Department of Cancer Biology

Division of Oncology
癌細胞シグナル研究分野

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Structural and functional analysis of the protooncogenes reveals that a large majority of them encode protein-tyrosine kinases or transcription factors. The protooncogenes are important not only for development of malignant tumors but also for the regulation of growth and function of normal cells. Our current interest is to characterize the cellular signaling mediated by protein tyrosine phosphorylation in central nervous system and cancer cells. We are also interested in the function of antioncogene products and serine/threonine phosphorylation-mediated signaling that are involved in regulation of cell cycle progression.

1. Roles of protein-tyrosine kinases and phosphatases in the central nervous system

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The Src-family protein-tyrosine kinases (PTKs) are implicated in various neural functions such as synaptic plasticity, formation of neural network and myelination. Glutamate is a major excitatory neurotransmitter in the central nervous system. Two types of glutamate receptors, ionotropic and metabotropic receptors, have been described and are implicated in synaptic plasticity, synaptogenesis, and excitotoxity. Long-term potentiation (LTP) in the glutamatergic system is one of the most striking examples of synaptic plasticity for supporting memory. Several lines of evidence suggest that protein-tyrosine kinases play regulatory roles in LTP induction. There are data showing that 1) the *N*-methyl-D-aspartate (NMDA) type of ionotropic glutamate receptors are highly tyrosine phosphorylated in neuronal cells; 2) Src interacts both physically and functionally with NMDA receptors (NMDAR); 3) specific inhibitors for protein-tyrosine kinases attenuate the induction of LTP. Moreover, LTP induction in the hippocampus is also attenuated in $fyn^{-/-}$ mice. Our own studies have shown that the Src-family kinase Fyn is involved in tyrosine phosphorylation of NR2A/2B subunits of NMDAR. A Postsynaptic-density protein PSD-95 that directly interacts with NMDAR facilitates the tyrosine phosphorylation events. To analyze the biological significance of tyrosine phosphorylation of the NMDAR, we have determined tyrosine phosphorylation sites on NR2A/B. Phosphorylation at Tyr-1472 on NR2B, which is a major tyrosine phosphorylation site, is significantly enhanced after induction of LTP in the hippocampal CA1 region, suggesting that Tyr-1472 phosphorylation on NR2B is important for synaptic plasticity. To further establish biological signifi-

cance of Tyr-1472 phosphorylation, mice with Tyr-1472 to Phe mutation were generated. Electrophysiological and behavioral analyses of the knock-in mutant mice are in progress. We also show that Tyr-1325 on NR2A is a major tyrosine phosphorylation site. Roles of other tyrosine phosphorylation sites on NR2A/2B are going to be analyzed using the same strategy for Tyr-1472 of NR2B.

Accumulating evidence shows that not only tyrosine phosphorylation but also tyrosine dephosphorylation regulates synaptic plasticity. We have shown that protein-tyrosine phosphatase PTPMEG is associated with glutamate receptors, NMDAR and GluRδ2, via its PDZ domain. PTPMEG is a family member of band 4.1 domain-containing protein-tyrosine phosphatases, and is expressed prominently in the brain. To know the physiological roles of PTPMEG, especially in the tyrosine phosphorylation events of NMDAR, we generated PTPMEG$^{-/-}$ mice. We are currently analyzing their histological and behavioral phenotypes.

Among the Src-family kinases, which are expressed in the brain, Lyn is specifically expressed in the granular layers of the cerebellum as well as in basal ganglia and cerebral cortex. Possible involvement of Lyn in neural functions such LTP and LTD (long-term depression) is under investigation by using *lyn*$^{-/-}$ mice. To date, we have found that locomotor activity of *lyn*$^{-/-}$ mice is impaired due to the constitutive activation of NMDAR signaling.

To further study the role of protein-tyrosine kinases in neural function and development of CNS, we have been searching for novel protein-tyrosine kinases. We identified AATYK2/KPI-2 through a database search, and after characterizing it, we have renamed it BREK. We showed that BREK, and family members AATYK1 and AATYK3, are serine/threonine kinases, although AATYK1 was reported to be a tyrosine kinase. All three members are expressed specifically in the central nervous system. Ectopic expression of a kinase-native form of BREK suppresses NGF-induced neurite outgrowth and MAPK activation in PC12 cells, suggesting that BREK inhibits the NGF-TrkA signaling.

In parallel of these studies, to uncover the signaling pathways in which the Src-family and BREK are involved, we have been trying to identify binding partners, substrates and transcriptional target genes of these kinases in the brain. We have already identified a number of novel proteins that could be associated with or phosphorylated by the Src-family kinases. Characterization of these putative binding partners and substrates is in progress. One of them is p250GAP, a novel brain-enriched GTPase-activating protein for Rho-family proteins that interacts with the NR2B subunit of NMDAR *in vivo*. Within neurons, p250GAP was highly concentrated in the postsynaptic density and colocalized with NR2B and PSD-95. p250GAP promoted GTP hydrolysis of Cdc42 and RhoA *in vitro* and *in vivo*. When overexpressed in neuroblastoma cells, p250GAP suppresses the activities of Rho-family proteins, which results in alteration of neurite outgrowth. NMDAR stimulation leads to dephosphorylation and redistribution of p250GAP in hippocampal slices. Taken together, p250GAP is likely to be involved in NMDA receptor activity-dependent actin reorganization in dendritic spines. The Cbl-family ubiquitin ligases are also associated with and phosphorylated by the Src-family kinases. Among the family members, Cbl and Cbl-b are expressed in the brain with distinct expression profiles. We found that Cbl-family proteins suppress the signaling through various tyrosine kinases such as Src, ErbB-2, and ErbB-4 through ubiquitination of these kinases. Roles of Cbl and Cbl-b in neural functions are under investigation.

2. Elucidation of the biological role of Tob family proteins and Tob-mediated signaling pathway

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By screening a cDNA expression library with autophosphorylated c-erbB-2 protein, we isolated cDNA clones coding for its possible substrates. One of the genes thus identified, termed *tob*, encodes a 45kDa protein with homology to the growth suppressing proteins Btg1 and PC3. We also cloned novel genes homologous to *tob* that were termed *tob-2* and *ana*. Microinjection experiments showed that exogenously expressed Tob, Tob-2, and ANA proteins suppress growth of NIH3T3 cells. We also found that Tob is rapidly phosphorylated by Erk1 and Erk2 upon growth factor stimulation. Oncogenic Ras-induced transformation and growth factor-induced cell proliferation are efficiently suppressed by mutant Tob that carries alanines but not glutamates, mimicry of phospho-serines at the phosphorylation sites. Intriguingly, cyclin D 1 expression is elevated in serum-starved *tob*$^{-/-}$ cells. Thus, Tob inhibits cell growth by suppressing cyclin D1 expression, which is cancelled by Erk1- and Erk2-mediated Tob phoshorylation. We propose that Tob is critically in-

volved in the control of early G1 progression.

To elucidate physiological function of Tob, we generated *tob*-deficient mice. *tob*-deficient mice had a greater bone mass resulting from increased number of osteoblasts. Orthotopic bone formation in response to BMP2 was elevated in *tob*-deficient mice. Overproduction of Tob repressed BMP2-induced, Smad-mediated transcriptional activation. The results indicate that Tob negatively regulates osteoblast proliferation and differentiation by suppressing the activity of the receptor-regulated Smad proteins. We also found that aged mice lacking Tob expression develop a variety of tumors, such as hemangiosarcomas and hepatocarcinomas, at high frequency. Intraperitoneal injection, at two weeks after birth, of the carcinogen diethylnitrosamine (DEN) led to more frequent generation of liver tumors in *tob*-deficient mice than in wild -type mice. $tob^{-/-}p53^{-/-}$ mice show accelerated tumor formation in comparison with single null mice. Levels of *tob* mRNA are often decreased in human cancers, implying *tob* in cancer development. Furthermore, embryonic fibroblasts deficient for *tob* displayed a marked increase in chromosomal aberration, including breakage, translocation, and aneuploidy after the treatment of DEN. To circumvent functional redundancy between Tob and Tob-2, disruption of the *tob*-2 gene and subsequent generation of double knockouts (*tob*-/-*tob*2-/-) were performed. Analysis of their phenotypes is underway.

Among the Tob family proteins, Tob and Tob 2 proteins contain a putative nuclear localization signal (NLS) that is present near the amino-terminus. We showed that Tob is a nuclear protein by immunostaining. Nuclear localization of Tob is impaired by mutation of the putative NLS. Furthermore, Tob NLS sequence alone could drive nuclear import of the EGFP-LacZ fusion protein, indicating that the NLS of Tob is functional. Unlike wild-type Tob, Tob NLS mutant protein is poorly antiproliferative, suggesting that subcellular localization of Tob is related to the regulation of cell growth. Because the Tob family proteins interact with various transcription factors, such as Caf1, Tob is likely involved in transcription regulation. We assume that Tob acts as a transcriptional co-repressor. Indeed, expression of cyclin D1 mRNA is increased in the absence of Tob and Tob suppresses the cyclin D 1 promoter activity through an interaction with histone deacetylase.

Other studies to establish biological significance of the *tob* family members are widely spread. (1) Generation of *caf1*-deficient mice. *caf1* -deficient males are sterile owing to oligo-astheno-teratozoospermia, suggesting that Caf1, a CCR4-associated transcriptional cofactor, is essential for spermatogenesis. Maturation of spermatids is unsynchronized and impaired in $caf1^{-/-}$ seminiferous tubules. Transplantation of spermatogonial stem cells from male $caf1^{-/-}$ mice to seminiferous tubules of c-*kit* mutant mice restores spermatogenesis, suggesting that the function of testicular somatic cells is damaged in the $caf1^{-/-}$ condition. Importantly, the testicular phenotypes of $caf1^{-/-}$ mice are similar to those of $RXRb^{-/-}$ mice. We further show that Caf1 binds the AF-1 domain of RXRb and RXRb malfunctions in the absence of Caf1. Therefore, Caf1 appears to function as a new coregulator of RXRb in testicular somatic cells and is thus involved in spermatogenesis. There are data suggesting that Caf1 is associated with deadenylase activity, suggesting that Tob-Caf1 complex may be involved in translational regulation. To elucidate functional relation between Tob and Caf1 in vivo, tob-/-caf1-/- mice are generated.(2) Search for other molecules that interact with Tob. We performed yeast two-hybrid screening using Tob as a bait, and identified novel serine/threonine kinase and a molecule responsible for the stability of Tob protein. (3) As the other projects, generation of *ana*-deficient mice and characterization of a large protein complex containing Tob are now in progress.

3. Role of chromokinesin Kid and mitotic kinases in spindle formation and chromosome segregation.

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Mitosis is a process whereby a complete copy of the genetic information is distributed to each new cell during cell division. This process is critically important, with even small errors leading to aneuploidy or cell death. The development of chromosomal and/or centrosomal abnormalities is often observed in tumor cells and a specific chromosome or centrosomal abnormality may often be one of the first events in the development of a cancer. It is well known that microtubule-associated motor proteins are involved in spindle formation and chromosome movements in mitosis, and that orchestrated regulation by many mitotic kinases are important for the progression of each step of mitosis.

