Department of Cancer Biology

Division of Oncology
癌細胞シグナル研究分野

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Protein-tyrosine kinases are important not only for the development of malignant tumors but also for the regulation of growth and function of normal cells. Our current interest is to characterize cell signaling downstream of protein-tyrosine kinases that are relevant respectively to cancer development and to neuronal function. We are also interested in protein kinase signaling that is involved in the regulation of cell cycle progression, particularly that of mitotsis.

1. The biological role of Tob family proteins and CCR4/Not complex

Toru Suzuki, Junko Tsuzuku, Mitsuhiro Yoneda, Masahiro Morita, Kentaro Ito, Kazuya Kitazawa, Shin-ichi Ogawa, Yusuke Nakajima and Tadashi Yamamoto

In search of signaling molecules donstream of receptor-tyrosine kinases, we identified Tob, a 45kDa protein displaying homology with the growth suppressing proteins, Btg1 and Btg2/PC 3. These proteins together with Tob2 and ANA, which we identified successively, compose an anti-proliferative Tob/Btg family

To elucidate the physiological function of the Tob/Btg family proteins, we generated mice lacking *tob*, *tob2*, or *ana*. (i) *tob*-deficient mice ($tob^{-/-}$) had a greater bone mass resulting from increased number of osteoblasts in comparison with wild-type mice. We also found that aged $tob^{-/-}$ mice develop a variety of tumors. (ii) In contrast, mice lacking *tob2* had decreased bone mass, and the number of osteoclasts differentiated from bone marrow cells was increased. We further showed that Tob2 negatively regulates formation of osteoclasts by suppressing RANKL expression through its interaction with VDR. (iii) Unlike the other Tob family proteins, Ana is specifically expressed in type II alveolar epithelial cells. *ana*-deficient mice developed spontaneous tumors including lung adenocarcinoma. We also found that expression of *ana* gene was largely reduced in almost all of the lung cancer cell lines and clinical samples of lung adenocarcinoma examined. Currently, we are investigating the molecular mechanism by which Ana negatively regulates development of lung cancer.

Next, we found that Tob plays a role in DNA damage response. UV-induced stress promotes proteasome-dependent degradation of Tob, triggering an apoptotic signal. Tob with either short deletion or a tag sequence at the C-terminus is resistant to UV-induced degradation. Introduction of the degradation-resistant Tob impaired UV-induced apoptosis. Reciprocally, suppression of Tob by small-interfering RNA (siRNA) resulted in frequent induction of apoptosis irrespective of the presence of functional p53 even

at UV doses that do not promote Tob degradation. Finally, *tob*-deficient ($tob^{-/-}$) mice and primary embryonic fibroblasts (MEFs) from $tob^{-/-}$ mice exhibit increased sensitivity to UV irradiation. Thus, proteosomal clearance of Tob provides a novel p53-independent pathway for UV-induced apoptosis.

As Tob is involved in various biological events, we sought for the mechanisms by which Tob functions. To do so we searched for molecules which interact with Tob by applying proteomics. We immunoprecipitated the Tob-interacting proteins from HeLa cells and the precipitates were analyzed by mass spectrometry. We found that the Tob complex contains the components of the CCR4-NOT deadenylase complex, Cnot1, Cnot2, Cnot3, Cnot6/CCR4 and Cnot7/Caf1. Interestingly, Tob appears to suppress the deadenylase activity of the CCR4-Not complex.

Recently we identified and characterized CNot6L, which is also a homolog of yeast CCR 4. Cnot6L is localized mainly in the cytoplasm and displays deadenylase activity both *in vitro* and *in vivo*. Suppression of Cnot6L by RNA interference (RNAi) results in growth retardation of NIH3T3 cells that is accompanied by elevation of both $p27^{Kip1}$ mRNA and $p27^{Kip1}$ protein. 3'UTR of $p27^{Kip1}$ mRNA is recognized directly or indirectly by Cnot6L, which suggests that Cnot6 L controls the length of poly(A) of $p27^{Kip1}$ mRNA, and thereby the stability of the mRNA Currently, we are examining the biological functions of Cnot6L by generating mice in which the cnot6L gene is disrupted. We are also generating mice lacking the *cnot3*, *cnot6*, *cnot7*, *cnot8*, *cnot9* or *cnot10* gene. Among these, *cnot7* mice are defective for sperm formation, due to the inability of sertoli cells to nurture the spermatogenesis. *cnot3*-deficient mice are embryonic lethal. $cnot3+/-$ mice are born alive, but are small and lean. Preliminarily, we showed that Cnot3 is regulatory to the CCR4-NOT deadenylase and reduction of Cnot3 affects the length of poly(A) tail of some mRNAs such as those encoding proteins involved in triglyceride and glucose turnover. Furthermore, RNAi-mediated Cnot1 or Cnot2 knockdown in HeLa cells induced growth arrest and apoptosis. The underlying mechanisms are under investigation.

We also performed yeast two-hybrid screening using Tob as a bait, and then identified a serine/threonine kinase Ndr2. Interestingly, a yeast homolog of Ndr2 (Dbf2) is reported to be associated with the CCR4-Not complex. By analyzing MEFs from $ndr2-/-$ mice and NIH3T3 cells treated with specific siRNA, we obtained data suggesting that Ndr2 is involved in cell cycle regulation and cell migration.

