome comparison between *Helicobacter pylori* strains suggests that restriction modification gene complexes are mobile genetic elements

Ayaka Nobusato, Ikuo Uchiyama[4], Seishi Ohashi, Ichizo Kobayashi:[4]National Institute for Basic Biology, Okazaki National Research Institutes

Comparison of genome sequences of two closely related cellular organisms, two *Helicobacter pylori* strains, at single base pair level suggested the presence of a novel mechanism for bacterial gene mobility --- insertion with long target duplication. It is formally similar to transposon insertion, but the target duplication is much longer. Restriction and/

or modification enzyme gene homologues are often within or adjacent to the insertion. This genome rearrangement is proposed to have resulted from attack of restriction enzyme on the chromosome. Horizontal transfer of restriction modification genes in *H. pylori* is suggested from analysis of their GC contents, codon use bias and sequence alignments.

b. Comparison between *Pyrococcus horikoshii* and *Pyrococcus abyssi* genome sequences reveals linkage of restriction-modification genes with large genome polymorphisms

Akito Chinen, Ikuo Uchiyama[4] and Ichizo Kobayashi: [4]National Institute for Basic Biology, Okazaki National Research Institutes

The complete genome sequences of *Pyrococcus horikoshii* and *Pyrococcus abyssi*, two species in a genus of hyperthermophilic archaea, were compared to detect large genome polymorphisms linked with restriction-modification gene homologues. Sequence alignments, GC content analysis, and codon usage analysis demonstrated the diversity of these homologues and revealed a possible case of relatively recent acquisition (horizontal transfer). In two cases out of the six large polymorphisms identified, there was insertion of a DNA segment with a modification gene homologue, accompanied by target deletion (*simple substitution*). In two other cases, homologous DNA segments carrying a modification gene homologue were present at different locations in the two genomes (*transposition*). In both cases, substitution (insertion/ deletion) in one of the two loci was accompanied by *inversion* of adjacent chromosomal segment. In the fifth case, substitution by a DNA segment carrying type I restriction, modification, and specificity gene homologues was likewise accompanied by adjacent inversion. In the last case, two homologous DNA segments, were found at different loci in the two genomes (*transposition*), but only one of them had insertion of a modification homologue and an unknown ORF. The possible relationship of these polymorphisms to attack by restriction enzymes on the chromosome was considered.

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

Ikuo Uchiyama[4], Toshio Higuchi[5], Ichizo Kobayashi: [4]National Institute for Basic Biology, Okazaki National Research Institutes and [5]INTEC Web and Genome Informatics Cooperation

Recently, complete sequences of two or more microbial genomes that are closely related to each other have been determined, and detailed comparison of such genomes becomes a useful approach for elucidating principles and mechanisms of genome evolution. For such an analysis, it is required not only to compare the sequences themselves but also to compare various aspects of sequence features, combining with the alignment of the genomic sequences. To this goal, we are developing a comparative genome analysis tool (CGAT) for closely related microorganisms. In this tool, an user can compare several feature segments identified in each genome by various sequence analysis programs, by overlaying them onto the alignment of homologous or orthologous segments identified by all-against-all homology search.

d. Coevolution of bacterial restriction modification systems and bacteriophages --- a theoretical study

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We constructed a mathematical model for population dynamics of bacteria carrying various restriction modification systems and bacteriophages carrying various restriction sites. We looked for conditions for maintenance of many restriction modification systems in a bacterial genome.

3. Recombination machines --- homologous, site-specific and illegitimate --- in interaction with restriction modification systems and other elements

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

a. Bacterial homologous recombination in fight and collaboration with selfish restriction modification genes

Naofumi Handa, Asao Ichige, Kohji Kusano and Ichizo Kobayashi

Several rec genes were found to defend bacteria against the RM gene complexes by repairing cleaved chromosomes, as detected by pulsed-field gel electrophoresis. Genetic and physical analyses indicate that the interaction of Chi sequence with RecBCD enzyme, plays an important role. RecBCD enzyme destroys restricted "non-self" DNA but repairs restricted "self" DNA marked by Chi sequence. RecF pathway, the other recombination pathway in *E. coli*, also resisted the post- segregational killing.

b. Chi-star, one of a class of sequences defining the *E. coli* recombination hotspot chi

Deana A. Arnold[8], Naofumi Handa, Ichizo Koba-

yashi, Steve C. Kowalczykowski[8]:[8]University of California at Davis

In wild-type *Escherichia coli*, recognition of the recombination hotspot, chi (5′-GCTGGTGG-3′), by the RecBCD enzyme is central to homologous recombination. However, in the *rec*C star class of RecBCD mutants, stimulation of recombination by the canonical chi sequence is not detectable, but the levels of homologous recombination are nearly wild-type in some assays. *In vivo* studies demonstrate that a member of this class of mutants, the *recC1004* allele, encodes an enzyme that responds to a novel variant of chi, termed chi-star (5′-GCTGGTGCTCG-3′). We established that, *in vitro*, the chi-star sequence is recognized more efficiently by the $RecBC^{1004D}$ enzyme than is the wild-type chi. This is manifest by both a greater modification of nuclease activity and a higher stimulation of RecA protein-mediated joint molecule formation at chi-star than at chi. In addition, we showed that this novel, eleven-nucleotide chi-star sequence also regulates the wild-type RecBCD enzyme, supporting the notion that variants of the canonical chi constitute a class of sequences that regulate the recombination function of RecBCD enzyme

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

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

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

d. Double-strand-break-repair type homologous recombination by bacteriophages

Noriko Kobayashi-Takahashi and Ichizo Kobayashi

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

e. Alleviation of restriction by bacteriophage recombination functions

Naofumi Handa and Ichizo Kobayashi

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

f. Spontaneous accumulation of large linear forms of Escherichia coli chromosome

Naofumi Handa and Ichizo Kobayashi

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

g. Homology-associated non-homologous recombination

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

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

h. Random-walk model for interference in meiotic recombination

Youhei Fujitani[9], Shintaro Mori[10], Junji Kawai[9], and Ichizo Kobayashi:[9]Department of Applied

Physics & Physico-Informatics, Faculty of Science & Technology, Keio University and [10]School of Science, Kitasato University

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

4. Basic studies for gene therapy by mutation correction

Asami Ino and Ichizo Kobayashi

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

5. Analysis of genes and genomes in bacteria and yeast

a. Hda, a novel DnaA-related protein, necessary for the regulation of the replication cycle in *E. coli*

Jun-ichi Kato and Tsutomu Katayama[11]: [11]Department of Molecular Microbiology, Kyushu University Graduate School of Pharmaceutical Sciences