We previously cloned cDNAs for a gene termed *kid* that encodes a novel member of the kinesin family of proteins. The kinesin-like motor domain of Kid is present in its amino-terminal half and has the plus-end directed motor activity. Its carboxyl-terminal half contains a

helix-hairpin-helix DNA binding motif and is able to bind to specific DNA sequences similar to a part of α-satellite DNA. During mitosis, Kid is localized on chromosome, centrosome, and spindle and is essential for chromosome alignment at metaphase plate. Overexpression of Kid, the motor domain, or the DNA binding domain abrogated chromosomal segregation. We showed that Kid was phosphorylated at multiple sites during mitosis. We identified a Cdc2 kinase phosphorylation site on Kid and showed that Kid with a point mutation at the site failed to localize to chromosome, although the mutant Kid retains the ability to bind to mitotic chromosomes. Our studies revealed that in addition to a motor domain, Kid contains a second MT binding site within its stalk region that enhances the affinity for microtubule and that Cdc2-mediated phosphorylation down regulates this additional association. These results indicate that Kid is one of the important target of Cdc2 kinase, through which chromosome movement along spindle microtubule during prometaphase and metaphase is regulated. To further investigate whether Kid has any functions for spindle formation through mitosis, we depleted Kid from HeLa cells using RNA interference (RNAi). We have data suggesting that Kid is also responsible for maintenance of the length of spindle MTs during metaphase and metaphase. In addition, to further study the mechanisms for regulating localization, function, and degradation of Kid, we are searching for Kid binding proteins by yeast two-hybrid screening and by purification of Kid complex from cultured cells.

We have previously identified genes encoding human serine/threonine protein kinases with significant homology to a *Drosophila* tumor suppressor gene *lats*. We named these two genes *hLATS1* and *hLATS2*, both encode serine/threonine kinases that share high homology with the myotonic dystrophy protein kinase (DMPK) family, many of which are known to be involved in various mitotic events. FISH analysis revealed that the *hLATS1* and *hLATS2* genes are localized to chromosome 6q24-25.1 and 13q 11-12, respectively. In these regions, loss of heterozygosity (LOH) is observed in various cancers including breast cancers, hepatocellular carcinomas, and renal carcinomas. To examine the role of hLATS2 in tumorigenesis and cell cycle regulation, we have searched for molecules that interact with hLATS2 and identified some centrosome-localized proteins such as Kendrin, and several LIM domain-containing proteins including Ajuba. Interestingly, hLATS2 and Ajuba were co-localized with the centrosomes during mitosis and hLATS2 was implicated in the mitotic phosphorylation of Ajuba. Ajuba-depleted cells were accumulated at prometaphase-metaphase, indicating the requirement of Ajuba in the progression of mitosis. RNAi experiments further revealed that LATS2 and Ajuba were required for the accumulation of γ-tubulin at the centrosomes and subsequent spindle organization at G2/M phase transition. The precise role of hLATS2 and Ajuba in centrosome regulation is under investigation. We also explore the role of other hLATS2 associated molecules and other mitotic kinases in the regulation of mitotic progression.

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Cell surface is an interphase for communication of cells with extracellular environment and signals are transmitted in both directions through the cell membrane. Molecules on the surface and their ligands in the extracellular milieu are frequently regulated by proteolysis. In addition to degradation, such pericellular proteolysis includes processing that mediates activation, inactivation and functional conversion of the molecules there. Since the signals traverse the cell membrane regulate various cellular functions such as proliferation, apoptosis, differentiation, and invasion, abnormality in the pericellular proteolysis contributes to the behavior of cancer cells. The aim of our study is to shed light on the proteolytic events on cell surface that regulate the cell functions and their relevance to various diseases including cancer.

1. Constitutive and induced CD44 shedding by ADAM-like proteases and MT1-MMP

Hiroyuki Nakamura, Naoko Suenaga, Kaori Taniwaki, Hirokazu Matsuki, Kayoko Yonezawa, Masato Fujii, Yasunori Okada and Motoharu Seiki

The extracellular matrix (ECM) not only constitutes the framework of tissues but also regulates various cellular functions, such as proliferation, differentiation, apoptosis, and migration. CD44 is an adhesion molecule that acts as a major receptor for hyaluronan (HA), an abundant glycosaminoglycan in the ECM. HA fills intercellular spaces in various tissues and has been implicated in many biological processes including inflammation, wound healing, remodeling of tissue, cell migration and invasion. CD44 binds HA at the globular lectin-like domain and regulates various cell physiologies via mechanisms that are not yet fully understood. The ligand-binding domain is linked to the cell surface through a stem sequence that follows the transmembrane domain. CD44 also has a cytoplasmic tail that acts as an interface for interaction with the actin cytoskeleton and in the assembly of signaling molecules regulating the actin dynamics. The basic and most common form of CD44 is referred to as the hematopoietic type (CD44H). Alternative splicing generates further diversification by adding variable exon-coded sequences to the stem. Further variation of CD 44 is conferred by extensive glycosylation at multiple sites including variant exon-coded sequences. Although the core protein of CD44H is 37 kDa, it usually appears as a molecule of 80-100 kDa as a result of heavy glycosylation.

Other than HA, ligands in the extracellular space such as type I collagen, fibronectin, fibrin, laminin, and chondroitin sulfate are reported to bind CD44. However, CD44 is not merely a receptor for ECM molecules, in that it also acts as a platform for signal transmission by assembling

bioactive molecules on the cell surface, such as growth factors (bFGF, FGF-8, and HB-EGF), cytokines (osteopontin), receptors (ErbB4), and matrix metalloproteinases (MMPs). For example, MMP-7 binds to a heparan-sulfate proteoglycan (HSPG) in the v3 region of a variant form of CD 44 and cleaves HB-EGF that may activate ErbB4. MMP-7 also induces cell migration through the processing of osteopontin. MMP-9 is another soluble MMP that binds to CD44 and the bound form of MMP-9 is reported to activate the latent form of TGF-β and stimulate tumor invasion and angiogenesis.

Proteins that bind CD44 may in turn be regulated through the actin cytoskeleton, because CD 44 is connected to the structure via the cytoplasmic domain. Membrane-type 1 MMP (MT1-MMP) has a strong invasion-promoting activity that is frequently employed by malignant tumors. To degrade the ECM barrier in the direction of cell locomotion, MT1-MMP has to attain a polarized localization at the leading edge. CD 44, which localizes at the ruffling edge of migrating cells, was found to bind MT1-MMP and regulate its localization to the edge through the hemopexin-like domain of MT1-MMP and the stem region of CD44H.

The shedding of CD44 is a frequent event observed in many types of cells and shed CD44 has been detected in culture supernatants, arthritic synovial fluid, and plasma. It has also been reported that higher levels of shed CD44 were detected in serum from patients bearing malignant cancer with metastasis. The proteases responsible for the shedding are mainly metalloproteinases because synthetic metalloproteinase inhibitors having a broad spectrum inhibit the shedding almost completely, though some serine proteinases may also participate. MT1-MMP was though to be an enzyme responsible for the shedding because it binds CD44H, has an ability to cleave it at least *in vitro*, and expression of MT1-MMP increases CD44 shedding in the cells. However, it was not necessarily clear whether MT1-MMP directly shed CD44 at a cellular level and whether it is the enzyme solely responsible for the shedding.

In this study, we found that expression of MT 1-MMP increased the shedding of 65-70 kDa CD 44H fragments that are commonly produced by many types of cells and generated two additional smaller fragments using a human melanoma cell line. We purified the three groups of heterogeneously glycosylated fragments and determined the cleavage sites by mass spectrometry. Cleavage site-specific antibodies were then generated and used to monitor the shedding at each site. Shedding near the membrane site that generates 65-70 kDa fragments occurs constitutively and the sensitivity of the shedding to TIMPs suggested ADAM-like proteases for the cleavage. Expression of MT1-MMP increased shedding at all three sites including the site of processing by the ADAM-like proteases. MT1-MMP itself cleaved the most N-terminal position that generates 37-40 kDa fragments. CD44 shedding at these sites was detected in human tumors with increased shedding of the fragments cleaved at the MT1-MMP-sensitive site.

2. Sequence-specific silencing of MT1-MMP expression suppresses tumor cell migration and invasion: Importance of MT1-MMP as a therapeutic target for invasive tumors

Jyunko Ueda, Masahiro Kajita, Naoko Suenaga, Katsuyuki Fujii, and Motoharu Seiki

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that collectively degrade most components of the extracellular matrix (ECM). Recent studies have expanded on their substrates to include non-ECM molecules such as growth factors, cytokines, receptors, and cell adhesion molecules. Thus, MMPs regulate a wide array of bioactive molecules in the extracellular milieu by degradation or processing. To date, 23 MMPs have been identified in mammals. They differ in substrate specificity and the control of gene expression. Tumor cells frequently overexpress multiple MMPs which are believed to play roles collectively in the invasive and metastatic growth of tumors.

Membrane-type MMPs (MT-MMPs), a subgroup of the MMP family composed of six members, are expressed on the surface of producer cells through a C-terminal sequence that acts either as a type I transmembrane domain or as a signal for GPI-anchoring. As they localize on the surface of the cell, MT-MMPs are responsible for proteolysis in the pericellular space and eventually affect various cellular functions. MT1 -MMP is of particular interest because it is expressed most frequently in human tumors. MT1-MMP degrades ECM molecules such as collagen types I, II, and III, fibronectin, laminin-1 and -5, vitronectin and aggrecan etc.. It also recruits proMMP-2 to the cell surface (Seiki, 2002) and causes its activation by cleaving the propeptide sequence. Since MMP-2 can degrade native type IV collagen, MT1-MMP and MMP-2 assembled on the tumor cell surface forms a potent system to invade the basement membrane and interstitial stroma. The activation of MMP-2 in tumor tissues correlates well with the expression levels of MT1-MMP and local invasion by tumor cells. MT1-MMP acts also as a processing enzyme for CD44, the γ2 chain of laminin-5, the integrin αv

chain, and tissue transglutaminase (tTG), and modulates the cell migration property as a result. Cytokine inactivation may also be caused by MT1-MMP on the cell surface. As expected from its proteolytic activity and expression in aggressive tumors, MT1-MMP, when overexpressed, strongly promotes cellular invasion in vitro and experimental metastasis. However, it is not clear whether endogenously expressed MT1-MMP is a critical part of the cellular invasiveness in the presence of other MMPs.

In this study, we used three human tumor cell lines, fibrosarcoma HT1080, gastric carcinoma MKN-28 and TMK-1 cells, and examined the specific roles of the MT1-MMP endogenously expressed in two of these cell lines. To knockout the expression of MT1-MMP, we used RNA silencing technology, in which sequence-specific degradation of mRNA is induced by double stranded RNA (dsRNA). Expression of MT1-MMP was successfully down-regulated to less than 10-20% by treating the cells with the dsRNA specific to MT1-MMP. Using the cells, MT1-MMP was demonstrated to be essential for activation of proMMP-2, migration on a HA-coated matrix, and invasion into Matrigel. Thus, our results provide a basis for the specific targeting of MT1-MMP for cancer therapy.