2. Roles of protein kinases in the central nervous system

Takanobu Nakazawa, Kazumasa Yokoyama, Takeshi Inoue, Naosuke Hoshina, and Tadashi Yamamoto

The Src-family protein-tyrosine kinases are implicated in various neural functions such as formation of neural network, myelination, and synaptic plasticity. To analyze the roles of Src and Fyn, we have been focusing on various substrates of these kinases, including *N*-methyl-D-aspartate (NMDA) type of ionotropic glutamate receptors. NMDA receptors play important roles in learning, memory formation, and emotional behaviors. Our own studies have shown that NR2A and NR2B subunits of NMDA receptors are the major substrates of Fyn and Src. We previously identified Tyr-1472 phosphorylation on NR2B, which is the major tyrosine phosphorylation site of NR2B, as a key mediator of fear-related learning and emotional behavior in the amygdala. On NR2A, we have identified Tyr-1325 as a major tyrosine phosphorylation site, however the significance of the phosphorylation event *in vivo* has remained unclear. To establish the biological significance of Tyr-1325 phosphorylation, we generated mice expressing mutant NR2A with a Tyr-1325-Phe (Y1325F) mutation to prevent phosphorylation of this site *in vivo*. The homozygous knock-in mice show significantly less immobility in the tail suspension test and in the forced swim test. As these tests are widely used for assessing antidepressant-like activity, we conclude that the YF mutation induces antidepressant-like effects in mice. In the striatum of the knock-in mice, DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, 32 kD) phosphorylation at Thr-34, which is important for the regulation of depression-related behaviors, is increased. Together with the finding that the Tyr-1325 phosphorylation site is required for Src-mediated potentiation of the recombinant NMDA receptor channel, our current data argue that the Tyr-1325 phosphorylation regulates NMDA receptor channel properties and the NMDA receptor-mediated downstream signaling to modulate depression-related behavior. In parallel of these studies, to uncover Src- and Fyn-mediated signaling pathways, we have been trying to identify binding partners and substrates of these kinases in the brain. As a result, we have identified a number of putative mediators of Fyn- and Src-mediated signaling, including p250GAP, TCGAP, Nogo-A, and Rho-GEFs, whose characterization is in progress.

In addition to characterization of the Src/Fyn substrates, we have been analyzing the develop-

mental phenotypes of *fyn*-deficient mice in the brain. We found that *fyn* deficiency on the C57 BL/6 genetic background resulted in premature death due to severe hydrocephalus, with neonatal onset. One week after birth, *fyn*-deficient mice showed enlarged lateral ventricles with thinner cerebral cortices and degenerating axons in the corpus callosum. Before the onset of myelination, the number of oligodendrocytes was reduced and their morphogenesis was impaired in the cerebral cortex. These results demonstrate that Fyn is essential for normal brain development and suggest that defects in oligodendrocyte development cause degeneration of cortical axons and subsequent hydrocephalus in *fyn*-deficient mice.

3. Roles of chromokinesin Kid and mitotic kinase Plk1 in execution of cell division

Miho Ohsugi, Noriko Tokai-Nishizumi, Naoki Oshiori, Natsuko Masuda, Xue Li, Shu-Jen Shiu, and Tadashi Yamamoto

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

i) The chromokinesin Kid

The human chromokinesin Kid/Kinesin-10 is a member of the chromosome-associated kinesin family identified in our lab in 1996. Kid has been implicated in generating the polar ejection force that pushes the chromosome arms away from the spindle poles toward the spindle equator, as well as in the maintenance of spindle length during prometaphase and metaphase. We previously showed that chromosome localization of Kid during prometaphase and metaphase requires Cdc2/cyclin B-mediated phosphorylation on Thr463. Our also found that the mitotic chromosome loading of Kid is mediated by importin α/β We defined two functional nuclear localization signals (NLSs) in Kid and found that Importin α directly associates with Kid via NLSs and that Ran-GTP-mediated dissociation of importin α/β from Kid promotes the accumulation of Kid on chromosomes.

During anaphase and telophase, Kid shows unique localization in the interstices between adjacent chromosomes. By analyzing mice and cultured cells lacking expression of Kid, we showed that Kid deficiency causes defects in compact clustering of the anaphase chromosomes. This often leads to micro- or multinuclear formation at oocyte meiosis II and the first couple of mitoses after fertilization, causing embryonic death in mice. Later somatic mitoses are not fatally affected by the absence of Kid, suggesting that cell divisions under significant influence of the ooplasm specifically require Kid to prevent formation of multinucleated cells.

ii) Mitotic kinase Plk1 and its substrates

Plk1 (polo-like kinase 1) is a highly conserved serine/threonine kinase that plays multiple pivotal roles in mitosis, meiosis, and also in oncogenesis. However, the exact mechanisms of Plk 1's actions remain to be elucidated. Previously, we identified the centrosomal Plk1 substrate, Kizuna (Kiz), which when depleted causes fragmentation and dissociation of the pericentriolar material from centrioles at prometaphase, resulting in multipolar spindles. Using a yeast two-hybrid screen for Kiz-interacting proteins, we identified an uncharacterized centrosome protein named Ckip. RNA interferance (RNAi) experiments demonstrate that Ckip is required for centrosomal localization of Kiz. In addition, our data suggest that Ckip also plays other important roles in regulating microtubule nucleation activity of the centrosomes. We are currently investigating other newly identified substrates of Plk1, which will uncover the molecular mechanisms underlying the Plk1-mediated control of cell divisions and oncogenesis.

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Department of Cancer Biology

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腫瘍細胞社会学分野

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Membrane proteins and their ligands including signaling molecules and extracellular matrix proteins mediate complex arrays of cell signaling. Fate and activities of these proteins are often regulated by proteases in the extracellular space. We are currently focused on studying biological roles of membrane-anchored type of matrix metalloproteinases (MT-MMPs) which are potent modulators of pericellular microenvironment and eventually regulate varieties of cellular functions such as proliferation, migration, apoptosis, and differentiation. Since uncontrolled expression of MT-MMPs in tumors contributes to their malignant characteristics, they are potential targets for cancer therapy.