The ATP-bound DnaA protein binds to the origin of replication on the *E. coli* chromosome to initiate DNA replication. DnaA belongs to a chaperone ATPase-related family, AAA+, as do certain other proteins essential for the initiation of DNA replication in eukaryotes. We have identified a novel AAA+ member, Hda, involved in the conversion of the active ATP-bound DnaA protein to the inactive ADP-bound form *in vivo* and *in vitro*. The *hda* gene is required for cell viability by preventing over-initiation of chromosomal replication. We propose that the once-per-cell-cycle rule of replication depends on the timely interaction of AAA+ proteins that comprise the apparatus regulating the activity of the initiator.

b. Extension of the functions of *E. coli* database, "PEC (Profiling of *E. coli* chromosome)"

Yukiko Yamazaki[12], Takehiro Yamakawa[12], Tohru Ikegami, and Jun-ichi Kato:[12]Center for Genetic Resource Information, National Institute of Genetics

The functions of the database "*Profiling of Escherichia coli chromosome* (PEC) database < http://shigen.lab.nig.ac.jp/ecoli/pec/>" has been extended to compile any relevant information that could help to characterize the *E. coli* genome, especially with respect to discovering the function of each gene. Some overviews are also provided. The database is intended to provide an interface comprehensible to most experimental researchers.

c. Systematic identification of novel essential genes of *E. coli*

Jun-ichi Kato and Yasuko Imai

To clarify the minimal gene set of cellular organisms, we are going to identify all of *E. coli* genes essential for cell growth. We identify novel essential genes by complementation of the chromosomal regions, which could not be deleted in the project of systematic construction of deletion mutants. About ten genes have been identified so far and the analyses of their functions are in progress.

d. Fission yeast Mog1p homologue, which interacts with the small GTPase, Ran, is required for mitosis-to-interphase transition and poly(A)$^+$ RNA metabolism

Kazuo Tatebayashi, Tokio Tani[13] and Hideo Ikeda[14]:[13]Graduate School of Science, Kyusyu University and [14]Center for Basic Research, Kitasato Institute

We have cloned and characterized the *Schizosaccharomyces pombe* gene *mog1*$^+$, which encodes a protein with homology to the *Saccharomyces cerevisiae* Mog1p participating in the Ran-GTPase system. *mog1*$^+$ is essential for cell viability and required for the mitosis-to-interphase transition, as the *mog1-1* mutant arrests at restrictive temperatures as septated, binucleated cells with highly condensed chromosomes and an aberrant nuclear envelope. Surprisingly, unlike Δ*mog1* mutation in *S. cerevisiae*, the *mog1-1* mutation causes nucleolar accumulation of poly(A)$^+$ RNA at the restrictive temperature in *S.*

pombe, but the signals do not overlap with the fibrillarin-rich region of the nucleolus. Thus, we found that *mog1*$^+$ is required for the mitosis-to-interphase transition and a class of RNA metabolism.

e. Isolation and characterization of the fission yeast mutants which are aberrant in chromosome segregation and sensitive to radiation and hydroxyurea

Kazuo Tatebayashi

To understand molecular mechanisms of crosstalk among DNA replication, DNA repair and mitosis, I have isolated three mutants of fission yeast that exhibit defective chromosome segregation and sensitivity to radiation and hydroxyurea. Among the mutants, mutant 199 shows pleiotropic phenotypes. In addition to defective chromosome segregation and sensitivity to radiation, the mutant 199 cells are sensitive to Na^+ or K^+, and become swollen or misshaped at high temperature. Interestingly, the temperature-sensitivity is rescued by the fission yeast homolog of Arp9p, which is shared in two chromatin-remodeling complexes, RSC and Swi/Snf, suggesting that the gene 199 may be involved in various cellular processes through chromatin-remodeling.

f. Genome analysis of methicillin-resistant Staphyloccoccus aureus, a major hospital pathogen

Makoto Kuroda[15], Toshiko Ohta[16], Ikuo Uchiyama[4], Tadashi Baba[15], Harumi Yuzawa[15], Ichizo Kobayashi, Longzhu Cui[15], Akio Oguchi[17], Kenichi Aoki[17], Yoshimi Nagai[17], JianQi Lian[15], Teruyo Ito[15], Mutsumi Kanamori[16], Hiroyuki Matsumi Kanamori[16], Hiroyuki Matsumaru[16], Atsushi Maruyama[16], Hiroyuki Murakami[16], Akira Hosoyama[17], Yoko Mizutani-Ui, Noriko Kobayashi, Toshihiro Tanaka[17], Toshihiro Sawano[17], Ryu-ichi Inoue[18], Chikara Kaito[18], Kazuhisa Sekimizu[18], Hideki Hirakawa[19], Satoru Kuhara[19], Susumu Goto[20], Junko Yabuzaki[20], Minoru Kanehisa[20], Atsushi Yamashita[10], Kenshiro Oshima[10], Keiko Furuya[10], Chie Yoshino[10], Tadayoshi Shiba[10], Masahira Hattori[21], Naotake Osagasawa[22], Hideo Hayashi[23], Keiichi Hiramatsu[15] : [4]National Institute for Basic Biology, Okazaki National Research Institutes, [10]School of Science, Kitasato University, [15]Department of Bacteriology, Juntendo University, [16]University of Tsukuba, College of Medical Technology and Nursing, [17]National Institute of Technology and Evaluation, [18]Graduate School of Pharmaceutical Sciences, University of Tokyo, [19]Faculty of Agriculture, Kyushu University, [20]Institute for Chemical Research, Kyoto University, [21]Human Genome Research Group, RIKEN Genomic Sciences Center, [22]Nara Institute of Science and Technology, Graduate School of Biological Sciences, Institute of Casic Medical Sciences, [23]University of Tsukuba

Staphylococcus aureus is one of the major pathogens causing both community-acquired and hospital-acquired infections. It produces variety of toxins that elicit both regional and systemic inflammation in human body. Many of the toxins are known as superantigens that cause unique disease entities such as toxic shock syndrome and staphylococcal scarlet fever. *S. aureus* possesses extreme flexibility in adapting to antibiotic pressure, and has acquired resistance to practically all antibiotics so far introduced in clinical practice. Whole genome analysis is a necessary step towards future development of countermeasures for the infection caused by *Staphylococcus aureus*.