3. Absence of mechanical allodynia and Aβ-fiber sprouting after sciatic nerve injury in mice lacking membrane-type 5 matrix metalloproteinase

Kiyoshi Komori, Takahiro Nonaka, Akiko Okada, Hiroaki Kinoh, Hiromi Hayashita-Kinoh, Nobuaki Yoshida, Ikuo Yana, Motoharu Seiki

MT5-MMP (MMP-24) was identified as a brain-specific MMP and its expression was specifically detected in neurons of both the central and peripheral nervous systems. During embryonic and early postnatal development in rodents, MT5-MMP is abundantly expressed throughout the nervous system. Subsequently in adults, significant expression levels are maintained in regions of neuronal plasticity, such as the cerebellum and hippocampus. The temporal and spatial regulation of the expression suggests that MT5-MMP plays roles in neuronal circuit formation and plasticity. To gain further information on the roles of MT5-MMP in vivo, we generated MT5-MMP-deficient mice by targeted disruption of the gene (*mmp-24*).

MT5-MMP-deficient mice were born without obvious morphological abnormalities. No apparent histological defects were observed in the nervous system either. However, MT5-MMP-deficient mice did not develop neuropathic pain with mechanical allodynia after sciatic nerve injury, though responses to acute noxious stimuli were normal. Neuropathic pain induced by peripheral nerve lesions is known to accompany structural reorganization of the nervous system. Intraneural injection of cholera toxin B subunit (CTB), a transganglionic tracer, into the injured sciatic nerve of wild type mice revealed that the myelinated Aβ-fiber primary afferents sprouted from laminae III-VI of the dorsal horn of the spinal cord and invaded lamina II. However, no such sprouting and invasion of Aβ-fibers were observed in MT5-MMP-deficient mice. These findings suggest that MT5-MMP is essential for the development of mechanical allodynia and plays an important role in neuronal plasticity in this mouse model.

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Department of Cancer Biology

Division of Cancer Genomics
癌遺伝形質分野

Associate Professor Hiroaki Miki, Ph.D.
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助教授 理学博士 三木裕明
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We have been investigating the mechanism of how Rho-family small GTPases such as Cdc42, Rac, and Rho regulate cell morphology and motility. Recently, we identified Eps8 and SF3a66 as main binding partners for IRSp53, an effecter protein for Cdc42 and Rac. We have also been examining the regulatory mechanism of microtubule organization in response to Wnt-signal, which plays a critical role in morphogenesis and development of human cancer.

1. Positive feedback regulation of Rac via the IRSp53/Eps8 complex is important for cell motility

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IRSp53 has been characterized as an adaptor protein that links Rho-family small GTPases such as Cdc42 and Rac to reorganization of the actin cytoskeleton. IRSp53 possesses a SH3 domain at its C-terminus, through which it binds to WAVE2, a WASP family actin regulating protein, and induces actin polymerization through Arp2/3 complex. We then searched for other binding partners for the IRSp53 SH3 domain and identified Eps8 as the most major binding protein in NIH3T3 fibroblast cells. We confirmed that IRSp53 and Eps8 form an *in vivo* complex and that Eps8 binds directly to the IRSp53 SH3 domain through its N-terminal proline-rich sequence. Thus far, Eps8 has been shown to form a trimolecular complex with Abi-1 and Sos-1, which shows GEF activity specific for Rac. This led us to investigate whether IRSp53 affects the Eps8-mediated Rac activation, and found that the interaction of IRSp53 with Eps8 augments the Rac activation by reinforcing the formation of the Eps8/Abi-1/Sos-1 Rac-GEF complex. As we characterized IRSp53 as the molecule that functions downstream of Rac in the previous study, these results suggest the possibility that Rac may be able to activate Rac itself through IRSp53 and Eps8. Indeed, we observed that the expression of activated mutant of Rac induced significant activation of wild-type Rac. Interestingly, the complex formation between IRSp53 and Eps8, as determined by fluorescent resonance energy transfer (FRET) analysis, occurred at the leading edge of motile cells, and the motility and invasiveness of HT 1080 fibrosarcoma cells was suppressed by inhibiting the complex formation. These findings implicate the importance of the IRSp53/Eps8 complex in Rac-induced Rac-activation and cell motility.

2. A novel function of SF3a66 in regulation of microtubules.

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A comprehensive search for novel binding partner(s) for the IRSp53 SH3 domain revealed that the three major binding proteins (SF3a60, SF3a66, and SF3a120) in N1E-115 neuroblastoma cells were the components of the SF3a RNA splicing factor, which plays essential roles during pre-mRNA splicing by forming U2snRNP. Interestingly, the ectopic expression of SF3a66 in N1E-115 cells specifically induced neurite formation as did IRSp53, whereas SF3a60 and SF3a120 did not show any significant effect, suggesting an unexpected function of SF3a66 in regulation of cell morphology.

In order to investigate the mechanism of SF3a 66-induced neurite formation, we generated various truncated constructs of SF3a66 and subjected them to morphological analyses. It was found that the partial fragment containing amino acids 105-216 in SF3a66 was sufficient for neurite formation and then we performed a binding protein analysis by affinity-chromatography with the GST-fusion proteins of the region. Mass spectrometric analysis revealed that the most major binding protein of 55-kDa was beta-tubulin. Indeed, we confirmed that purified SF3 a66 proteins associate with microtubules that were reconstituted from purified alpha/beta-tubulin heterodimers. Electron microscopic examination revealed that SF3a66 bundles microtubules. These results suggest the possibility that SF3a66 is a novel microtubule regulating protein.

3. A novel Wnt-signaling pathway that regulates the dynamics of microtubules.

Takeshi Terabayashi, Hideki Yamaguchi, and Hiroaki Miki

It has been established that Wnts exert their effects through the activation (stabilization) of β-catenin, which forms a protein complex with transcription factors such as LEF/TCF and induces specific gene-expression. We have found that ectopic expression of Dishevelled, a critical Wnt-signal transducer, in neuroblastoma cells resulted not only in the accumulation of β-catenin in the nucleus but also in significant neurite formation. We then generated various truncated constructs of Dishevelled and performed similar expression analyses in neuroblastoma cells. The results indicated that the neurite formation occurred in a manner independent of the accumulation of β-catenin. As the morphology of cells are determined by the cytoskeleton, we then focused on possible effects of Dishevelled on the cytoskeleton and found that microtubules in the Dishevelled-expressing cells were stabilized, that is, they became resistant to microtubule-depolymerizing drug nocodazole. Interestingly, the partial fragment of Dishevelled that induced neurite-formation but lacked the ability to activate β-catenin also induced microtubule-stabilization. Dishevelled is therefore thought to regulate morphology of neuroblastoma cells through the stabilization of microtubules, which seems to be independent of the β-catenin function. We are now trying to elucidate the molecular mechanism of how the novel Wnt-signaling pathway leads to microtubule-stabilization.

4. Identification of p50 as a negative regulator of Dishevelled

Yosuke Funato, Kei Takenaka, and Hiroaki Miki

As described above, Dishevelled is a critical signal transducer of the Wnt-signal. To search for novel binding partner(s) of Dishevelled under a physiological condition, we generated a cell line that stably and moderately expresses FLAG-tagged Dishevelled and performed an immunoprecipitation with anti-FLAG antibody. Among the co-precipitated proteins with FLAG-Dishevelled, we found a protein that migrated around the 50-kDa region (p50) as the most major one. We first confirmed that endogenous proteins of Dishevelled and p50 form an in vivo complex by immunoprecipitation analysis. p50 was found to bind to the basic-PDZ domain in Dishevelled, where binding to Frat/GBP, an important regulator of the Wnt-signal, occurs. Indeed, the co-expression of p50 completely suppressed Dishevelled-induced beta-catenin accumulation, morphological change of cells, and phosphorylation of Dishevelled itself. These results implicate that p50 is a physiological binding partner of Dishevelled and inhibits its function.

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Department of Cancer Biology

Division of Pathology
人癌病因遺伝子分野

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Division of Pathology has worked on the pathogenetic mechanisms and diagnosis of various human lymphoid diseases through the analysis of pathology specimens. Elucidation of the events hidden in such specimens is the main interest in this division. We have worked on human lymphoid diseases and viral infections involving lymphoid tissues/cells. Meanwhile, this division is dissolving in 2004 because of the retirement of the professor and the transfer of other faculty members to Laboratory of Tumor Cell Biology, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The Tokyo University.

I. Malignant lymphomas

1. Construction of a data base on malignant lymphoma cases

Shigeo Mori, Tomoko Nakajima

In recent 17 years we have engaged in pathological diagnosis of around 4500 cases of human lymphoid diseases. Clinical and pathological records of those cases are now filed in a data base. In addition, around 1500 fresh-frozen specimens have been analyzed for immunohistochemical study and those are also filed. Eight hundred cutaneous lymphomas were already filed a few years ago. Together, those account for around 3-5% of lymphoma cases of this country. Most of those fresh-frozen samples are stocked in −80℃ freezer. Permission was granted from IRB on most of those specimens to be used for the study of pathogenesis. A part of those fresh specimens have been transplanted into SCID mice and around 30 cases have been maintained for certain period of time to obtain enough amount of fresh-frozen specimens for further detailed analysis. Those specimens are also filed.

2. New markers for lymphoma diagnosis

Kinji Itoh, Takayuki Kanno, Shigeo Mori

In addition to the recent identification of Methionine Aminopeptidase 2 (METAP2) as a immunohistologic marker for germinal center B cells, Kinji Ito identified another lipid metabolism-associated well-known enzyme as a specific marker for the germinal center B cells (submitted).

3. Identification of possible lymphoid disease -associated cDNA fragments

Shigeo Mori, Tomoko Nakajima, Hidenobu Takahashi

We identified over 300 cDNA fragments by subtracting cDNAs of inflammatory lymphoid diseases including necrotizing lymphadenitis, sarcoidosis, EBV-negative Hodgkin's disease

and a few other lymphoid inflammatory diseases by cDNAs derived from ordinal reactive lymphoid hyperplasias. A part of those are worth for further study since some can be disease specific.

4. Trial to establish U50HG knock-out mice.

Yuichi Soeno, Hitoshi Satoh, Ritsuko Tanaka, Shigeo Mori

U50HG, the host gene of human U50 snoRNA host gene, was first identified from the chromosomal translocation-site of diffuse large B cell lymphoma bearing t (3; 6) translocation. In this case, U50HG gene was shown to fuse with the Bcl6 gene locating at 3q23. Since U50HG is found lost in some other lymhoma cases, while no reports ever referred to the knocking-out of snoRNA/snoRNA host genes, we are tempted to work for the production of U50HG-knock out mice. We found in mice there are two host genes as the human U50 orthologue and thus now we are trying to make constract for the knocking out of mouse U50HG.