1. Development of a new tracking tool for the human monomeric laminin-gamma 2 chain *in vitro* and *in vivo*

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Laminins are assembled from three polypeptide chains designated α, β, and γ. There are several isoforms of each type of chain that associate in different combinations to constitute the burgeoning laminin protein family. For example, laminin 5 (Ln-5), the most abundant form of most of the basement membrane (BM) in adult tissues, contains α3, β3, and γ2 chains. Among these polypeptide chains, γ2 (Ln-γ2) has been found to be frequently expressed as a monomer in several types of malignant cancer cells based on experiments showing the lack of simultaneous expression of Ln-α3 and Ln-β3 chains. Expression of monomeric Ln-γ2 is particularly predominant in the budding cells of tumor masses as observed in esophageal, stomach, colon and cervical carcinomas, though it is not detectable in normal tissues. These observations strongly emphasize the importance of monomeric Ln-γ2 as a specific marker for invasive carcinomas.

In addition, monomeric Ln-γ2 may also play an important role as a modulator of tumor cell behavior. Ln-γ2 has an EGF-like repeat in domain III (DIII) and this portion can be clipped out from Ln-γ2 by the action of MMPs, such as MMP-2 and MT1-MMP. The released DIII fragment can act as a ligand of the EGF receptor (EGFR) and elicit receptor-mediated intracellular signals. Interestingly, the monomeric form of Ln-γ2 shows ten fold higher sensitivity to MT1-MMP than the γ2 chain in human Ln-5 (Ln-5). Thus, the monomeric form of Ln-γ2 expressed in human tumors may contribute to tumor cell behavior by activating EGFR. Indeed, aggressive melanoma Mum2B cells that express monomeric Ln-γ2 and MT1-MMP can form a tube-like network known as vascular mimicry when the cells are cultured in collagen gel. Knockdown of MT1-MMP expression by siRNA or inhibition of tyrosine kinase activity of EGFR suppressed the tube formation. In spite of the importance of monomeric Ln-γ2 as a marker in tumors and as a tumor cell regulator, we do not have simple

methods to detect it directly and sensitively because all the available antibodies against Ln-γ2 also react with Ln-5. Thus, the presence of monomeric Ln-γ2 can only be inferred by confirming the lack of Ln-α3 and Ln-β3.

In this study, we developed a monoclonal antibody (mAb) that reacts selectively with the monomeric form of Ln-γ2. For this purpose mice were immunized with purified monomeric human Ln-γ2 and antibodies produced by hybridoma clones were screened by enzyme-linked immuno-sorbent assay (ELISA) using monomeric Ln-γ2 and Ln-5. Most of the hydridoma supernatants that recognized the monomer also recognized Ln-5 with the exception of one (mAb 1H3) that reacted preferentially with monomeric Ln-γ2. We evaluated this antibody as a practical tool to monitor monomeric Ln-γ2 *in vitro* and *in vivo*.

2. The Second Dimer Interface of MT1-MMP, the Transmembrane Domain, Is Essential for ProMMP-2 Activation on the Cell Surface

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MT1-MMP is a type I transmembrane proteinase that promotes cell migration in tissue. MT1-MMP is implicated in many physiological and pathological conditions including wound healing, bone development, lung development, angiogenesis, cancer invasion and growth, rheumatoid arthritis, and atherosclerosis. MT1-MMP promotes cellular invasion by degrading barrier extracellular matrix components including collagens I, II, III, fibronectin, laminins, vitronectin, and aggrecan; by activating other MMPs, namely proMMP-2 and proMMP-13; by shedding cell adhesion molecules such as CD44 and syndecan 1; and by activating extracellular signal-regulated kinase (ERK) through as yet undefined mechanisms.

Having such diverse functions, MT1-MMP is regulated by different mechanisms including gene expression, activation of the zymogen, inhibition by endogenous inhibitors, including tissue inhibitor of metalloproteinases (TIMPs), RECK, and Testicans, localization to the leading edge of migrating cells, including lamellipodia and invadopodia, autolytic degradation and processing, endocytosis through clathrin- and caveolae-dependent mechanisms, palmitoylation at its cytoplasmic domain, recycling, and lysosomal degradation. Such regulation is thought to be essential to coordinate MT1-MMP activity with cellular events, enabling it to promote cell invasiveness.

ProMMP-2 activation is one of the MT1-MMP functions thought to be important in cancer invasion and growth, where its significance lays particularly on basement membrane degradation as MT1-MMP itself cannot degrade collagen IV, a major component of the matrix but activated MMP-2 does. In this activation process, MT1-MMP forms a complex with its endogenous inhibitor, TIMP-2. TIMP-2 binds to the catalytic site of MT1-MMP through its inhibitory site in the N-terminal domain, leaving the exposed C-terminal domain of TIMP-2 to interact with the hemopexin (Hpx) domain of proMMP-2. Thus the MT1-MMP-TIMP-2 complex acts as a receptor for proMMP-2. To activate proMMP-2 in this complex, a second MT1-MMP, which is free of TIMP-2, needs to be positioned in close proximity to the trimolecular complex. This is achieved by the formation of an MT1-MMP homodimer complex.

Another important biological activity of MT1-MMP is collagen degradation. Among MMP family members, at least six enzymes can degrade fibrillar type I collagen, namely MMP-1, MMP-2, MMP-8, MMP-13, MT1-MMP, and MT 2-MMP. MT1-MMP and MT2-MMP are membrane bound collagen-degrading enzymes, but MT2-MMP is the weakest of the collagenolytic MMPs, exhibiting 1/100 of the activity of MT1-MMP. Thus MT1-MMP is likely to be the major pericellular collagenase. MT1-MMP-null mice exhibit phenotypes thought to be due to a lack of cellular collagenolytic activity. We have recently shown that MT1-MMP dimer formation is essential for collagen degradation on the cell surface, suggesting that dimerization of MT1-MMPmay be an important regulatory mechanism to activate MT1-MMP on the cell surface for both collagenolysis and proMMP-2 activation.