Whole genome sequences of two related *Staphylococcus aureus* strains N315 and Mu50 were determined by shot-gun random sequencing strategy. N315 and Mu50 were determined by shot-gun random sequencing strategy. N315 is an MRSA strain isolated in 1982. Mu50 is an MRSA strain with vancomycin resistance (MIC=8 mg/l) isolated in 1997. The open reading frames (*orfs*) were identified by using GAMBLER and GLIMMER programs, and annotation of each *orf* was performed using BLAST homology search, motif analysis, and protein localization prediction (PSORT)

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

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

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小林一三：細菌ゲノムを造り替えていた制限酵素修飾酵素遺伝子．蛋白質核酸酵素、臨時増刊号「ゲノムサイエンスの新たなる挑戦」刊行中．

Department of Basic Medical Sciences
Division of Molecular and Developmental Biology

Our long-term goal is to understand the molecular mechanisms which coordinately regulate growth and differentiation of metazoan cells with emphasis on intracellular signal transduction, transcription and DNA replication. For this purpose, we are using systems ranging from E. coli, yeasts, frog, zebrafish, and culture cells of lymphocytic and hematopoietic lineages as well as animal models. The major research areas of interest are on: 1) transcriptional regulation of Th1 and Th2-specific cytokine genes in activated T cells, 2) roles of cytokines and their receptors in growth and differentiation, 3) regulation of DNA replication and cell cycle 4) roles of nuclear structures in differentiation and development and 5) development of sensory organs. On the basis of these efforts, we intend to develop technologies to manipulate growth and differentiation of various stem cells with high fidelity, which is important for cell and gene therapy.

I. Regulation of lymphocyte differentiation and activation by transcription factors

CD4 naive precursor T cells are the population of CD4 positive T cells that have matured in thymus and have never been exposed to real antigens. They are characterized by low CD44 and high CD62L. Recognition of antigens by naive precursor T cells leads to extensive proliferation and production of cytokines. Among various cytokines, activated naive precursor T cells produce mainly IL-2. Concomitantly, activated naive precursor T cells differentiate into one of the two T helper subsets, Th1 and Th2. Th1 and Th2 cells are characterized by the spectrum of cytokines they produce. Th1 cells are one of the important producers of interferon γ (IFNγ) and lymphotoxin (LT), while Th2 cells produce Interleukin (IL)-4, IL-5, IL-6, IL-10 and IL-13. Resting T helper subsets are characterized by high CD44 and low CD62L and they are designated as memory CD4 T cells. Th1 cytokine, such as IFNγ, is crucial for the activation of macrophages and cellular immune responses against intracellular pathogens. Th2 cytokines such as IL-4, IL-5 and IL-13 regulate B cell activation, antibody production and differentiation and activation of eosinophils. Thus, Th2 cells play important roles in protection against extracellular pathogens, especially helminth parasites. However, inappropriate activation of these T helper subsets can cause various immunological disorders. Many organ specific autoimmune diseases such as rheumatoid arthritis and multiple sclerosis are suggested to be triggered by the Th1 cells recognizing tissue-specific self antigens. Activation and expansion of Th2 cells against particular antigens are probably the most critical culprits of allergic responses such as asthma and allergic dermatitis.

The differentiation of T helper subsets requires DNA replication and cell proliferation. In addition, it is regulated by various parameters incurred by stimuli, such as the strength and nature of the signal through TCR, type of costimulatory signals, type of antigen presenting cells including dendritic cell subsets etc. Among various conditions, two cytokines are crucial to regulate the process. IL-12 produced by antigen presenting cells skew the differentiation process toward Th1 cells, while IL-4 is effective to induce Th2 cells. Our study is centered on the role of

transcription factors such as STAT6, GATA3 and NFAT family proteins inTh2 differentiation pathway, with particular focus on their role in the remodeling of chromatin structures during induction of Th2 subset specific gene expression. Furthermore, we are studying the role of STAT6 in other tissues in allergic responses.

1. Chromatin remodeling of the mouse IL-4 and IL-13 loci in Th2 differentiation

Naofumi Takemoto[1], Shoichiro Miyatake[1], Yumiko Kamogawa[1], Miho Nagoya, Sahori Namiki[1], Naoko Arai[2] and Ken-ichi Arai[1]:[1]CREST (Japan Science and Technology) and [2]Department of Immunology, DNAX Research Institute

Many cytokine genes are located on the human chromosome 5 and its syntenic region is found on the mouse chromosome 11. Three of the Th2 specific cytokine genes, IL-4, IL-13 and IL-5 are clustered on these chromosomes. IL-4 and IL-13 genes are only 12 kb apart, whereas IL-5 and IL-13 genes are separated by rad50 gene which is about 120 kb long. We identified three DNase I hypersensitive sites (HSS) in the intergenic region of IL-4 and IL-13 genes. Two of them, HSS1 and HSS2 are detected in both resting and activated Th2 cells but not in naive precursor cells and Th1 cells. The appearance of these Th2 specific HSS is not associated with the actual transcription of IL-4 or IL-13 genes, suggesting that these HSS reflect Th2 cell-specific chromatin structures. We could show that both Th2 differentiation and the induction of Th2-specific chromatin structures can be achieved by activation of STAT6 or exogenous expression of GATA3 even in the Th1 skewing condition. These data strongly suggest that GATA3 induced by STAT6 is the key regulator for Th2 specific chromatin remodeling. One putative GATA binding site was identified within the region containing HSS2. The binding of GATA3 to this site *in vitro* was shown by EMSA. We also identified a Th2-specific DNA binding complex which recognizes a putative AP-1 binding site within the region containing HSS1. Since AP-1 DNA binding activity can be detected in both Th1 and Th2 cells, this Th2 specific complex is different from the known AP-1 complexes. Identification of the functional domains in GATA3 has been carried out by introducing various mutant GATA3 into *in vitro* differentiating precursor T cells. We showed that one of the two zinc fingers, located closer to N terminus, is dispensable for the expression of IL-4 but not for IL-5. Th2 specific HSS are also induced by the GATA3 mutant lacking this finger. Since it was reported previously that this zinc finger as well as other fingers of GATA3 are required for its DNA binding activity, DNA binding activity of GATA3 may not be required for remodeling of the Th2 cell-specific chromatin structures. On the other hand, transcription of IL-5 may require GATA3 DNA binding activity. Further biochemical analyses of GATA3 and associating molecules are in progress to clarify the role of GATA3 in chromatin remodeling.