5. Hodgkin's lymphoma (HL) and anaplastic large cell lymphoma (ALCL)

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(1) AP-1 mediated relief of repressive activity of the CD30 promoter microsatellite in Hodgkin and Reed-Sternberg cells

Overexpression of CD30 is the hallmark of Hodgkin and Reed-Sternberg (H-RS) cells and drives constitutive NF-κB activation that is the molecular basis for the pathophysiology of Hodgkin lymphoma (HL). Transcription of the CD30 gene is controlled by the core promoter that is driven by Sp-1 and the microsatellite sequences (MS) that represses core promoter activity. To understand the mechanism(s) of CD30 overexpression in H-RS cells, we structurally and functionally characterized the CD30MS. Although the CD30MS of H-RS cell lines was polymorphic, it was not truncated compared with that of control cells. A strong core promoter activity and constitutive Sp-1 binding were revealed in all cell lines examined irrespective of the levels of CD30 expression. In transient reporter gene assays, all MS clones derived from H-RS cell lines repressed the core promoter activity in unrelated cell lines, but not in the H-RS cell lines. An AP-1 binding site was found in the MS at nucleotide position of -377 to -371, the presence of which was found to relieve repression of the core promoter in H-RS cell lines but not in other tumor cell lines. H-RS cell lines showed constitutive and strong AP-1 binding activity, but other cell lines did not. The AP-1 complex contained JunB, whose overexpression activated reporter constructs driven by the CD30 promoter including the MS, and was dependent on the AP-1 site. JunB expression was detected in H-RS cells *in vitro* and *in vivo*, but not in reactive cells or tumor cells of non-Hodgkin's lymphoma of diffuse large B cell type. Taken together, overexpression and binding of JunB to the AP-1 site appear to relieve the repression of the core promoter by the CD30 MS in H-RS cells, which provide one basis for the constitutive overexpression of CD30 in HL (Watanabe M et al., Am J Pathol 163, 633, 2003).

(2) The NPM-ALK oncoprotein abrogates CD 30 signaling and constitutive NF-κB activation in anaplastic large cell lymphoma

NPM-ALK characterizes ALCL as does the high expression of CD30, a feature shared with H-RS cells of classic Hodgkin's lymphoma. In H -RS cells, ligand-independent signaling by overexpressed CD30 drives constitutive NF-κB activation, which is absent in ALCL cells. Here we show that NPM-ALK impedes CD30 signaling and NF-κB activation, dependent on both ALK kinase activity and the N-terminal NPM domain. NPM-ALK transduction into H-RS cell lines abrogates recruitment and aggregation of TRAF proteins, inducing an ALCL-like morphology and phenotype. TRAF2 associates with NPM-ALK at a consensus binding motif located in the kinase domain. Thus, NPM-ALK abrogates CD30-driven NF-κB activation and can also induce an ALCL phenotype, distinguishing ALCL cells from H-RS cells of T-cell origin. The chimeric oncoprotein NPM-ALK has transforming capacity through its tyrosine kinase activity. This study provides evidence for a novel function of this chimeric tyrosine kinase oncoprotein, presumably based on the chaperone activity of the partner domain; a new insight into the function of chimeric tyrosine kinases in lymphomagenesis. Furthermore, NPM-ALK induction of an ALCL-like phenotype in H-RS cells of T lymphocyte origin provides insight into the pathol-

ogy of HL and ALCL, and the foundation for a new conceptual classification of lymphomas with high CD30 expression. (Horie et al., Cancer Cell, in press)

II. Viral Pathogenesis of Human retroviruses, HTLV-1 and HIV

Momoko Shoda, Tsukasa Koiwa, Akiko Hamano, Takaomi Ishida, Masae Nagai-Maruyama, Toshiki Watanabe, Emi Itoh[8], Shinya Watanabe[8], Akihiko Okayama[9], Kazunari Yamaguchi[10], Yasuteru Yamada[11], Shimeru Kamihira[11], Jun Tanaka[12], Yoichiro Iwakura[12], Masanori Hayami[13], Kazuo Suzuki[14], David Cooper[14]: [8]Division of Cancer Genomics, [9]Department of Internal Medicine, Miyazaki Medical College, [10]Blood Transfusion Service, Kumamoto University School of Medicine, [11]Depertmemt of Laboratory Medicine, Nagasaki University School of Medicine, [12]Center for Experimental Medicine, Division of Cell Biology, [13]Kyoto University Institute of Virus Research, [14]St. Vincent Hospital, Sydney.

1. Joint Study of Predisposing Factors for ATL development (JSPFAD)

As a member of the nation-wide collaborative study, our group has contributed to the work by serving as a processing center of the collected peripheral blood samples. After separating plasma and isolating PBMC, frozen pellets and extracted genomic DNA were stored to establish a bio-resource bank for HTLV-1-infected peoples. Furthermore, a reliable method to quantify the provirus DNA copy number in the PBMC was established using a real-time PCR system. In 2003, more than 400 samples were collected, and the proviral load in the PBMC was measured. Samples with higher proviral load (i.e., more than 20 copies per 100 PBMC) were subjected to analysis of clonality of the infected cells by Southern blotting. Results showed without exception that samples with more than 40 copies /100 PBMC showed mono-or bi-clonal expansion of HTLV-1-infected cells, indicating that proviral load more than 40 copies/100 PBMC is diagnostic for ATL (Aizawa et al., manuscript in preparation).

2. Expression profile analysis of Adult T cell leukemia cells by synthetic DNA microarray

In collaboration with JSPFAD and DR. Shinya Watanabe's group (Tokyo Medical and Dental University), we have been studying the expression profiles of ATL cells using as controls various cell lines, normal activated T cells, leukemic cells of other categories and unstimulated, resting PBMC. Array system was developed Dr. S Watanabe's group. The system is called as Synthetic DNA array. ATL samples were mainly provided by members of JSPFAD. Cluster analysis of the results clearly showed group of genes that are distinctly expressed in ATL cells. Genes whose expression characterizes ATL were narrowed to 66. Studies are now under way to develop a diagnostic system to identify transformed ATL cells in the virus carriers PBMC, as well as those to reveal genetic events pivotal to transformation of HTLV-1-infected T cells.

3. Inhibition of constitutive NF-κB activation by a novel NF-κB inhibitor DHMEQ as a strategy for molecular target therapy and chemoprevention of adult T-cell leukemia

Adult T-cell leukemia (ATL) is notorious for a poor prognosis. ATL is refractory to all modalities of currently available combination chemotherapy with a median survival time of about one year. Establishing a molecularly targeted therapy and preventive intervention in ATL development is essential. Mechanisms of transformation of virus-infected T lymphocytes are largely unknown, although a high provirus load is considered to be a risk factor for development of ATL. The tumor cells are characterized by strong constitutive activation of NF-κB. Taking advantage of p65NF-κB activation in ATL cells and virus-infected T cells in the peripheral blood, we tested NK-κB inhibitor DHMEQ. Results showed efficient induction of apoptosis of ATL cells and rescue of xenografted SCID mice without significant toxicity. NF-κB inhibition triggered antiproliferative and proapoptotic events, delineated by global transcriptional profiling and protein expression analyses. Furthermore, DHMEQ can reduce the provirus load in HTLV-1 carriers' PBMC, showing little cytotoxicity for normal PBMC and transient NF-κB inhibition. These results provide proof of principle for the therapeutic use of selective NF-κB inhibitors in ATL and DHMEQ may be a promising compound for translation of this strategy into clinical medicine. We conclude that this study provides the basis for a novel molecularly targeted therapy and preventive intervention for ATL. (Watanabe M et al., submitted)

5. CpG methylation of LTR as a regulatory mechanism of latency of human retroviruses

Transcriptional repression of heterologous genetic elements such as proviruses is often ob-

served concomitantly with their integration into chromatin in the host cell genome. Deacetylation of nucleosomal histones and methylation of cytosines, particularly in the dinucleotide 5′ CpG, negatively influence transcriptional activity in cis. Therefore, we have been investigating the regulatory roles of CpG methylation and chromatin structures in latency induction and reactivation of human retroviruses, HIV and HTLV-1.

a) CpG methylation-dependent and -independent regulation of HIV latency

(1) Reactivation of latent HIV is associated with CpG demethylation

The incidence of AIDS and AIDS-related mortality has decreased with availability of highly active anti-retroviral therapy. This has led to considerable optimism, however, it became evident that complete eradication of latent HIV in the reservoir pool is very difficult. Thus, invention of a novel therapy to overcome treatment resistance of latent HIV and to achieve containment or purging of HIV depends on a better understanding of mechanisms involved in latency and reactivation of HIV. DNA methylation has been implicated in HIV latency. Cytokines such as TNF-＜can induce HIV gene expression in HIV-infected T cell lines as well as in latently infected lymphocytes in vivo. Using HIV-infected T-cell lines and HIV transgenic mice, we obtained evidence that LPS-induced reactivation of heavily methylated provirus is cell cycle-dependent and is associated with demethylation of specific CpG sites located in the binding sites for CREB/ATF family transcription factors. Our evidence shows binding of a common factor(s) (other than known CREB/ATF factors) to sequences around these CpG sites, irrespective of the methylation status. Since protein binding can specify sites of DNA demethylation, our results suggest a mechanism for reactivation where extracellular signal-induced DNA replication results in demethylation of CpG sites that are protected from maintenance methylation by binding of CREB/ATF-like factors. The present study provides novel clues to delineation of the mechanism for signal-mediated demethylation and reactivation of HIV, and to design of a treatment strategy to contain or purge HIV (Tanaka et al., AIDS17, 167, 2003, Ishida et al., submitted to J Virol).

(2) Repression of HIV gene expression by the "repressive histone code"

Analysis of CpG methylation of integrated provirus in autopsied samples showed almost total absence of CpG methylation of the LTR. The results suggested other mechanisms than CpG methylation to stably repress viral gene expression. We have found a latently infected cell line, OM10.1, where the provirus LTR is totally unmethylated. Chromatin immunoprecipitation analysis (CHiP) revealed histone modification at the nucleosome B region that is compatible with the repressive histone code (inclusion of H1, absence of H3 acetylation), which changed into a permissive one afterTNF-＜ stimulation and induction of viral gene expression (absence of H1, hyperacetylation of H3 and H4). These results suggest presence of CpG methylation independent mechanisms for viral latency that is controlled by chromatin structure controlled by histone modification (Ishida et al., manuscript in preparation).

(3) Mechanisms of repression of HIV gene expression in vivo

To examine mechanisms of repression of viral gene expression, we studied samples obtained animal model of SHIV infection and those from Sydney cohort studies in collaboration with Dr. Hayami's group in Kyoto University and Dr. Cooper's group in St. Vincent Hospital, respectively. Results of monkey infection model revealed very low levels of CpG methylation of SHIV LTR region, even in the absence of plasma viremia. However, the levels of methylation showed an increase after 72 weeks of infection. The cytosine methylation was frequently in the non-canonical sites, that is out side of CpG motif, which are considered a target site of de novo methylation by Dnmt3. Human cohort samples are now under characterization. (Ishida et al., manuscript inpreparation).

b) Involvement of CpG methylation in the latency of HTLV-1

(1) Demethylation of 5'-LTR of the integrated HTLV-1 provirus in carriers with increased levels of provirus load

Since polyclonal expansion of HTLV-1-infected T cells has been well documented in patients with TSP/HAM or HU and dual carriers of HTLV-1 and *S. stercoralis*, we next investigated methylation status of 5′-LTR of provirus in the PBMC of these individuals. Results showed demethylation of the 5′-LTR which is correlated with the levels of provirus load in the PBMC which was determined by quantitative real time PCR method. These results suggested demethylation induced by signaling of some extracellular stimuli, since CpG methylation of the

provirus LTR in MT-1 cells showed demethylation following reactivation of virus gene expression by TPA treatment. As expected, stimulation by a combination of anti-CD3 antibody and IL-2 resulted in demethylation of CpG sites in the 5′-LTR in TL-om1 cells. Thus, polyclonal expansion of HTLV-1-infected T cells in these individuals appear to results from TCR and IL-2 signal-mediated growth of HTLV-1-infected cells that is associated with demethylation of 5′-LTR, where reactivation of virus expression and production of Tax protein may have conferred growth advantage on these cells (Koiwa et al., manuscript inpreparation).