We have previously reported that dimerization of MT1- MMP is driven by homodimeric complex formation of the Hpx domains and that this interaction is crucial for proMMP-2 activation and collagen degradation on the cell surface. It was also reported that the Hpx and cytoplasmic domains can drive dimerization for proMMP-2 activation. On the other hand, it was recently reported that an Hpx domain-deleted MT1-MMP mutant retains the ability to activate proMMP-2, suggesting that Hpx domain-dependent dimer formation may not play a role in this process.

In this study, we have reevaluated the role of the Hpx, linker-2, transmembrane (TM), and cy-

toplasmic domains in proMMP-2 activation and the collagenolytic activity of MT1-MMP and found that MT1-MMP has two modes of dimer formation: Hpx domain- and TM domain-dependent dimerization. For proMMP-2 activation, TM dependent dimerization is essential, whereas Hpx-dependent dimerization is essential for collagenolytic activity. Inhibition of Hpx domain-dependent dimerization by co-expressing the Hpx domain resulted in inhibition of TM-dependent dimer formation, proMMP-2 activation, and collagenolytic activity. Our finding reveals an additional molecular arrangement contributing to MT1-MMP function on the cell surface.

3. Spontaneous transformation of human granulosa cell tumours into an aggressive phenotype: a metastasis model cell line

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Granulosa Cell Tumors (GCTs) are a relatively uncommon neoplasm; the incidence of GCTs ranges from 1.6-3.0% in all cases of ovarian tumour and comprises about 10% of all cases of ovarian cancer. They belong to the sex-cord stromal tumours, and are classified as juvenile or adult, although the majority of GCTs occur in menopausal women. GCTs are known to retain numerous characteristics of native granulosa cells, such as the expression of active FSH receptor, inhibin, and estrogen. Although GCTs have a malignant potential, they are often indolent and have a propensity for late recurrence. Up to 53% of all cases lead to metastases within 5 years and studies encompassing long-term follow-up have shown high mortality rates, with about 50% of women dying from the disease within 20 years of diagnosis. Although there have been extensive studies on the biology of normal granulosa cells, much knowledge of the molecular mechanism by which transition from promotion to the progression stage occurs in GCTs remains unknown.

To date, only seven human granulosa cell lines have been established, although several animal granulose cell-derived cell lines have been reported. Of these, KGN cells were generated from a GCT that recurred in the pelvic region, and were shown to have detectable aromatase activity. KGN cells have an abnormal karyotype (45, XX, 7q-, -22) which is probably related to the tumourigenic character of this granulosa cell, as frequent abnormalities in chromosome 7 have been reported in ovarian tumours. Interestingly, KGN cells revealed a unique characteristic and grew progressively faster during passages in our preliminary experiment.

Here, we investigated the specific characteristics of KGN cells towards understanding the molecular pathogenesis of GCTs. Because KGN cells grew much faster after passages in culture, we investigated their cellular characteristics, such as proliferation capacity and invasiveness, during passages *in vitro*. We then investigated the behavior of these cells *in vivo* with the use of subcutaneous xenografts at different passages.

4. The cytoplasmic tail of MT1-MMP regulates macrophage motility independently from its protease activity

Takeharu Sakamoto and Motoharu Seiki

Membrane type-1 matrix metalloproteinase (MT1-MMP) cleaves multiple proteins in the pericellular milieu and has been characterized as a potent modulator of the cell environment. Processing of proteins by MT1-MMP alters their activities and thereby regulates a variety of cellular functions, such as motility, invasion, growth, differentiation, apoptosis, and morphology etc. Mice deficient in MT1-MMP expression ($MT1^{-/-}$) display multiple defects reflecting the importance of MT1-MMP in different cell types, such as fibroblasts, muscle cells, endothelial cells, osteoblasts, osteoclasts, and adipocytes etc.

A major substrate of MT1-MMP is collagen I, which is the most abundant constituent of the tissue extracellular matrix (ECM). MT1-MMP is unique as a membrane-anchored collagenase and its activity is important for a variety of cell functions that require collagenolysis, such as cell growth, invasion, and differentiation. Other MT 1-MMP substrates include ECM proteins, membrane proteins, and a variety of other proteins located in the cells' vicinity. Since the biological output of MT1-MMP is believed to be mediated by its protease activity, most MT1-MMP studies have focused on exploring mechanisms regulating the protease activity on the cell surface, identifying important substrates, and characterizing the biological consequence of the cleavage of substrate proteins (Itoh & Seiki 2006; Overall & Dean 2006). This line of inquiry is still important for understanding the biological roles of MT1-MMP. Aside from these protease-dependent functions, little is known about non-proteolytic functions MT1-MMP.

In this study, we analyze the recruitment of macrophages to sites of inflammation and demonstrate that infiltration of macrophages into the inflammatory site is significantly disturbed in $MT1^{-/-}$ mice. Since macrophage movement into a site of inflammation is a multi-step process that involves many factors and cells, our observation may reflect a complex interplay between different types of cells exhibiting defects caused by MT1-MMP-deficiency. However, a simpler hypothesis is that MT1-MMP might affect macrophage function directly. We analyze the roles of MT1-MMP expressed in macrophages by isolating such cells from wild type (WT) and $MT1^{-/-}$ mice. While MT1-MMP is required for macrophage invasion acting as a protease, it also promotes the motility of the cells. Interestingly, the motility-stimulating activity of MT1-MMP is independent of the protease activity, but dependent upon the CP tail. Thus, our study uncovers dual functions of MT1-MMP to stimulate macrophage invasion; degradation of the ECM by its protease activity and migration-boosting activity mediated by a CP-dependent mechanism.

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Human cancers develop and progress toward malignancy through accumulation of multiple genetic alterations. Elucidation of these alterations is essential to provide molecular targets for prevention, diagnosis, and treatment of cancer. Our current interest is to understand the role of cell adhesion in cancer invasion and metastasis, as well as in mast cell function. To this end, an immunoglobulin superfamily cell adhesion molecule, CADM1/TSLC1, and its cascade were identified and are being characterized. Genetic and epigenetic abnormalities in human tumors are also being investigated.