2. The role of STAT6 in Th2 differentiation and the pathology of airway hyperresponsiveness

Yumiko Kamogawa[1], Shoichiro Miyatake[1], Miho Nagoya, Sahori Namiki[1], Naofumi Takemoto and Ken-ichi Arai[1]

IL-4 is a critical cytokine for the differentiation of Th2 cells which may trigger allergic responses. It has been shown that IL-4 and IL-13, Th2 specific cytokines, may play important roles in the pathology of asthma not only through induction of Th2 cells but also through directly affecting airway tissues such as airway epithelial cells and smooth muscle cells. STAT6 is an exclusive signaling molecule which functions downstream of IL-4 and IL-13 receptors. Thus, it is one of the attractive drug targets to suppress both IL-4 and IL-13 pathways, thereby reducing Th2 differentiation and suppressing the direct effects of these cytokines on airway tissues. This may lead to reduced airway hyperresponsiveness.

We have been utilizing STAT6-hormone binding domain of estrogen receptor fusion protein (STAT6-ER) to elucidate the role of STAT6. Estrogen analog 4-hydroxy tamoxifen (4-HT) can induce dimerization of the hormone binding domains, thereby resulting in the dimerization and activation of STAT6 without affecting other signaling pathways functioning in the downstream of IL-4 or IL-13 receptors. We could show that up-regulation of CD23 and MHC II in B cells can be induced by the activation of STAT6 alone. In addition, the differentiation of Th2 cells can be induced by the activation of STAT6 together with TCR signaling. We introduced STAT6-ER into airway epithelial cell lines and detected the induction of several chemokines upon STAT6 activation, suggesting a critical role of STAT6 in IL-4 and IL-13 receptor signaling in airway epithelium. To analyze the role of STAT6 in the intact animal, STAT6-ER transgenic mice are being produced.

Dominant negative STAT6 fused to the hormone binding domain of the estrogen receptor (dnSTAT6-ER) was also constructed. We could show that biological effects of STAT6 described above are suppressed in 4-HT dependent manner when dnSTAT6-ER was exogenously expressed. The suppressing effect of dnSTAT6-ER was not observed in other STAT family pathways, suggesting that the specificity is maintained in spite of the structural similarity of STAT family proteins. We are trying to produce transgenic mice expressing dnSTAT6-ER in order to evaluate its suppressing activity on various biological responses involving STAT6, especially allergic responses and airway hyperresponsiveness.

3. The differential functions of NFAT family proteins in T cell activation

Jingtao Chen, Shoichiro Miyatake[1] and Ken-ichi Arai[1]

NFAT family proteins have been shown to be required for the transcriptional activation of various cytokine genes in mature T cell lines, where three NFAT genes, NFATp, NFATc and NFATx are expressed. NFATp is the most abundant, while NFATc is upregulated by the activation through TCR and the expression level of NFATx is relatively low. Potent immunosuppressants, cyclosporin A (CsA) and FK506, exert their functions through inhibiting Ca-dependent phosphatase, calcineurin, thereby suppressing the activation of NFAT proteins. However, gene disruption experiments of each NFAT family member did not reveal absolute requirements of NFAT proteins for the transcriptional activation of various cytokine genes, suggesting that each NFAT protein may have overlapping functions. Furthermore, these experiments have suggested inhibitory functions of NFATp and NFATx in cytokine gene expression in T cells.

Deletion analysis of the NFAT homology domain (NHD) of NFATx lead to generation of a constitutively active mutant whose transcriptional activity is comparable to that of the activated wild type protein. To elucidate specific functions of NFATx in normal T cells, transgenic mice expressing this constitutively active NFATx in T lineage cells were produced. CD4 T cells isolated from the transgenic mice can express several cytokines including IL-2 and proliferate by PMA treatment alone. In addition, the cytokine production and proliferation are resistant to CsA treatment. These data strongly suggest that NFATx can stimulate transcription of several cytokine genes and cell proliferation. We are applying the same strategy to other NFAT family members to analyze their specific functions. NFATx is expressed at a high level in double positive population in thymus. The number of double positive thymocytes in NFATx -/- mice was reduced, indicating the requirement of NFATx for the survival of double positive thymocytes. Analysis of thymocytes from this transgenic mice expressing a "gain-of-function" mutant will complement the data from a "loss-of-function" mutant and may reveal additional roles of NFATx in thymic development.

II. Signaling mechanisms of cytokine receptors and during development

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

1. Nuclear transport of Stat5

Zeng Rong, Yutaka Aoki, Saori Sato[1], Ken-ichi Arai[1] and Sumiko Watanabe

Stats play various critical roles in cytokine and growth factor signaling. In response to factor stimuli, Stats are phosphorylated and translocate to the nucleus, followed by activation of target genes. A portion of the Stats are degraded but most are dephosphorylated and relocate back to the cytoplasm where it can be reactivated. Using Ba/F3 cells expressing the hGM-CSF receptor (hGMR), we found that nuclear export of Stat5B by factor-depletion was inhibited by leptomycin B (LMB), a specific inhibitor of nuclear export receptor CRM1. CRM1 binds to target proteins through the leucine-rich short stretch termed NES (nuclear export sequence). Mutation of putative NES of Stats resulted in disruption of translocation of Stat5B to cytoplasm after cytokine depletion. Interestingly, addition of LMB in the absence of cytokine lead to accumulation of Stat5B in nucleus in Ba/F3 and COS7 cells, suggesting that Stat5B shuttles between nucleus and cytoplasm in the CRM1-dependent manner regardless of cytokine stimulation. The mutant Stat5B lacking tyrosine 699, which is essential for Stat dimerization, behaves as the wild type, suggesting that shuttling between nucleus and cytoplasm in the absence of GM-CSF occurs in a monomer state. Using a series of mutant Stat5B, we identified a part of coiled coil (amino acids 138-165) as a critical region for monomer nuclear import. Interestingly, the N-terminal 104 amino acids are essential for dimer import of Stat5B. Taken together, we propose that two different import systems and the CRM1-dependent export system are coupled for regulation of Stat subcellular localization under various physiological states.