III. Cytogenetic Studies

1. Detection of the integrated feline leukemia viruses in feline lymphoid tumor cell lines by fluorescence *in situ* hybridization

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Feline leukemia virus (FeLV) is an oncogenic type-C retrovirus associated with lymphoid and hematopoietic malignancies in cats. The FeLV-induced tumors are thought to be caused, at least in part, by somatically acquired insertional mutagenesis in which the integrated provirus activates a proto-oncogene or disrupts a tumor suppressor gene. To enumerate and map the acquired proviral insertions in the genome of feline lymphoma cell lines infected with (FeLV), fluorescence *in situ* hybridization was applied on the metaphases from a feline thymic lymphoma cell line (FT-1) at first using an 8.4kb FeLV-A/Glasgow-1 proviral genome as a probe. Six loci of chromosomal regions, A2p22, B2p14, B4p15, D4q23, E1p13, and E2p13, were suspected to be positive for FeLV integration. Simultaneously, southern blot hybridization using a probe specific for the long terminal repeat U3 region of exogenous FeLV revealed multiple copies (at least six copies) of integrated FeLV proviral genomes in FT-1 cells. Two other lymphoid cell lines, FL-74 and KO-1, were tested and also suspected to contain six possible integration sites. Mapped integration sites were varied in three cell lines.

2. Chromosomal assignment of novel genes in mouse and rat

Hitoshi Satoh, Masahiko Fujiwara[15]: [15]Pathology Division, KOTOBIKEN Medical Laboratory Inc.

The following six novel genes were mapped this year: Murine *Ankrd2* (ankyrin repeat domain 2 (strech responsive muscle)) to mouse chromosome 19C3-D1 and rat *Ankrd2* to rat chromosome 1q51-q53. The rat *TSLC1* and two *TSLC1*-like tumor suppressor genes, *TSLL1* and *TSLL2*, were assigned to rat chromosomes, 8q13-q22, 13q24, and 1q21.3-q22.1, respectively. Mouse calcium-calmodulin-dependent kinase I-like kinase (*CKLiK*) gene to mouse chromosome 2A1-A3.

IV. Service activities of the Histopathology and Cytogenetics Service Laboratory

Tomoko Nakajima, Hitoshi Satoh, Shigeo Mori

Following histochemical technical services were conducted during April 2002-March 2004. Frozen blocks: 35 specimens; Paraffin blocks: 632 specimens; Thin section cutting: 3,303 sections; Hematoxylin and Eosin staining: 1,393 slides; Immunohistochemical staining: 288 cases.

One case of request for chromosome analysis was processed. The first query was presented July 8, 2002. The question was to make sure the origin of a tumor mass in muscle tissue of the lower limbs of a SCID mouse yielded after injection of ES cells derived from the crab-eating macaque, *Macaca fascicularis*. As a result, the tumor cells were consisted from macaque chromosomes, the chromosome number was normal (2n $=42$) but contained structural abnormalities. Then the second request was presented Sept 9, 2002. Second question was when the mutant cell line was arose. Tracing search made the final conclusion that the original macaque ES cell line, CMK6, had normal karyotype and the late passage of the cell line showed mosaic kayotype of 42, XY and 42, XY, $+10$, -18, therefore, the mutation was occurred during the maintenance of the cell lines *in vitro* and clonally expanded in SCID mouse.

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分子発癌分野

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Gene expression is largely regulated by signal transduction triggered by various stimulations. Several lines of evidence indicate that genetic defects of molecules involved in the signal transduction or the gene expression lead to abnormal cell differentiation or tumor formation. Thus, we are mainly focusing on three such proteins including Tumor necrosis factor receptor-associated factor (TRAF), E coli. Ras-like protein (ERA), and WT-1. Our current studies indicate that these three proteins play critical roles in cell proliferation and differentiation. Therefore, elucidation of how these proteins work is definitely required for understanding onset of diseases.

1. The molecular mechanism of the regulation of Rel/NFκB transcription factor

Kazuyuki Meguro, Jin Gohda, Taishin Akiyama and Jun-ichiro Inoue

Transcription factor Rel/NFκB binds specifically to a decameric motif of nucleotide, κB site, and activates transcription. The κB sites are present in the enhancers of many genes including the immunoglobulin κ light chain gene, Interleukin-2 receptor α-chain gene, β-interferon gene and LTR of human immunodeficiency virus, which are involved in the regulation of the immune system. Recent studies showed that the activation of Rel/NFκB links to the anti-apoptotic signal. Thus, it is essential to understand the molecular mechanisms by which Rel/NFκB transcription factor is regulated. The activation of Rel/NFκB has been demonstrated to be carried out post-translationally upon extracellular stimuli through membrane receptors such as interleukin-1 receptor, tumor necrosis factor (TNF) α receptor or CD40. Rel/NFκB forms a complex with regulatory protein, IκB, and is sequestered in the cytoplasm prior to stimulation. Upon stimulation, IκB is rapidly phosphorylated on two specific serine residues by IκB kinase (IKK) complex followed by Lys 48-linked ubiquitination and proteasome-dependent degradation. Rel/NFκB subsequently translocates to the nucleus to activate transcription of target genes. This project is to identify a molecule that transduces signal from membrane receptor (IL-1 receptor, TNF receptor) to Rel/NFκB /IκB complex. We have previously identified upstream activators of Rel/NFκB, tumor necrosis factor receptor-associated factor (TRAF) 5 and TRAF6. We have also found that MAPKKK, TAK1 is downstream of TRAF6 and becomes activated upon dimerization of TRAF6 and that Ubc13/Uev1A complex, together with TRAF6, catalyses the formation of a Lys63 (K63)-linked polyubiquitin chain that conjugates TRAF6 and mediates IKK activation through unique proteasome-independent mechanism. TRAF6 becomes ubiquitinated upon IL-1 stimulation. We also found that TRAF6 becomes conjugated by a Lys48 (K

48) polyubiquitin chain and degraded upon stimulation. Therefore, it is possible that poly ubiquitin chain regulates TRAF6 both positively and negatively. To elucidate mechanisms of the ubiquitination-mediated regulation of TRAF6, we are trying to identify ubiquitination sites of TRAF6. Several lines of evidence suggest that TRAF6 is able to activate some kinase other than TAK1. To identify such kinases, we have set up cell-free system in which IKK becomes activated upon addition of recombinant TRAF6 protein. By using this system, we are trying to identify the molecule(s) that function between TRAF6 and the IKK complex by biochemical protein purification. To elucidate how TRAF6 transduces signals, crystallographic studies are required. To do this end, we have prepared large amount of the purified recombinant TRAF 6 protein produced by baculovirus expression system. We are trying to set up experimental condition for crystallization.

2. Role of TRAF6 in osteoclastogenesis

Jin Gohda, Kazuyuki Meguro, Taishin Akiyama and Jun-ichiro Inoue

We have previously identified TRAF6 as a putative signal transducer of CD40 by yeast two-hybrid system. However, it is not known whether TRAF6 plays pivotal roles in immune system in vivo, and whether it has additional functions yet to be identified. Thus, we have generated TRAF6-deficient mice by homologous recombination-based gene knockout. TRAF6-/- mice exhibit severe osteopetrosis due to the lack of osteoclasts in bone. Ex vivo co-culture experiments revealed the inability of osteoclast precursor cells derived from TRAF6-/- mice to differentiate to functional osteoclasts in response to osteoclast differentiation factor (ODF), indicating that RANK, a receptor of ODF, utilizes TRAF6 for signal transduction. The number of sIgM+B 220+ B cells is markedly reduced in both spleen and bone marrow, whereas T cell development is normal in the absence of TRAF6. However, thymocytes from TRAF6 deficient mice do not proliferate in response to IL-1. IL-1 does not activate JNK and NFκB of TRAF6 deficient embryonic fibroblast, while TNFa does. These results indicate that TRAF6 is essential for IL-1 signaling. Furthermore, TRAF6-/- mice are defective in lymph-node organogenesis. Thus, TRAF6 is an essential component of ODF signaling pathway, and also show that TRAF6 plays pivotal roles in immune and inflammatory systems *in vivo*. To understand how TRAF6 activates downstream pathways that lead to osteoclastogenesis, we tried to identify functional domains of TRAF6 under physiological conditions established by appropriate expression of TRAF6 mutants in TRAF6-deficient cells. In IL-1 and LPS signaling pathways, the RING finger and first zinc finger domains are not required for NFκB activation but required for full activation of MAPK. However, IL-1 and LPS signals utilize distinct regions within the zinc finger domains of TRAF6 to activate NFκB. By expressing various TRAF6 mutants in TRAF6-deficient progenitors of osteoclasts, we found that the RING finger domain is not required for differentiation of splenocytes to multinuclear osteoclasts but essential for osteoclast maturation. Thus, TRAF6 plays essential roles in both differentiation and maturation of osteoclasts by activating various kinases via its multiple domains. Although we learned that RANK-TRAF6 linkage is essential for osteoclastogenesis, why CD40 and IL-1 signals, which activate TRAF6, are unable to induce formation of osteoclasts remains to be elucidated. To address this question, we have generated various RANK mutants to find out the structural characteristics that are specifically required for osteoclastogenesis.

3. Regulation of TRAF6 by TRAF6-binding protein, TIFA

Takayuki Matsumura, Jin Gohda, Taishin Akiyama, Kentaro Semba and Jun-ichiro Inoue

TRAF6 transduces signals from members of the Toll/IL-1R family by interacting with IL-1 receptor-associated kinase-1 (IRAK-1). However, the molecular mechanisms underlying regulation of the IRAK-1/TRAF6 interaction are largely unknown. To understand the molecular mechanisms of TRAF6-mediated signals, we have searched for the TRAF6-interacting protein by the yeast two-hybrid system. We have identified TIFA, a TRAF-interacting protein with a forkhead-associated (FHA) domain. The FHA domain is a motif known to bind directly to phosphothreonine and phosphoserine. In transient transfection assays, TIFA activates NFκB and JNK. However, TIFA carrying a mutation that abolishes TRAF6 binding or mutations in the FHA domain that are known to abolish FHA domain binding to phospho-peptide fails to activate NFκB and JNK. TIFA, when overexpressed, binds both TRAF6 and IRAK-1 and significantly enhances the IRAK-1/TRAF6 interaction. Furthermore, analysis of endogenous proteins indicates that TIFA associates with TRAF6 constitutively, whereas it associates with IRAK-1 in an IL-1 stimulation-dependent manner *in vivo*. A dominant negative mutant of TIFA blocks NFκB activation induced by signaling from both IL-1R

and TLR4. Thus, TIFA is likely to mediate IRAK -1/TRAF6 interaction upon IL-1 stimulation and LPS stimulation. Recently, we have identified TIFAB, a TIFA-related protein that associates with TIFA but not with TRAF6. TIFAB forms a oligomeric complex with TIFA and TRAF6 and blocks TIFA-induced NFκB activation. Thus, TIFAB could be a negative regulator of TIFA.