1. The biological role of CADM1/TSLC1 protein in epithelial structure

Mika Sakurai-Yageta, Yumi Tsuboi, Miwako Iwai, Shigefumi Murakami, Takeshi Ito and Yoshinori Murakami:

CADM1/TSLC1 is an immunoglobulin superfamily cell adhesion molecule and primarily involved in epithelial cell adhesion, while loss of its function could lead cancer cells to invasion and metastasis. We have previously shown that CADM1 associates with a family of actin-binding proteins, including 4.1B/DAL-1 and 4.1 N, and members of scaffold proteins such as membrane protein palmitoylated 1 (MPP1), MPP 2, MPP3, and CASK. These complexes localize along the cell membrane and play a critical role in the formation of epithelia-like cell structure. To further investigate the function of this cascade, additional binding proteins to CADM1 are being identified by proteomics approaches. Chemical library is also being screened to identify specific inhibitors of this cascade using *in vitro* cell-based system. In addition, stability of CADM1 protein within the cell membrane is being analyzed using photo-bleaching assay. Transcriptional regulation of the *CADM1* gene is also investigated to understand the physiological mechanisms of gene expression and to intervene in its expression.

2. Patho-physiological function of the CADM 1 protein in animal models

Masayoshi Nagata, Taketo Kawai, Hiromi Ichihara, Tomoko Masuda, and Yoshinori Murakami

To understand the physiological role of CADM1 protein in animal models, *Cadm1*-deficient mice were generated by disrupting exon 1 of the gene. We have previously reported that *Cadm1*$^{-/-}$ mice showed male infertility due to disruption of adhesion of spermatocytes to Sertoli cells in the seminiferous tubules. We have also found that about 30% of *Cadm1*$^{-/-}$ mice spontaneously develop lung adenomas and adenocarcinomas at 15 months of age. *Cadm1*$^{+/-}$ mice also showed significantly increased incidence of lung tumor development. CADM1 expression was only lost in the tumors probably

due to two-hit inactivation of the gene. These results indicate that the *CADM1* is a potent suppressor gene of lung tumor. Additional molecular alterations in lung tumors are being investigated to understand the the molecular mechanisms of lung tumorigenesis in *Cadm1*$^{-/-}$ and *Cadm1*$^{+/-}$ mice.

3. Analyses of genetic and epigenetic abnormalities in human tumors

Takashi Obana, Yasuhiro Ebihara, Mika Takahashi and Yoshinori Murakami

To understand the molecular features of multistage carcinogenesis in human, promoter methylation of the *CADM1* genes, as well as mutations of several key molecules, was examined in various tumors, including lung cancer, renal cancer, head and neck cancer, breast cancer and neurpblastoma. In 103 non-small cell lung cancers (NSCLC), the *EGFR* mutation was detected in 27 (26%) tumors, while the *KRAS2* mutation was detected in 11 (11%) tumors. However, these mutational events are independent of promoter methylation in the *CADM1* or *4.1B* genes, supporting a hypothesis that CADM1 is involved in invasion or metastasis, a late event in lung tumor progression. Loss of expression of the *CADM1* gene was also observed preferentially in unfavorable neuroblastoma or metastatic nasopharyngeal carcinoma, providing a possible diagnostic tool to predict patients with poor prognosis in collaboration with others.

4. Analysis of CADM1-mediated cellular adhesion that promotes cell type-specific function by approaches of experimental pathology

Akihiko Ito, Mitsuru Hagiyama, Naoki Ichiyanagi, Keiko Kimura

CADM1 plays an important role not only in tumor suppression but also in cellular adhesion that mediates cell and tissue type-specific functions. Among a variety of adhesions mediated by CADM1, we have mainly investigated physiological and pathological aspects of cellular adhesion between pancreatic islet cells and nerve cells, between malignant pleural mesothelioma (MPM) cells and non-neoplastic mesothelial cells, and between nerve cells and a soluble form of CADM1.

We have newly demonstrated that CADM1 is expressed in pancreatic islet cells and that the adhesion between islet cells and autonomic neurons through homophilic interaction of CADM1 is involved in Ca^{++} mobilization of islet cells triggered by nerve activation. Interestingly, CADM1 is expressed in 6 of 7 islet cell tumors (ICTs) functional in hormone secretion, whereas 12 of 14 ICTs lacking CADM1 expression are non-functional in terms of hormone secretion. These results suggest that CADM1 would participate in autonomic regulation of pancreatic hormone secretion.

Since CADM1 is also expressed in a subset of MPM, but not in non-neoplastic mesothelial cells, we have investigated possible role of CADM1 in adhesion between MPM cells and non-neoplastic mesothelial cells. When CADM1 was introduced into MPM cells lacking endogenous CADM1, these MPM cells showed more efficient adhesion and faster growth on monolayers of mesothelial cells in comparison with parental cells. However, such difference was not observed when the cells were cultured on monolayers of lung fibroblasts. Thus, CADM1 appears to play a role in disseminated growth of MPM cells over the pleural surface, which is one of the characteristic features of human MPM.

In addition to the membrane-bound CADM1 (mCADM1) on neurites, we have newly shown that a soluble isoform of CADM1 (sCADM1) is present in mouse brain stroma. Possible function of sCADM1 is subsequently examined by implanting superior cervical ganglion (SCG) cells into collagen gels together with cells secreting sCADM1 and those with truncated sCADM1. Bodian staining of the co-culture collagen gels revealed that most SCG neurites turned toward the source of sCADM1, but not that of truncated sCADM1. Immunofluorescence double-staining signals for sCADM1 and mCADM1 were co-localized with each other on the neurite surface. These findings imply that sCADM1 would be a directional guidance cue of neurite outgrowth by serving as an anchor to which mCADM1 on the neurites binds.