2. Cell cycle dependent interaction of Mad2 with conserved box1/2 region of hGM-CSF receptor common βc

Mitsuo Takeda[1], Ken-ichi Arai[1] and Sumiko Watanabe

Box1 is a conserved motif in the membrane proximal region of cytokine receptors, including the GM-CSF receptor. Studies using mutants of the hGMR βc as well as dominant negative Jak2 showed an essential role of Jak2 in hGMR signaling through its interaction with the box1 region of βc. Since overexpression of the wild type Jak2 inhibits hGM-CSF-induced proliferation, molecules other than Jak2 interacting with

the box1 region may play roles in cell proliferation. Therefore, we searched for molecules interacting with the box1/2 region by pull-down assay using GST fused with the βc box1/2 region and the Ba/F3 cell lysate. Microsequence of a protein which associates with the box1/2 region but not with the hGMR α cytoplasmic region revealed that it is the mouse homologue of Mad2 protein which plays an important role in regulation of cell division. Peptides corresponding to the box1/2 region sequence also bind to Mad2 and a mutation of the conserved amino acid of box1 decreased Mad2 interaction. Deletion analysis of Mad2 indicated that interaction with box1/2 occurs through the C-terminal portion of Mad2. Association of Mad2 with other proteins is known to be regulated during cell cycle. Indeed, its binding affinity to box1/2 increased in the late M phase. These results suggest a possibility that GM-CSF regulates the M phase checkpoint through interaction with Mad2.

3. Cloning of factors involved in anti-apoptosis or proliferation using dimerization libraries

MD Golam Mohi, Ken-ichi Arai[1] and Sumiko Watanabe

We showed previously that Jak or Stat can be activated by induced dimerization using GyraseB/coumermycin or ER/tamoxifen dimerization system. It now appears that many signaling molecules or transcription factors are activated by dimerization. Therefore, we applied these inducible dimerization systems to isolate new genes involved in factor-dependent cell proliferation or anti-apoptosis. For this purpose, we constructed GyrB or ER fused cDNA library in retroviral vectors. These cDNA libraries were introduced into Ba/F3 cells and the clones were selected on the basis of their ability to support factor-independent proliferation or survival. The cDNA inserts were rescued from the isolated clones by PCR using primers designed from the retroviral LTR promoter regions. The cDNA inserts were then sequenced, and are now being analysed by transfection into Ba/F3 cells.

4. Analyses of the promoter regions of the human IL-3 receptor α chain (IL-3Rα) and GM-CSF receptor α chain (GM-CSFRα) genes

Eiji Akagawa, Ken-ichi Arai[1] and Sumiko Watanabe

The receptors for IL-3 and GM-CSF consist of α and β subunits. Using transgenic mice expressing hGMR, we previously showed that features of biological activities of cytokines are determined primarily by expression pattern of their ligand binding unit, α subunit, rather than specificity of signal transduction pathways of each cytokine. We have analyzed mechanism of transcriptional regulation of the hIL-3R α and hGMR α subunits. RT-PCR analysis showed different expression pattern of IL-3 and GM-CSF receptors in various cell lines. We determined sequences of the 1.5 kb genomic DNA spanning the promoter region of IL-3Rα gene. 5'-RACE and primer extension analyses indicated the presence of multiple transcription start sites. Using transient transfection assays, two regions (-363 ~ -331 and -124 ~ -76) of the hIL-3Rα promoter have been implicated in promoter enhancing activity. Electrophoresis mobility shift assays revealed specific binding of unknown protein(s) and Sp1, respectively, to these regions, suggesting roles of these proteins in hIL-3Rα gene induction. On the other hand, in the 2.5kb hGMR α promoter segment, the promoter-proximal PU-1 binding site was found to have transcriptional enhancing activity. These results suggested distinct mechanisms of transcriptional activation for expression of the IL-3 and GM-CSF receptors.

5. Analyses of transgenic mice expressing mutant hGM-CSF receptor

Eishun Muto, Yutaka Aoki, Koichiro Tsuji[3], Ken-ichi Arai[1] and Sumiko Watanabe:[3]Division of Division of Cellular Therapy, Institute of Medical Science, University of Tokyo

Using Tg mice expressing hGMR (wild-Tg), we previously showed that hGM-CSF is not a GM-lineage promoting factor but a strong proliferation promoting factor of all the lineages examined. GM-CSF induced not only GM colonies, but also the erythroid colonies were observed within bone marrow cells of wild-Tg mice. In contrast, thymocyte of wild-Tg mice ceased development at the pre-T cell stage by the addition of GM-CSF, and thus it is speculated that the GM-CSF may negatively regulate T cell development at a certain stage in wild-Tg mice. To analyse signaling requirement for these activities, we generated Tg mice expressing Fall hGM-CSF mutant receptor, in which all the cytoplasmic tyrosine residues were replaced by phenylalanine. Methyl cellulose assays of bone marrow cells from Fall-Tg, wild-Tg and their litter mate showed that Fall induced lower but still significant numbers of myeloid colonies. Interestingly, no erythroid colonies were observed in BM cells of Fall-mice. *In vivo* administration of hGM-CSF to wild-Tg mice resulted in disappearance of double positive cells in thymocyte, whereas no effects were seen with Fall-Tg mice thymocyte under the same condition. *In vitro* FTOC culture further confirmed these results. Taken together, our results suggest that signaling pathways stimulated through the tyrosine residues of hGMR may be required for erythroid colony formation and inhibition of thymocyte development.

6. Role of Stat3 in eye development

Akihiko Muto, Ken-ichi Arai and Sumiko Watanabe

Stat3 is a transcription factor known to be activated by various cytokines. We found that Stat3 protein was expressed in mouse lens and retina and the expression pattern in retina dramatically changed during development. In lens, Stat3 was mainly detected in epithelial cells in any stages. On the other hand, Stat3 was ubiquitously expressed in whole retinal cells at early developmental stage (E11), whereas in later stages (from E15 to P1) its expression became significant in the inner (ganglion) layer. In adult mice, Stat3 was exclusively detected in inner and outer plexiform layers, ganglion layer, nerve fiber and retinal inner segment. To analyze the role of Stat3 in eyes, we generated retina or lens-specific stat3-KO mice by using the Cre-loxP system. Morphology of the eyes and the expression of several retinal cell-type specific markers in the KO-mice were indistinguishable from those in control littermates. More detailed functional analyses are in progress.

7. Alteration of eye development by lens-specific expression of diphtheria toxin A

Ryo Kurita, Rika Ishida, Chiaki Abe, Hiroshi Sagara[4], Ken-ichi Arai[1] and Sumiko Watanabe: [4]Division of Fine Morphology, Institute of Medical Science, University of Tokyo

To understand the role of lens in development of other modules of eye, we designed a series of experiments in which to perturb lens development by expressing genes of interest under the control of the αA-crystallin promoter. We isolated the zebrafish counter part of αA-crystallin coding region by degenerate PCR and used this fragment to clone the 5′ promoter region by screening genomic libraries. We fused the 2.5 kb 5′ promoter region with EGFP (αAcrys-EGFP). Injection of the αAcrys-EGFP construct at the two cell stage revealed that this segment is sufficient for lens-specific expression of EGFP. Using this assay system, we mapped a minimal region responsible for lens-specific expression. This region contains a sequence homologous to that of 5′ promoter region of the chicken αA-crystallin gene which includes a L-maf binding site. To perturb lens formation, we expressed diphtheria toxin A fragment under the regulation of the isolated αA-crystallin promoter. A plasmid containing αA-crystallin promoter-diphtheria toxin A was injected into eggs at the two cell stage. At 54 hours post fertilization, structural abnormalities of the lens was observed. Several vacuoles were observed within lens epithelial cells and an abnormal spaces were detected between the lens epithelium and fibers. Detailed phenotypes of the lens as well as other modules of the eye and the expression pattern of diphtheria toxin A are being investigated.