4. Role of TRAF6 in TLR signaling

Jin Ghoda, Taishin Akiyama and Jun-ichiro Inoue

Toll-like receptors (TLRs) signaling pathways are mediated by the TIR domain containing adopter molecules, MyD88, TIRAP, and TRIF. TRAF6 was shown to activate NFκB and MAPKs downstream of these TIR domain proteins to induce inflammatory cytokines expression. However, the precise role of TRAF6 in individual TLR signaling has never been analyzed genetically. By analyzing macrophages isolated from TRAF6-deficient mice, we demonstrate here the following: In the absence of TRAF6, (1) TLR5, TLR7, and TLR9 ligands failed to induce NFκB and MAPKs activations and inflammatory cytokine production; (2) TLR2 and TLR4 ligands produced normal level of cytokines, although NFκB and MAPKs activations were delayed; (3) The responses to TLR3 ligand were normal. These results indicate the presence of unidentified TRAF6-independent pathway that leads to the production of inflammatory cytokines specifically downstream of MyD88/TRIP complex and that TRAF6 is dispensable for the TRIF signaling..

5. Identification and functional characterization of vertebrate homologues of *E. coli* Ras-like protein (ERA) and Developmentally Regulated GTP-binding protein (DRG)

Taishin Akiyama, Taizo Ishikawa, Kosuke Ishikawa, Sakura Azuma, Jin Gohda, Kentaro Semba and Jun-ichiro Inoue

In this year, subcellular localization of ERA has been determined. Immunoflourescence analysis using anti-ERA specific antibody revealed that ERA is in mitochondria. When we fused the N-terminal region of ERA to GFP, GFP was localized in mitochondiria, indicating that N-terminal region of ERA acts as a mitochondria localization signal. Furthermore, sonication and protenaseK treatment of mitochondria fraction of Hela cell lysates revealed that ERA attaches to the matrix side of mitochondria inner membrane.

drg gene was identified by subtractive cDNA cloning as a gene highly expressed in mouse embryonic brain. The DRG protein has G-motifs that are known to constitute the core of the GTPase domain, although only GTP-binding activity has been shown for DRG. On the basis of structural features, DRG is classified as a small branch of the Obg family, which forms one large branch of the GTPase superfamily (Leipe et al., 2002). Although other large branches such as the classic translation factor family (EF-Tu/EF-κ subfamily, etc.) and the extended Ras-like family (Ras/Rab subfamily, etc.) have been well characterized, the physiological roles of the Obg family members remain to be elucidated. Genes homologous to the mouse *drg* were subsequently identified in a wide variety of species in eucaryotes and archaea, suggesting that DRG may play a fundamental cellular role. Two distinct members, DRG2 and DRG1, comprise DRG family in eucaryotes. However, all archaea's complete genome sequences available on the Entrez Genome database contain only a single *drg* related gene, to which it is difficult to assign *drg 2* or *drg1*. It is likely that *drg* gene was separated into two groups in evolutional processes. Therefore, the two DRG proteins in eucaryotes may serve distinct functions. However, no clear difference has been reported in their expression patterns. Thus, we first cloned *Xenopus drg2 (Xdrg2)* and examined the temporal and spatial expression patterns of *Xdrg2* mRNA in comparison to those of *Xdrg1*. Both *Xdrg2 and Xdrg1* are induced at late gastrula and subsequently increase during later stages of embryos (stage 13-41). Whole-mount *in situ* hybridization showed that *Xdrg2 and Xdrg1* expression patterns are almost identical except that only *Xdrg2* expression is detected in the stage 22 pronephric anlage. Strong transcripts of both genes are also observed at this stage in neural crest cells, blood islands and developing eyes, and in brain, eyes, otic vesicle, branchial arches, pronephroses, spinal cord, notochord, head mesenchyme and somites at stages 27 and 32. Northern blot analysis of adult tissues revealed that both genes are expressed highly in ovary and testis and rather moderately in other organs, except that *Xdrg1* transcripts are scarcely detected in heart, lung and liver. Accordingly, transcription or stability of *Xdrg2 and Xdrg1* mRNAs may be regulated by different mechanisms. In addition, by generating recombinant XDRG2 and XDRG1 proteins, we found the RNA binding activity of these proteins *in vitro*. Our results suggest that the DRG proteins may play their physiological roles via RNA binding.

6. Synthesis of compounds which specifically

regulates the activity of Rel/NFκB transcription factors

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Chemical compounds are designed and synthesized to test their ability to modulate the signal transduction pathways linked to the physiological response of cells. One of the research targets is the signaling linked to Rel/NFκB activation. We have previously synthesized various compounds comprising dimethylaminopyridine and histidine. One of the compounds inhibits the activation of Rel/NFκB by TNF-α only when the compound is within the cell. This year we have synthesized oligonucleotides 26-mers including single 5-formyl-2′-deoxy-uridine or 5-formyl-2′-*O*-methyluridine in place of thymidine at the κB site. One of the 26-mer with 5-formyl-2′-deoxy-uridine was critically discriminated by the NFκB p50 homodimer. These findings should provide a better understanding of the NFκB-DNA interaction and give us basic information for drug design. Our another trial is to synthesize a number of derivatives of bleomycin conjugated with oligonucleotides that preferentially associate with κB motif present in the enhancer of several genes to form a triple helix structure and cut out double strand DNA. Our goal of this project is to use this compound for the inactivation of human immunodeficiency virus (HIV), since HIV has tandem copies of κB sites in its own LTR. We have already succeeded in cutting out double strand DNA by forming triple helix structure using the oligonucleotides derived from the regulatory element of interleukin-2 receptor α-chain. We are trying to apply this system to HIV.

7. Function of a chimeric EWS-WT1 oncoprotein

Kentaro Semba, Shinya Watanabe[3], Sakura Azuma and Jun-ichiro Inoue: [3]Department of Clinical Informatics, Tokyo Medical Dental University School of Medicine

WT1 gene was originally identified as a tumor suppressor gene for Wilms' tumor, a pediatric kidney tumor. Several lines of evidence show that WT1 is required for development of various organs including kidney, gonad, spleen, heart and retina. WT1 is a transcription factor, which binds to the specific DNA sequence via its zinc finger domain and then regulates transcription of target genes. WT1 is also involved in DSRCT, desmoplastic small round cell tumor. In this tumor, the WT1 gene is fused to the EWS gene as a result of chromosomal translocation between chromosome 11 and 22. This fusion gene product consists of three of the four zinc fingers of WT1 and transcriptional activation domain of EWS, and thus it acts as a transcriptional activator. We are searching for target genes of EWS-WT1 using synthetic polynucleotide microarray, which covers more than 20,000 human genes. Recently, we found that two genes encoding membrane proteins, TALLA1 and BAI1 were highly expressed in EWS-WT1-expressing cell lines and in DSRCT tumor specimens. BAI1 was originally identified as a target of p53. This seven-transmembrane protein is thought to inhibit angiogenesis mediated by the extracellular thrombospondin domain. The reason why the oncogenic EWS-WT1 protein induces anti-oncogenic BAI1 is currently under investigation. Another candidate is TALLA1, a member of tetraspanin family. This family member usually forms a complex with integrin and modulates integrin-mediated signals. Since TALLA1 also binds to PI4K, TALLA1 itself may directly mediate signals. We are interested in the function of TALLA1 in movement and attachment of cancer cells.

8. Function of WT1 in sex determination

Kentaro Semba, Shinya Watanabe[3], Sakura Azuma and Jun-ichiro Inoue: [3]Department of Clinical Informatics, Tokyo Medical Dental University School of Medicine

In humans, as in other mammals, sex determination is controlled by a dominant switch termed TDF for Testis Determining Factor. The SRY gene is thought to be the TDF, which encodes a transcription factor with one HMG box as a DNA binding domain. Mutations in the SRY gene have been identified in 15% cases of XY sex reversal in humans. Introduction of mouse SRY gene (Sry) into XX female mice induced testis differentiation and subsequent male development. However, little is known about mechanism of transcriptional regulation by SRY. WT1 mutations have frequently been observed in Denys-Drash syndrome (DDS) patients with urogenital malformation. During analysis of WT 1-associated proteins, we found that WT1 bound to several types of transcription factors. One of them was Sox30, which encodes a novel transcription factor with one HMG box. Further analysis showed that WT1 bound to its HMG box. This observation prompted us to analyze

interaction between WT1 and SRY. To date, we showed i) WT1 binds to SRY *in vitro* and in cultured cells, ii) this binding is mediated by the zinc finger domain of WT1 and the HMG box of SRY, iii) WT1 and SRY synergistically activates transcription from a promoter which contains SRY binding sequence, while WT1 mutants found in DDS did not show this activity, iv) the SRY gene itself is a candidate for target genes which are regulated by WT1 and SRY synergy, v) WT1 is recruited on SRY-binding sequence in a SRY-dependent manner, while recruitment of DDS mutants is significantly reduced, vi) one SRY mutant (L163ter) found in a Japanese family with complete gonadal dysgenesis had reduced affinity with WT1. These observations suggest that WT1 and SRY interaction plays an important role in early gonadal development and its disease. We are currently searching for the target genes which are induced by coexpression of WT1 and SRY.

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Our major projects are to clarify (1) signal transduction systems in cell growth, reorganization of cytoskeleton and morphogenesis (2) roles of inositolphospholipids as biomodulators. Currently we are focusing on the clarification of mechanisms in cortical actin reorganization and cell movement, and the roles of inositolphospholipid-mediated signallings in trafficking and a variety of physiological functions using knockout mice.

1. Phospholipase Cδ1 Is Required for Skin Stem Cell Lineage Commitment

Yoshikazu Nakamura[1], Kiyoko Fukami[1], Haiyan Yu[1], Kei Takenaka[1], Yuki Kataoka[2], Yuji Shirakata[3], Shin-Ichi Nishikawa[4], Koji Hashimoto[3], Nobuaki Yoshida[2], and Tadaomi Takenawa[1]: [2]Div. Gene Expression and Regulation, [3]Department of Dermatology, Ehime University School of Medicine, [4]Department of Molecular Genetics, Kyoto University Graduate School of Medicine

Phosphoinositide-specific phospholipase C (PLC) is a key enzyme in phosphoinositide turnover and is involved in a variety of physiological functions. Here we report that PLCδ1-deficient mice undergo progressive hair loss in the first postnatal hair cycle. Epidermal hyperplasia was observed, and many hairs in the skin of PLCδ1-deficient mice failed to penetrate the epidermis and became zig-zagged owing to occlusion of the hair canal. Subsequently, many cysts that developed as a result of transdifferentiation of the hair follicles into interfollicular epidermis as well as hyperplasia of sebaceous glands were observed. Molecular markers of interfollicular epidermis were expressed in these cysts. From these results, we conclude that PLCδ 1 is required for skin stem cell lineage commitment.