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Department of Cancer Biology

Division of Cellular and Molecular Biology
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. Our goal is to understand the molecular mechanisms of disease pathogenesis and oncogenesis by elucidating normal regulation of intracellular signal transduction and gene expression involved in cell proliferation and differentiation. We have identified and been interested in Tumor necrosis factor receptor-associated factor 6 (TRAF6), which transduces signal emanating from the TNFR superfamily and the TLR/IL-1R family leading to activation of transcription factor NF-κB and AP-1. By generating TRAF6-deficient mice, we found that TRAF6 is essential for osteoclastogenesis, self-tolerance, lymph node organogenesis and formation of skin appendices. We are currently focusing on how TRAF6 is involved in osteoclastogenesis and self-tolerance. In addition, NF-κB is thought to be constitutively activated in cancer cells and this activation could be involved in the malignancy of tumors. Thus, we are also investigating the molecular mechanisms and target genes of the constitutive activation of NF-κB.

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

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Transcription factor Rel/NFκB binds specifically to a decameric motif of nucleotide, κB site, and activates transcription. 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 Lys48-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. Several lines of evidence suggest that TRAF6 is able to activate some kinase other than TAK1. To identify such kinases, we are trying to identify the molecule(s) that function between TRAF6 and the IKK complex by biochemical protein purification.

2. Molecular mechanism regulating thymic microenvironment to establish self-tolerance

Taishin Akiyama, Junwen Qin, Yusuke Shimo, Daisuke Ohshima, Hiromi Yanai, Yuya Maruyama, Miho Shinzawa, Takuma Shiraishi and Jun-ichiro Inoue

Clonal deletion is one of the main mechanisms to maintain T-cell tolerance. T-cell clones that have high avidity for self-antigens are eliminated during their development in the thymus. Self-antigens are predominantly expressed and presented by epithelial cells (TECs) and dendritic cells. Medullary thymic epithelial cells (mTECs) have a unique property to promiscuously express peripheral tissue specific self-antigens (TSAs). Therefore, it has been proposed that developing T-cells encounter TSAs in the thymic medullar for clonal deletion. This hypothesis is supported by the study of autoimmune regulator (Aire), inactivation or deficiency of which results in autoimmune polyendocrinopathy-candidiasis ectodermal dystrophy. Aire is preferentially expressed in mTECs and lack of Aire in mice results in the defective expression of some TSAs.

Even though an increasing body of evidence indicates crucial roles of mTECs on establishing self-tolerance in thymus, signaling pathways underlying the differentiation and proliferation of mTECs expressing Aire and TSAs are poorly understood. In order to determine cytokines regulating the development of mTECs, we estimated the expressions of TNF receptor family in fetal thymic stroma, and found RANK and CD 40 as possible candidates. In fact, RANKL and CD40 double deficient mice showed a severe defect in mTEC development. Furthermore, recombinant RANKL or CD40L is sufficient for inducing the development of mTECs in fetal thymic stroma, which contains immature thymic epithelial cells. Furthermore, the development of mTECs induced by RANKL or CD40L was completely dependent on the presence of TRAF6 and functional NIK, which are signal-transducing molecules essential for mTEC development. These results indicate that two signals from RANK and CD40 cooperatively regulate the development of mTECs.

3. Molecular mechanism of RANK signaling in osteoclastogenesis

Jin Gohda, Yuu Taguchi, Yoko Kiga, Sayaka Yamane, and Jun-ichiro Inoue

Osteoclasts are responsible for bone resorption and play a critical role in bone homeostasis. Excess formation or activity of osteoclasts results in pathological bone resorption, such as that in postmenopausal osteoporosis, rheumatoid arthritis, Paget's disease and bone tumor metastasis. Therefore, a precise understanding of the mechanisms that regulate osteoclast formation is required for understanding the onset of skeletal diseases and for developing drugs to treat such disease.

Osteoclast differentiation is induced by Receptor activator of NFκB (RANK), which is a member of the tumor necrosis factor receptor superfamily (TNFRSF). Binding of RANK-Ligand to RANK recruits TRAF6 to the cytoplasmic region of RANK, and then TRAF6 activates downstream signaling pathways, including those that activate NF-κB and MAPKs. In addition to RANK signaling, osteoclastogenesis requires co-receptor-mediated signaling pathways from ITAM-containing receptors, which induce PLCγ2 activation followed by intracellular calcium oscillation, finally leading to activation of NFATc1, a master regulator of osteoclastogenesis. We previously identified a novel functional domain of RANK essential for osteoclastogenesis, named High conserved region in RANK (HCR). HCR is highly conserved in RANKs from various vertebrates and does not contain the TRAF6 binding sites. We are currently working on the molecular mechanisms of HCR-mediated signaling.

4. Molecular mechanisms of NF-κB activation in Adult T-cell leukemia

Jin Gohda, Yuri Shibata, Akihisa Hirosawa, and Jun-ichiro Inoue

Human T-cell leukemia virus type 1 (HTLV-1) infects and immortalizes CD4-positive T cells, and it is etiologically associated with Adult T-cell leukemia (ATL). NF-κB is constitutively activated during development of ATL, and its activation plays a crucial role in T-cell transformation and survival of leukemic cells. Therefore, clarification of the molecular mechanisms of

constitutive NFκB activation may lead to development of drugs to treat ATL. In early stage of ATL development, constitutive NFκB activation is caused by HTLV-1-derived oncoprotein, Tax. Tax directly associates with NEMO, a noncatalytic subunit of IκB kinase (IKK) complex, and induces activation of IKK leading to NFκB activation. In contrast, leukemic cells derived from ATL patients show constitutive NFκB activation despite the deficiency of *tax* gene or severe suppression of Tax expression. These facts suggest that constitutive NFκB activation in ATL is caused in Tax-dependent and Tax-independent manners.