III. Studies on regulation of DNA replication

DNA replication is one of the central events of cell growth, and needs to be precisely regulated to ensure coordinated and ordered duplication of the entire genome within a specific period of the cell cycle, namely S phase. It is also under strict regulation of various external signals such as growth/differentiation factors and DNA damaging agents. Ordered assembly of protein complexes at replication origins is likely to be prerequisite for initiation of DNA replication in eukaryotes as well, as has been demonstrated for prokaryotic replicons. Recent studies in yeasts and frog eggs indicate cell cycle stage-specific assembly of replication-competent complexes during G1 phase. These complexes, termed prereplicative complexes (preRC), need to be "triggered" for DNA replication to be initiated. This triggering signal appears to be activated by cell cycle signals during G1 phase or by growth factor stimulation. The Cdc7-Dbf4 kinase and Cyclin-dependent kinase complexes are known to play essential roles to fire the origins.

It is well established that Cdks are conserved widely in eukaryotes and play critical roles at various stages of cell cycle. *Saccharomyces cerevisiae* Cdc7 kinase is essential for initiation and progression of S phase. Its kinase activity, which is controlled by the regulatory subunit Dbf4, peaks at the G1/S boundary. This oscillation of Cdc7 kinase activity is caused mainly by altered abundance of the Dbf4 protein which is regulated at both transcriptional and post-translational levels. We previously showed that Cdc7-related kinases and their regulatory subunits are conserved in higher eukaryotes including human, mouse and frog. This suggests that the regulatory mechanisms of initiation of DNA replication by Cdc7-related kinases may be conserved among all eukaryotes.

This year, we tried to elucidate how Cdc7 and Cdk may collaborate to achieve precise regulation of G1-S transition. We have obtained two possibly important results suggestive of collaboration and close interaction of these two essential kinases. First, phosphorylation of MCM2, the critical subunit of Cdc7, is facilitated by prior phosphorylation of critical serine residues on MCM2 by Cdk. Second, early embryonic lethality of mouse Cdc7 knockout can be partially rescued by elevating Cdk activity. These results indicate collaboration of the two kinases for initiation of S phase.

We are also interested in modes of DNA replication observed after ongoing replication forks are stalled. We already reported that *E. coli* PriA protein, a Zinc finger helicase, plays crucial roles in recombi-

national repair of double-stranded DNA breaks. PriA protein, widely conserved in eubacteria, specifically binds to structures resembling recombination intermediates or stalled replication forks. On the basis of detailed structure-function studies of PriA, we ultimately hope to find a protein with similar activity from eukaryotes in order to obtain clues on modes of replication that occur on damaged DNAs or at stalled replication forks.

1. Structure and function studies of ASK, the regulatory subunit for mammalian Cdc7 kinase

Noriko Sato, Megumi Sato[1], Ken-ichi Arai[1] and Hisao Masai

Human Cdc7 (huCdc7)-ASK kinase complex, the human homologue of budding yeast Cdc7-Dbf4, is essential for chromosomal DNA replication in mammalian cells. ASK, whose expression fluctuates during cell cycle, binds and activates the huCdc7 catalytic subunit. In order to elucidate how huCdc7 kinase is regulated, we have generated a series of ASK mutants and analyzed their functions. We have identified three stretches of amino acids, namely Dbf4-motif-N, -M and C, which are conserved among the known Dbf4-related molecules. Small amino acid stretches containing either Dbf4-motif-M or Dbf4-motif-C can bind to huCdc7. For activation of huCdc7, the 177 amino acid region of ASK (minimum ASK) containing only motif-M and motif-C is sufficient. Mammalian ASK possesses a long C-terminal tail, which is not found in the yeast counterparts. This region can also bind to huCdc7 with high affinity and contains a cluster of autophosphorylation sites at its C-terminus. Cell cycle-specific phosphorylation of human MCM2 causes its characteristic mobility shift on SDS-PAGE during S to G2 phases. Overexpression of huCdc7 and ASK, either full-length or the minimum form, in COS7 cells can cause similar mobility shift of the endogenous MCM2. Furthermore, overexpression of minimum ASK in 293 cells enhances endogenous Cdc7 kinase activity. Currently, we are investigating the effect of overexpression of various mutant ASKs on DNA synthesis and cell cycle progression of mammalian cells.

2. Growth regulation of ASK, a regulatory subunit for human Cdc7-related kinase, is mediated by a 65 base-pair segment containing putative Sp1 sites and unknown repressive elements

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Transcription of ASK, a regulatory subunit for human Cdc7 kinase, is repressed in quiescent cells and is induced by growth stimulation during the cell cycle progression. In order to analyze modes of the transcriptional regulation of the ASK gene, we have isolated the promoter region of the ASK gene, and showed that it lacks a canonical TATA box but contains a cluster of E2F and Sp1 binding motifs near the transcription start sites. Using the luciferase reporter gene, we confirmed the induction of promoter activity in serum-stimulated NIH3T3 cells in transient transfection assays. Cotransfection of a plasmid expressing an E2F transcription factor into Ba/F3 cells, mouse IL-3 dependent proB cells, together with this reporter plasmid led to promoter activation without IL-3 stimulation. Furthermore, endogenous ASK mRNA was induced by infection of a recombinant adenovirus expressing E2F1. However, introduction of point mutations into putative E2F binding sites resulted in only slightly reduced response to serum stimulation and ectopically expressed E2F1, and did not result in constitutive activation, which is often observed with other E2F-regulated promoters. Promoter analyses using various deletion constructs have indicated that the most proximal Sp1 site plays a critical role in responses to E2F1 as well as to serum stimulation. These results suggest that transcription of the ASK gene during the transition from quiescence to proliferation is regulated by cooperation of E2F and Sp1. Furthermore, the 63 bp segment containing the essential Sp1 site and the major transcription initiation site, which is highly conserved between human and mouse, is sufficient for responses to serum stimulation and E2F.