2. WAVE2 involved in directed cell migration is required for cardiovascular development

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WAVE2, a Wiskott-Aldrich syndrome protein (WASP)-related protein, is crucial for Rac-induced membrane ruffling, which is important in cell motility[1–4]. Cell movement is essential for morphogenesis; however, it is unclear how cell movement is regulated or related to morphogenesis. Here we show the physiological functions of WAVE2 by disruption of the *WAVE2* gene in mice. WAVE2 was expressed predominantly in vascular endothelial cells during embryogenesis. *WAVE2*$^{-/-}$ embryos showed hemorrhages and died around E10. WAVE2 deficiency had no significant effect on vasculogenesis, but it decreased sprouting and branching of endothelial cells from existing vessels during angiogenesis. In *WAVE2*$^{-/-}$ endothelial cells, cell polarity formed in response to vascular endothelial growth factor, but formation of lamellipodia at leading edges and capillaries was severely impaired. These findings suggest that WAVE2-regulated actin reorganization is required for proper cell movement and that lack of functional WAVE2 impairs angiogenesis *in vivo*.

3. Essential role of the C. elegans Arp2/3 complex in cell migration during ventral enclosure.

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Migration of cells through the reorganization of the actin cytoskeleton is essential for morphogenesis of multicellular animals. In a cell culture system, the actin-related protein (Arp) 2/3 complex functions as a nucleation core for actin polymerization when activated by the members of the WASP (Wiskott-Aldrich syndrome protein) family. However, the regulation of cell motility in vivo remains poorly understood. Here we report that homologues of the mammalian Arp2/3 complex and N-WASP in Caenorhabditis elegans play an important role in hypodermal cell migration during morphogenesis, a process known as ventral enclosure. In the absence of one of any of the C. elegans Arp2/3 complex subunits (ARX-1, ARX-2, ARX-4, ARX-5, ARX-6 or ARX-7) or of N-WASP (WSP-1), hypodermal cell migration led by actin-rich filopodia formation is inhibited during ventral enclosure owing to the reduction of filamentous actin formation. However, there is no effect on differentiation of hypodermal cells and dorsal intercalation. Disruption of the function of ARX-1 and WSP-1 in hypodermal cells also resulted in hypodermal cell arrest during ventral enclosure, suggesting that their function is cell autonomous. WSP-1 protein activated Arp2/3-mediated actin polymerization in vitro. Consistent with these results, the Arp2/3 complex and WSP-1 colocalized at the leading edge of migrating hypodermal cells. The stable localization of WSP-1 was dependent on the presence of Arp2/3 complex, suggesting an interaction between the Arp2/3 complex and WSP-1 in vivo.

4. SKIP negatively regulates insulin-induced GLUT4 translocation and membrane ruffling formation

Takeshi Ijuin and Tadaomi Takenawa

Skeletal muscle and kidney enriched inositol phosphatase (SKIP) is an inositol polyphosphate 5-phosphatase that hydrolyzes phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P_3) to downregulate intracellular levels. In this study, we show that SKIP inhibits phosphoinositide 3-kinase (PI 3-kinase) signaling in insulin-stimulated CHO cells. Ectopic expression of SKIP did not inhibit insulin-induced PI(3,4,5)P_3 generation but did rapidly decrease insulin-induced intracellular PI(3,4,5)P_3 levels compared to control cells. Further, insulin-induced phosphorylation of some downstream targets such as Akt and p 70 S6 kinase was markedly inhibited by the ectopic expression of SKIP, whereas phosphorylation of MAP-kinase was not. In contrast, downregulation of intracellular SKIP levels by antisense oligonucleotides dramatically enhanced Akt (PKB) phosphorylation in response to insulin, suggesting that endogenous SKIP downregulates insulin signaling. SKIP also markedly inhibited GLUT4 translocation and membrane ruffle formation. We conclude that SKIP preferentially regulates glucose transport and actin cytoskeletal re-arrangement among a variety of PI (3,4,5)P_3 downstream events.

5. Phospholipase Cdelta4 is required for Ca^{2+} mobilization essential for acrosome reaction in sperm.

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Zona pellucida (ZP)-induced acrosome reaction in sperm is a required step for mammalian fertilization. However, the precise mechanism of the acrosome reaction remains unclear. We previously reported that PLCdelta4 is involved in the ZP-induced acrosome reaction in mouse sperm. Here we have monitored Ca^{2+} responses in single sperm, and we report that the $[Ca^{2+}]i$ increase in response to ZP, which is essential for driving the acrosome reaction in vivo, is absent in PLCdelta4$^{-/-}$ sperm. Progesterone, another physiological inducer of the acrosome reaction, failed to induce sustained $[Ca^{2+}]i$ increases in PLCdelta4$^{-/-}$ sperm, and consequently the acrosome reaction was partially inhibited. In addition, we observed oscillatory $[Ca^{2+}]i$ increases in wild-type sperm in response to these acrosome inducers. Calcium imaging studies revealed that the $[Ca^{2+}]i$ increases induced by exposure to ZP and progesterone started at different sites within the sperm head, indicating that these agonists induce the acrosome reaction via different Ca^{2+} mechanisms. Furthermore, store-operated channel (SOC) activity was severely impaired in PLCdelta4$^{-/-}$ sperm. These results indicate that PLCdelta4 is an important enzyme for intracellular $[Ca^{2+}]i$ mobilization in the ZP-induced acrosome reaction and for sustained $[Ca^{2+}]i$ increases through SOC induced by ZP and progesterone in sperm.

6. Phospholipase C-L2, a novel PLC like protein, negatively regulates B cell activation

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Several Phospholipase C (PLC) isoforms have been found in immune cells and are known to play important functions dowmstream of immunogloblin (Ig) superfamily receptors by regulating the calcium/protein kinase C signaling pathway. PLC-L2 is a novel PLC like protein, which does not have PLC activity, though it is very homologous to PLC δ. PLC-L2 is highly expressed in hematopoetic cells. However, to date, its physiological roles in the immune system have not been clarified yet. To elucidate the function of PLC-L2 in the immune system, we generated the *PLC-L2* gene deficient mice. PLC-L2 deficient splenic mature B cells are hyper proliferative and show enhanced calcium influx in response to B cell receptor (BCR) cross-linking. PLC-L2 deficient splenice B cells also have enhanced Ig secretion *in vitro*. PLC-L2 deficient mice have stronger T-independent II antigen response compared to wild type mice *in vivo*. Higher titer of anti-nuclear antigen IgM is observed in aged PLC-L2 deficient mice, although they had showed no apparent autoimmune disease. Surprisingly, in contrast to conventional B cells, B1-a cells in the peritoneal cavity is significantly reduced. These results indicate that PLC-L2 negatively regulates B cell activation, suggesting that PLC-L2 is involved in B cell homeostasis and tolerance.

7. Translocation of N-WASP by Nuclear Localization and Export Signals into the Nucleus Modulates Expression of HSP90

Shiro Suetsugu and Tadaomi Takenawa

N-WASP regulates the actin cytoskeleton through activation of the Arp2/3 complex. N-WASP localizes at the cell periphery, where it controls actin polymerization downstream of signal molecules such as adapter proteins, Cdc 42, Src family kinases, and phosphoinositides. N -WASP also localizes in the nucleus; however, the role of N-WASP in the nucleus is unclear. Here, we show that localization of N-WASP is controlled through phosphorylation by Src family kinases in which phosphorylated N-WASP is exported from the nucleus in a nuclear export signal (NES) and leptomycin B-dependent manner. N-WASP had nuclear localization signal (NLS) at its basic region and NES close to the phosphorylation site by Src family kinases, indicating that phosphorylation controls the accessibility to the NES through conformational changes. Increased levels of unphosphorylated N-WASP in the nucleus suppressed expression of HSP90 and transcription from a heat shock element (HSE). N-WASP bound heat shock transcription factor (HSTF) and enhanced the HSTF association with HSE. In addition, nuclear N-WASP was present in the protein complex that associates with HSE, suggesting that N-WASP

participates in suppression of HSP90 transcription. Increased levels of unphosphorylated N-WASP also decreased the activities of Src family kinases in cells but not in experiments in vitro with pure N-WASP and Fyn. Because HSP90 is essential for the activities of Src family kinases, these results suggest that localization of N-WASP modulates Src kinase activity by regulating HSP90 expression.

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Carbohydrates bound to the polypeptide chains widely occur in the body, and their structures change during development and differentiation of the cells and under pathological conditions. Our major research interest is to elucidate direct and indirect roles of the carbohydrates in cell-cell interaction. For this end, we are currently focusing on structure and function of carbohydrate binding proteins and their ligands, regulation of protein structures and functions by glycosylation, and development of new methods and tools for protein-carbohydrate interaction.

1. Regulation of polylactosamine synthesis and branch formation of N-glycans *in vivo*

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β4GalT-1 knockout mice are quite useful not only for the functional analysis of the enzyme involved in physiological and pathological processes but also for the analysis of regulation of glycosylation *in vivo*. Previous *in vitro* studies using various human GalT enzymes suggested that the enzymes play important roles not only in the β1, 4-galactosylation of terminal GlcNAc residues of the trimannosyl core of N-glycans and of core 2 O-glycans but also in the synthesis of polylactosamines. However, distinct contribution of each GalT to the polylactosamine synthesis has so far been shown by *in vitro* studies using recombinant enzymes. Competition between different glycosyltransferases is one of the factors regulating glycosylation, and *in vitro* studies indicated that β4GalT interferes with addition of GlcNAc to the trimannosyl core of N-glycans by N-acetylglucosaminyltransferases IV and V (GnT-IV and V). Since mouse erythrocyte glycoproteins express a series of biantennary to tetraantennary glycans and polylactosamines, we analyzed in detail the structural change of β 4GalT-1 deficient mice by focusing on if the deficiency affects synthesis of the polylactosamines and the branched N-glycans in the erythrocyte glycoproteins. The results indicated that moderately decreased galactosylation occurs in β4Gal-T1$^{-/-}$ mice and the synthesis of polylactosamines in β4Gal-T1$^{-/-}$ mice is comparable to that in control β4Gal-T1$^{+/-}$ mice. The increased expression of more branched N-glycans was observed in β4Gal-T1$^{-/-}$ mice, and its extent was more remarkable in elder β4Gal-T1$^{-/-}$ mice (28 weeks old) than in younger β4Gal-T1$^{-/-}$ mice (6 to 9 weeks old). In relation to this issue, the less galactosylation of biantennary glycans were observed in the elder group, suggesting that β4 GalTs actually compete with N-acetylglucosaminyltransferases IV and V in erythroid cells. In contrast, approximately 80% of core 2 O-glycans were not β1, 4-galactosylated regardless of age of the knockout mice. These results suggest that β4GalT-1 expressed in erythroid cells may regulate a constant branch formation of N-glycans and plays a predominant role in β1, 4-galactosylation of core 2 O-glycan.