We have previously established a cell free system that activates IKK in response to Tax. Addition of recombinant Tax protein to cell extracts prepared from Jurkat cells led to the phosphorylation of IκBα. In contrast, Tax did not directly activate IKK complex purified from cell extracts, suggesting that Tax-induced IKK activation requires the unknown factor(s) other than the components of IKK complex, which is/are involved in polyubiquitination. We are currently purifying such factors from the cell extracts. On the other hand, to elucidate the molecular mechanism of Tax-independent NFκB activation in ATL, we are trying to establish the cell free system using cell extract from ATL-leukemic cells and identify IKK inducers from the cell extracts.

5. Role of TRAF6 in anti-viral response

Hiroyasu Konno, Takuya Yamamoto[3], Kohsuke Yamazaki, Jin Gohda, Taishin Akiyama, Kentaro Semba[1], Yasuko Tsunetsugu-Yokota[3] & Jun-ichiro Inoue: [3]Department of Immunology, National Institute of Infectious Diseases

Innate immune responses to viral infection begin when viral nucleic acids are recognized by the host, and this recognition is followed by the production of type I IFNs that block viral replication and the production of inflammatory cytokines that activate acquired immune responses against specific viral antigens. Viral nucleic acids are recognized by Toll-like receptor 7 (TLR7) and TLR9 in the endosomes of plasmacytoid dendritic cells (pDCs), as well as by RIG-I-like helicases (RLHs) and DNA sensors in the cytosol of conventional DCs (cDCs). Both types of recognition are essential for proper innate immune responses. We found that TRAF6, which is essential for TLR signaling, also plays a crucial role in RLH and DNA sensor signaling by contributing to the activation of transcription factors NF-κB and IRF7. Since TAK1 and MEKK 3, which act downstream of TRAF6 during TLR signaling, are found to be dispensable for RLH signaling, we hypothesize that in response to cytosolic nucleic acids sensors, TRAF6 mediates antiviral responses in a way different from that by TLR signaling. The findings of this study identify TRAF6 as a key molecule in innate and acquired immune responses to viral infection.

6. Role of TRAF6 in T cell proliferation

Hidehiko Motegi, Taishin Akiyama & Jun-ichiro Inoue

Recently, we found that CD4-positive T cells from TRAF6-deficient (*Traf6*$^{-/-}$) mice proliferated more extensively than wild-type (WT) CD4 -positive T cells in response to T-cell antigen receptor (TCR) stimulation. Furthermore, expression of protein and mRNA of Interleukin-2 (IL-2), which is essential for T cell proliferation, are also enhanced in *Traf6*$^{-/-}$ T cells. We are currently focusing on molecular mechanisms of such an abnormality in the absence of TRAF6.

7. Selective transmission of R5 HIV-1 over X4 HIV-1 at the dendritic cell-T cell infectious synapse is determined by the T cell activation state

Takuya Yamamoto[3], Jun-ichiro Inoue and Yasuko Tsunetsugu-Yokota[3]:

HIV-1 infects T cells and monocyte lineage cells, including macrophages and DCs, through CD4, the primary receptor for entry. The cellular tropism of HIV-1, i.e., macrophage (M)-tropic or T-cell line (T)-tropic, is determined by chemokine receptors. Depending on whether they mainly use the CCR5 or CXCR4 entry coreceptors, primary isolates are defined as R5 for M-tropic and X4 for T-tropic variants, respectively. When HIV-1 is isolated from individuals newly infected with the mixture of R5 and X4 variants, R5 is predominant, irrespective of the route of infection. To understand why this might be so, we used fluorescent recombinant X 4 and R5 HIV-1 consisting with a common HIV-1 genome structure except envelope, which allowed us to discriminate the HIV-1 transmitted from DCs infected with the two virus mixture to antigen-specific $CD4^+$ T cells by flow cytometry. We clearly showed that the selective expansion of R5 over X4 HIV-1 did occur, which was determined at an early entry step by the activation status of the $CD4^+$ T cells transmitted virus from DCs, but not by virus entry efficiency or productivity in DCs.

8. Constitutive activation of NF-κB in breast cancer cell lines

Noritaka Yamaguchi, Taku Ito, Mizuki Yamamoto, Sakura Azuma[1], Shinya Watanabe[4], Kentaro Semba[1] and Jun-ichiro Inoue: [1] Department of Life Science and Medical Bioscience, School of Science and Engineering, Waseda University, [4]Department of Clinical Genomics, Translational Research Center, Fukushima Medical University

Constitutive NF-κB activation is thought to be involved in survival, invasion, and metastasis in various types of cancers. However, neither the subtypes of breast cancer cells with constitutive NF-κB activation nor the molecular mechanisms leading to its constitutive activation have been clearly defined. Therefore, we quantitatively analyzed basal NF-κB activity in 35 human breast cancer cell lines and found that most of the cell lines with high constitutive NF-κB activation were categorized in a certain type of breast cancer cell line. Regarding the mechanisms, we checked expression of various genes involved in the NF-κB activation and found that some genes are up-regulated in cells with high NF-κB activity.

In additon, we have investigated target genes of constitutively activated NF-κB. To identify genes induced by constitutively activated NF-κB, we performed microarray analysis of a breast cancer cell line before and after the expression of the IκBαSR. Expression levels of several genes, which are known to be involved in tumorigenesis, were reduced by the expression of the IκBαSR, suggesting that these genes are candidate target genes of constitutively activated NF-κB.

9. Constitutive NF-κB activation in pancreatic cancer cell lines

Takashi Nishina, Noritaka Yamaguchi, Sakura Azuma[1], Shinya Watanabe[4], Kentaro Semba[1] and Jun-ichiro Inoue

Pancreatic cancer is one of the most malignant cancers with an extremely poor prognosis and lacks effective therapies. Although constitutive activation of NF-κB is thought to be involved in tumorigenesis in pancreatic cancer cells, neither the precise function of constitutive NF-κB activation nor the molecular mechanisms leading to its constitutive activation in these cancer cells have been fully understood. We analyzed expression levels of several genes, which are involved in the NF-κB activation and identified a gene whose expression level showed positive correlation with the level of the basal NF-κB activity. We are currently investigating a role of the identified gene in constitutive NF-κB activation.