The most striking property of E2F proteins is that they can drive quiescent cells into S phase. In order to examine whether deregulated expression of ASK protein can induce S phase in quiescent cells, we generated stable cell lines constitutively expressing ASK protein. The stable cell lines overexpressing ASK protein after factor depletion showed a population of S phase cells greater than that of the parental Ba/F3 cells under the same condition, suggesting that overexpression of ASK protein can partially bypass the mitogen requirement. Our data is consistent with the possibility that ASK is one of the critical targets of E2F for S phase induction.

3. Human Cdc7-related kinase complex: *In vitro* phosphorylation of MCM by concerted actions of Cdks and Cdc7

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We have expressed huCdc7 complexed with ASK regulatory subunit using the insect cell expression system. To facilitate purification of the kinase com-

plex, glutathione-S-transferase (GST) was fused to huCdc7 and GST-huCdc7-ASK complex was purified. GST-huCdc7 protein is inert as a kinase on its own, and phosphorylation absolutely depends on the presence of the ASK subunit. It autophosphorylates both subunits *in vitro* and phosphorylates a number of replication proteins to different extents. Among them, MCM2 protein, either in a free form or in a MCM2-4-6-7 complex, serves as an excellent substrate for huCdc7-ASK kinase complex *in vitro*. MCM4 and MCM6 are also phosphorylated by huCdc7 albeit to less extent. MCM2 and 4 in the MCM2-4-6-7 complex are phosphorylated by Cdks as well, and prior phosphorylation of the MCM2-4-6-7 complex by Cdks facilitates phosphorylation of MCM2 by huCdc7, suggesting collaboration between Cdks and Cdc7 in phosphorylation of MCM complex for initiation of S phase. Critical Cdk-mediated phosphorylation sites were mapped at serine 27 or serine 41 of mouse MCM2. Substitution of these serine residues by alanine resulted in very inefficient generation of the Cdc7-directed mobility-shifted form of MCM2 *in vivo* and *in vitro*, whereas that with glutamic acid lead to efficient phosphorylation of MCM2 by Cdc7 in the absence of Cdk. These results indicate that phosphorylation of MCM2 in MCM complex is achieved by concerted actions of Cdk and Cdc7 for efficient initiation of DNA replication.

4. Phosphorylation of MCM2 by Cdc7 kinase

Min-kwon Cho, Etsuko Matsui[1], Noriko Sato, Keiko Ogino[1], Tadayuki Takeda[1], Chika Taniyama[1], Yukio Ishimi[6], Ken-ichi Arai[1] and Hisao Masai

Genetic and biochemical evidence indicates that MCM2 is a critical substrate of Cdc7 kinase for firing of origins. Although both the free form of MCM2 and the one in a complex can be efficiently phosphorylated by Cdc7 *in vitro*, tryptic peptide mapping and mobility-shift on SDS-PAGE indicate phosphorylation of distinct residues on MCM2 in the complex. Analyses of MCM2 within the cells indicate that MCM2 in a complex is the major target of Cdc7 *in vivo*.

We have expressed and purified mouse MCM2-4-6-7 complexes containing a mutant MCM2 protein in which selected serine and threonine residues were replaced by alanine. We analyzed biochemical characteristics of these mutant MCM complexes and examined their phosphorylation by huCdc7-ASK kinase complex *in vitro*.

We have identified several *in vitro* phosphorylation sites on MCM2 using oligopeptides and recombinant proteins containing segments of MCM2 protein. Serine 17 of human MCM2 can be specifically phosphorylated by huCdc7 *in vitro*. A C-terminal region of the MCM box (F6), conserved in MCM family, and the region further C-terminal to it (NF3), which is unique to MCM2 but is conserved in MCM2 from other species, are also phosphorylated by huCdc7-ASK *in vitro*, which are confirmed by two-dimensional tryptic phosphopeptide mapping. Amino acid substitution of the corresponding serine/threonine residues in Cdc19 (fission yeast MCM2 homologue) with alanine or glutamic acid, respectively, resulted in loss of its function, as determined by the ability to complement the growth defect of *cdc19*(ts) mutant. Alanine substitutions of conserved serine/ threonine residues near the zinc-finger motif (F1) on full-length MCM2 resulted in loss of specific phosphorylation by huCdc7, and MCM2-4-6-7 complex containing the F1 mutation showed retarded migration of the complex on a native gel, indicating that F1 mutation causes alteration of complex structures.

Our results indicate that multiple residues on MCM2 are phosphorylated by Cdc7 and suggest a possibility that Cdc7 may regulate the subunit organization of the MCM complex.

5. Genetic studies on murine Cdc7-related kinase by the use of conditional knock-out ES cells and mice

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In order to address *in vivo* functions of mammalian Cdc7 kinase, we generated muCdc7-deficient mice. muCdc7 homozygous null mice are early embryonic lethal (between E3.5 and 6.5). We have established muCdc7-/- ES cell lines and muCdc7-/- mice whose viability is maintained by the Cre-removable muCdc7 transgene encoding one of the cDNA variant (p62 driven by EF1α promoter). muCdc7-/- ES cells carrying the transgene showed growth properties indistinguishable from those of the wild-type ES cells. The transgene in this mutant ES cells was efficiently excised upon infection of Cre-encoding adenovirus, and the cell growth was immediately retarded with concomitant loss of thymidine incorporation into mutant cells, which eventually lost viability. The cells arrest mostly with S phase DNA content with concomitant increased expression of p53. These results demonstrate that muCdc7 is essential for proliferation and DNA replication of ES cells. In order to examine interactions between CDK and Cdc7 pathways in cell proliferation and mouse development, we tried to generate muCdc7-/- p27-/- double knockout mice. Viable embryos were detected at E8.5. Furthermore, muCdc7-/- p27-/- blastocysts formed inner cell mass which showed significant level of BrdU uptake, whereas

muCdc7-/- p27+/+ blastocysts did not generate any visible inner cell mass nor DNA replication. These results indicate that increase of CDK activity can rescue early embryonic growth of muCdc7-/- embryos by partially restoring DNA replication activity in the absence of Cdc7 functions.

In contrast to the above mutant ES cells, the mutant mice with the identical genetic background were dead at one or two days after birth in about half the population, and those mice which survived were extremely small in size. The embryonic fibroblast cells derived from these mutant mice exhibited retarded growth *in vitro*, indicating that the transgene, although capable of rescuing the growth of muCdc7-/- ES cells, cannot completely rescue the loss of muCdc7 gene in more differentiated cells. We have identified many alternatively spliced forms of muCdc7 transcripts as well as the proteins derived from them, which are differentially expressed in various tissues. We are currently dissecting the roles of each variant in generation, proliferation and functions of various tissues.