2. Carbohydrate recognition molecules involved in mammalian fertilization

Etsuko Mori, Naoei Yoshitani, Chi Chih Lin, Seiichi Takasaki

Mammalian eggs are surrounded by an extracellular matrix called the zona pellucida (ZP) which consists of a few glycoproteins. The ZP plays important roles in sperm-egg binding, induction of sperm acrosome reaction, and block to polyspermy. On the basis of our previous results that multiple mechanisms recognizing glycans on the ZP are working in the process of sperm-egg interaction, we are trying to identify the putative carboydrate recognition molecules. The solubilized plasma membrane proteins of boar sperm were applied on the plastic well coated with various oligosaccharide probes, and the proteins bound to the well were analyzed by SDS-PAGE. We found that two proteins bind to the well coated with the probe containing galactosylated N-glycans. cDNA encoding one of the two putative carbohydrate binding proteins was cloned, and its sequence showed the protein is rich in cysteine residues. We produced the recombinant protein, and are analyzing its activity.

3. Impaired selectin-ligand biosynthesis and reduced inflammatory responses in β-1,4-galactosyltransferase-1-deficient mice

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Leukocyte recruitment in acute and chronic inflammation is characterized by sequential cell adhesion and activation events. It is known that E-, P- and L-selectins mediate initial leukocyte-endothelial cell adhesion events required for this process. Each selectin recognizes related but distinct counter-receptors displayed by leukocytes and/or the endothelium. These counter-receptors called selectin ligands correspond to specific glycoproteins whose activity is enabled by carefully controlled post-translational modifications. Carbohydrate chains such as sialyl Lewis x (sLe^x), which are mainly expressed at the terminal of N-acetyllactosamine repeats on core 2 O-linked glycans, are essential for binding to selectins. Several glycosyltransferases are successively acting to extend N-acetyllactosamine repeats and to synthesize sialyl Lewis x structure, and β-1,4-galactosyltransferase (GalT) plays a key role in these processes. However, seven members of GalT genes have been isolated so far and individual roles of them including selectin ligand biosynthesis remain to be elucidated. Using GalT-1-deficient mice, we evaluated the contribution of GalT-1 in selectin ligand biosynthesis. More than 80% of core 2 O-glycans in leukocyte membrane glycoproteins of GalT-1-deficient mice were not galactosylated by β-1,4 linkage and binding of soluble P-selectin to their leukocytes was significantly reduced, indicating that biosynthesis of selectin ligands for P-selectin was impaired. On the other hand, weight of peripheral lymph nodes and the in vivo lymphocyte homing assay were comparable between GalT-I-deficient and control mice. These results suggest that lymphocyte homing is not affected in GalT-I-deficient mice. Thus, core 2 O-glycans are dispensable for the biosynthesis of L-selectin ligands. Acute and chronic inflammatory responses were suppressed and infiltration of neutrophils into inflammatory sites was largely reduced during inflammatory responses in GalT-1-deficient mice. Our results clearly demonstrate that GalT-1 is a major galactosyltransferase responsible for P-selectin ligand biosynthesis and that inflammatory responses of GalT-1-deficient mice are impaired due to the defect in selectin ligand biosynthesis.

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Our major research interest is to elucidate the molecular mechanisms involved in signal transduction for malignant transformation and for tumor angiogenesis. During several years we have been mainly focusing on the functions of tyrosine kinases [VEGF receptors-1 and -2 (Flt-1/KDR), FGF receptor and Bcr-Abl], adaptor proteins, FRS2 and Vav, and VEGF-independent regulation of angiogenesis. This year we showed that the basal level of VEGF-VEGFR signaling is essential for tubular formation in vitro and that the BMP-4 plays an important role in physiological regression of vascular network in pupillary membrane of eye. We are also examining the biological role of FRS2 in embryogenesis, and our data suggest that this adaptor molecule is essential for FGF-receptor signaling in the anterior-posterior axis formation in early embryogenesis.

1. Bone Morphogenetic Protein 4 Mediates Apoptosis of Capillary Endothelial Cells during Rat Pupillary Membrane Regression.

Mari Kiyono and Masabumi Shibuya

Programmed capillary regression is essential for development, but little is known about the mechanism behind this phenomenon. In this study, we characterized the molecular determinants of capillary regression utilizing the pupillary membrane (PM) in the newborn rat's eye. We observed in the 1-day-culture system that apoptotic endothelial cells decrease in number with the addition of a natural antagonist, Noggin, strongly suggesting the involvement of the bone morphogenetic protein (BMP) family in PM regression. In addition, the lens-conditioned medium (Lens-CM) induced apoptosis of HUVE cells and inhibited endothelial tubulogenesis, which were completely blocked by both Noggin and the BMP4-specific neutralizing antibody. Activation of BMP4 pathway in endothelial cells was confirmed by both the up-regulation of Msx genes correlated with apoptosis and the translocation of Smad1 into the nucleus. We showed a transient expression of BMP4 in Lens-CM by immunoprecipitation assay. Furthermore, the transcorneal injection of BMP4 in rats enhanced the apoptosis of PMs, while that of Noggin attenuated it. These results indicate that BMP4 patways play pivotal roles in capillary regression in a paracrine manner between lens and PMs.

2. Vascular endothelial growth factor receptor -2: Its unique signaling and specific ligand, VEGF-E.

Masabumi Shibuya

Vascular endoghelial growth factor receptor-2 (VEGFR-2/KDR/Flk-1) is a high-affinity recep-

tor for vascular endothelial growth factor-A (VEGF-A), and mediates most of the endothelial growth and survival signals from VEGF-A. VEGFR-2 has a typical tyrosine kinase receptor structure with seven immunoglobulin (Ig)-like domains in the extracellular region, as well as a long kinase insert in the tyrosine kinase domain. We have recently shown that it utilizes a unique signaling system for DNA synthesis in vascular endothelial cells, i.e. a phospholipase Cγ-protein kinase C-Raf-MAP kinase pathway. Although VEGF-A binds two receptors, we found that VEGFR-1 and -2, a newly isolated ligand VEGF-E (Orf-virus-derived VEGF) binds and activates only VEGFR-2. Transgenic mice expressing VEGF-E_{NZ-7}, showed a dramatic increase in angiogenesis with very few side effects (such as edema and hemorrhagic spots), suggesting strong angiogenic signaling and a potential clinical utility of VEGF-E. VEGF family members bear three loops produced via three intramolecular disulfide bonds, and cooperation between loop-1 and loop-3 is necessary for the specific binding and activation of VEGFR-2 for angiogenesis. As it directly upregulates tumor angiogenesis, VEGFR-2 is an appropriate target for suppression of solid tumor growth using exogenous antibodies, small inhibitory molecules and *in vivo* stimulation of the immune system.

3. Induction of tube formation by angiopoietin -1 in endothelial cell/fibroblast co-culture is dependent on endogenous VEGF.

Momomi Saito, Maho Hamasaki and Masabumi Shibuya.

The angiopoietin-1 (Ang1)/Tie2 receptor system is known to be important for angiogenesis and vascular remodeling. However, its contribution to the survival and morphogenesis of endothelial cells is still not well elucidated. In this study, we analyzed the role of the Ang1/Tie2 pathway in cell survival and tube formation using a human umbilical vein endothelial (HUVE) cell and fibroblast co-culture system. In this system, which mimics angiogenesis in vivo, fibroblasts secrete a basal level of vascular endothelial growth factor (VEGF), and Ang1 stimulated tube formation. However, anti-VEGF or anti-VEGF receptor-2 neutralizing antibody blocked the Ang1-induced tube formation. Furthermore, other angiogenic factors such as hepatic growth factor (HGF) and basic fibroblast growth factor (bFGF) showed the same phenotype as Ang1, i.e., a stimulatory effect only in the presence of endogenous VEGF. The Ang1-promoted tube formation was mainly due to suppression of HUVE cell apoptosis in a PI3-kinase-dependent manner. These findings suggest that Ang1 stimulates tube formation *in vivo* via the PI3-kinase/Akt pathway, but this effect takes place only in a VEGF-dependent manner.

4. Signaling through FRS2 docking proteins

Signal transduction through tyrosine kinases has been studied extensively in the past two decades. We now understand that a lot of intracellular signaling proteins including docking proteins bind to receptor tyrosine kinases such as fibroblast growth factor (FGF) receptors. These signaling proteins activate a variety of signaling pathways in cells. However, to date, it is still unclear how each signaling protein plays a role *in vivo*. FRS2 family docking proteins, FRS2α and FRS2β, bind to FGF receptors or neurotrophin receptors upon stimulation with their ligands and become tyrosine phosphorylated. Tyrosine phosphorylated FRS2α serves as a platform for recruitment of multiple signaling proteins including Shp2, Grb2, Gab1 and Cbl. FRS2α then activates several signaling pathways: Shp2/Ras/ERK pathway, Grb2/Gab1/PI-3 kinase pathway and Grb2/Cbl ubiquitination/degradation pathway. Similarly, tyrosine phosphorylated FRS2β activates Shp2/Ras/ERK pathway upon stimulation with FGF.

In order to understand the biological role of FRS2 *in vivo*, we introduced several mutations on *Frs*α gene in mice.

a. The docking protein Frs2α is an essential component of multiple FGF responses during early mouse development.

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The docking protein Frs2α is a major mediator of FGF signaling. As FGF-signaling plays a critical role in early embryogenesis, deficiency in Frs2α results in impairment of several critical processes both in extra-embryonic and embryonic tissues of mutant embryos. We showed that *Frs2*α-null embryos fail to form anterior-posterior (A-P) axis and are developmentally retarded, resulting in embryonic lethality by E8.0. This year, we demonstrate that Frs2α is essential for FGF4-stimulated proliferation of self-

renewing trophoblast stem (TS) cells in the extraembryonic ectoderm (ExE) that gives rise to tissues of the placenta. Furthermore, we showed that *Bmp4* expression is rapidly induced in TS cells in response to FGF4 and is downregulated after removal of FGF4, suggesting that expression of *Bmp4* in ExE is tightly regulated by FGF 4. These experiments underscore the critical role of Frs2α in mediating multiple processes during embryonic development and reveal a potential new link between FGF and Bmp4-signaling pathways in early embryogenesis.

b. Analysis of the roles of Shp2-binding sites and Grb2-binding sites of Frs2α during mouse development

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In order to clarify the role of signaling pathways emanating from Shp2-binding sites and Grb2-binding sites of FRS2α, we generated mutant mice expressing the mutant Frs2α protein lacking Shp2-binding sites or Grb2-binding sites. Mutations on *Frs2*α gene were achieved by introducing point mutations on two Shp2-binding sites (Y436 and Y471, 2F mutant) or four Grb2-binding sites (Y196, Y306, Y349 and Y392, 4F mutant). We replaced these tyrosine residues to phenylalanine residues in *Frs2*α genome.

The $Frs2\alpha^{2F}/Frs2\alpha^{2F}$ homozygous mice had embryonic lethal phenotype, while all the $Frs2\alpha^{4F}/Frs2^{4F}$ homozygous mice were born without overt morphological abnormality. At E17.5, the $Frs2\alpha^{2F}/Frs2\alpha^{2F}$ embryos had deformity of the body, defects in eye, limb and tail, and failure of the closure of physiological hernia. These results indicate that the Shp2-binding sites of Frs2α play important roles for multiple developmental processes.

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