We are also developing more efficient systems for generating conditional knock-out ES cells and are trying to genetically dissect the functions of various cell cycle and DNA replication genes.

6. A novel mode of kinase activation through bipartite binding of a kinase activator to the catalytic subunit: Dissection of the regulatory subunit for Cdc7-related kinase essential for S phase

Keiko Ogino[1], Tadayuki Takeda[1], Etsuko Matsui[1], Chika Taniyama[1], Ken-ichi Arai[1] and Hisao Masai

Him1/Dfp1 protein of fission yeast, *Schizosaccharomyces pombe*, encodes the regulatory subunit for Hsk1 kinase, a homologue of budding yeast Cdc7 kinase essential for initiation and progression of S phase. Him1 protein, containing three conserved motifs, Dbf4-motif-N, Dbf4-motif-M, and Dbf4-motif-C, binds and activates Hsk1 kinase which phosphorylates MCM2 protein. We have shown that Dbf4-motif-M and Dbf4-motif-C are essential for mitotic functions of Him1/Dfp1 protein as well as for full-level activation of the Hsk1 kinase. Deletion or mutation of either of Dbf4-motif-M or Dbf4-motif-C results in impaired mitotic function and kinase activation, although binding to Hsk1 is not affected. *In vitro*, a small segment containing Dbf4-motif-M (60 amino acids) alone or Dbf4-motif-C (58 amino acids) alone binds to Hsk1. Thus, Him1/Dfp1 binds to Hsk1 through bipartite binding modules. Furthermore, a fusion polypeptide containing small segments of Dbf4-motif-M and Dbf4-motif-C could activate Hsk1 and was capable of rescuing the growth defect of *him1* null cells, and insertion of a long stretch of amino acids between the motif-M and motif-C could be tolerated for mitotic functions. *In vitro*, coexpression of two small segments of motif-M and motif-C could partially activate Hsk1. These results indicate a novel mode of kinase activation through bipartite binding of these conserved motifs to the catalytic subunit. Internal deletion of Dbf4-motif-N, which shares some similarity with the BRCT (BRCA C-terminal domain) motif, resulted in defect in hydroxyurea (HU)-induced checkpoint responses and sensitivity to methyl methane sulfonate (MMS), but mitotic functions and kinase activation were intact. Our results define the roles of the conserved motifs of Cdc7 regulatory subunits and suggest a molecular architecture of Cdc7-Dbf4 related kinase complexes at the origins.

7. A novel "initiation-type" mutant of fission yeast Cdc45 homologue reveals its essential role in loading of DNA polymerase α onto MCM

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Proteins involved in the initiation of DNA replication play critical roles in the loading and assembly of replication complexes at replication origins. To gain novel insights into the regulation of initiation, we screened for fission yeast temperature sensitive mutants which arrest at G1/S boundary. One of the isolated mutants, *goa1-U53*, arrests after START but before the hydroxyurea block point. This mutant is allelic to the CDC45 homologue of fission yeast (*sna41+*) and mutated in a motif highly conserved in Cdc45-related proteins. *goa1-U53* genetically interacts with DNA polymerase α temperature sensitive mutants. The gene product of the cloned cDNA complementing *goa1-U53* (Sna41p/SpCdc45p) interacts with Polαp throughout cell cycle and with Mis5p/Mcm6p in the chromatin fraction during S phase. Although Polαp loading onto the chromatin fraction, which occurs before START, is not affected, association of Polαp with Mis5p is absent in *goa1-U53*. These results support a model that loading of DNA polymerase α onto replication origins occurs through two distinct steps, namely START-independent loading onto the chromatin at early G1 and association with the replication complexes at the origins at G1/S boundary through S phase in a manner dependent on Cdc45 which interacts with both DNA polymerase α and MCM.

8. PriA protein in recombination-dependent DNA replication from stalled replication forks

Taku Tanaka, Chika Taniyama[1], Ken-ichi Arai[1], and Hisao Masai

E. coli PriA protein, a DEXH-type DNA helicase with

unique C2C2-type zinc finger-like motifs interrupting the helicase domains, is an essential component of the ϕX174-type primosome and plays critical roles in RecA-dependent inducible and constitutive stable DNA replication (iSDR and cSDR, respectively) as well as in recombination-dependent repair of double-stranded DNA breaks. We have postulated that PriA protein recognizes stalled replication forks to restore replication forks and repair legions in a recombination-dependent manner. We show here that mutant PriA proteins defective in ATP hydrolysis and helicase activities do not fully support iSDR or cSDR *in vivo*, although the ATPase/helicase-deficient mutants could support single-stranded DNA replication from the origin of ϕX174, as was reported previously. We have also identified a novel motif in the N-terminal segment of PriA, WYY motif, which, along with a C-terminal helicase conserved motif, is required for binding to a D-loop structure, mimicking the stalled replication forks. This motif is also required for iSDR and cSDR *in vivo*. Thus, ATPase/helicase and D-loop binding activities are both required for recombination-dependent DNA replication. We are currently fractionating fission yeast and mice tissue extracts to identify eukaryotic proteins which recognize the stalled replication forks.

9. Mapping of replication origins in human and mouse cytokine cluster regions

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The mode of activation of mammalian replication origins is still poorly understood. In spite of various evidence pointing to interplay between replication and transcription, the molecular basis for coordination between these two nucleic acid transactions is still largely an open question. The human chromosome 5 and mouse chromosome 11 contain the genome regions in which cytokine genes are present in clusters. We are particularly focusing on the human 5q locus containing tandemly arranged IL-3 and GM-CSF genes and mouse 11q containing IL-4 and IL-13 genes as models for the study of possible coupling between transcription and replication. Expression of IL-3 and GM-CSF in T cells is induced in response to T cell receptor stimulation. IL-4 and IL-13 belong to type 2 T helper cell-specific cytokines, and their expression is strictly cell-type specific. By utilizing an origin-enriched library as well as the competitive PCR method, we have identified a replication origin which is active in non-T cell lines at 4 kb downstream of human GM-CSF gene. We did similar mapping in T cell lines, and preliminary results indicate the presence of an origin in the region between the two genes, upstream of GM-CSF. We are currently mapping replication origins in the vicinity of the mouse IL-4 and IL-13 locus in Th2 cells, Th1 cells and non-T cells. We wish to extend these studies to eventually determine genome-wide distribution pattern of replication origins in various cell types.

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