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Department of Cancer Biology

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The major interest of this division is in molecular signals that regulate a variety of cellular activities. Our aim is to address how deregulated cellular signals give rise to neoplastic, immune, neural, metabolic, or developmental disorders. Our goal is to understand the molecular bases of tumorigenesis and the development of other intractable diseases as a path toward uncovering therapeutic targets. Currently, we are investigating regulatory mechanisms in protein-tyrosine kinase (PTK)-mediated signaling pathways and their pathophysiological roles in hematopoietic tumors and myasthenia.

1. Activation of the receptor tyrosine kinase MuSK by the cytoplasmic adaptor-like protein Dok-7.

Inoue, A., Setoguchi, K.[1], Matsubara, Y.[1], Okada, K.[1], Hamuro, J., Tezuka, T., Ikegami, T., Sato, N.[2], Iwakura, Y.[2], Higuchi, O., and Yamanashi, Y.: [1]Medical Research Institute, Tokyo Medical and Dental University, [2]Division of Cell Biology, Center for Experimental Medicine, IMSUT.

Protein-tyrosine kinases (PTKs) play crucial roles in a variety of signaling pathways that regulate proliferation, differentiation, motility, and other activities of cells. Therefore, deregulated PTK signals give rise to a wide range of diseases such as neoplastic disorders. To understand molecular bases of PTK-mediated signalings, we identified a common substrate of many PTKs as Dok-1 in 1997. Since then, the Dok-family has been expanded to seven members, Dok-1 to Dok-7, which share structural similarities characterized by the NH_2-terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains, followed by the SH2 target motifs in the COOH-terminal moiety, suggesting an adaptor function. Indeed, as mentioned later, Dok-1 and Dok-2 recruit p120 rasGAP upon tyrosine phosphorylation to suppress Ras-Erk signaling. Unlike the other members of the Dok-family proteins, Dok-7 is preferentially expressed in muscle tissues. Furthermore, we found that Dok-7 is specifically localized to the postsynaptic area of neuromuscular junction (NMJ), suggesting its role in NMJ formation and/or maintenance.

The NMJ is a synapse between a motor neuron and skeletal muscle, where the motor nerve terminal is apposed to the endplate. The contraction of skeletal muscle is controlled by the neurotransmitter acetylcholine (ACh), which is released from the presynaptic motor nerve terminal. To achieve efficient neuromuscular transmission, acetylcholine receptors (AChRs) must be densely clustered on the postsynaptic muscle membrane of the NMJ. Failure of AChR clustering is associated with disorders of neuromuscular transmission such as congenital myasthenic syndromes (CMS) and myasthenia gravis, which

are characterized by fatigable muscle weakness. The formation of NMJs is orchestrated by the muscle-specific receptor tyrosine kinase MuSK and by neural Agrin, an extracellular activator of MuSK. However, experimentally when motor nerves are ablated, AChRs form clusters in the central region of muscle in a manner dependent on MuSK. In addition, in vivo overexpression of MuSK causes neuromuscular synapse formation in the absence of Agrin, suggesting that muscle-intrinsic, cell-autonomous activation of MuSK may be adequate to trigger presynaptic and postsynaptic differentiation in vivo. However, the mechanisms by which MuSK is activated independently of nerve and Agrin have long been unclear.

Because both MuSK and the adaptor-like cytoplasmic protein Dok-7 are localized to the postsynaptic region of NMJ, we had previously examined their interaction and found, to our surprise, that Dok-7 can induce MuSK activation in cultured myotubes and plays an essential role in neuromuscular synaptogenesis, although the mechanisms by which Dok-7 regulates MuSK activity and promotes synapse formation had been unclear. In the current study, we found that Dok-7 directly interacts with the cytoplasmic portion of MuSK and activates the receptor tyrosine kinase, and that neural Agrin requires Dok-7 in order to activate MuSK. Indeed, in vivo overexpression of Dok-7 increased MuSK activation and promoted NMJ formation. Furthermore, Dok-7 was required for the localization of MuSK in the central region of muscle, which is essential for the correct formation of NMJs in this region. These observations indicate that Dok-7 positively regulates neuromuscular synaptogenesis by controlling MuSK activity, its distribution, and responsiveness to neural Agrin. The exceptional aspects of Dok-7-MuSK-Agrin signaling lie in the unusual role of adaptor-like protein in the activation of receptor tyrosine kinase; the adaptor-like cytoplasmic protein (Dok-7) activates the receptor tyrosine kinase (MuSK) in the absence of the extracellular activator (Agrin), but activation of MuSK by the extracellular activator (Agrin) requires the cytoplasmic protein (Dok-7). Furthermore, the indispensability of Agrin for NMJ formation in vivo strongly suggests that both Dok-7 and Agrin are required for full activation of MuSK to a level sufficient for NMJ formation. We are investigating the signaling mechanisms involving Dok-7, Agrin, MuSK, and LRP4, which forms a complex with MuSK and acts as an Agrin-binding module of the complex.

2. Pathophysiological mechanisms underlying DOK7 myasthenia.

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As mentioned above, impaired clustering of acetylcholine receptors (AChRs) can underlie NMJ disorders that are both autoimmune (anti-MuSK antibody-positive myasthenia gravis) and genetic (congenital myasthenic syndromes (CMS)) in origin. Therefore, our findings that Dok-7 activates MuSK to cluster AChRs and to form NMJs had suggested that *DOK7* is a candidate gene for mutations associated with CMS. Indeed, we previously demonstrated that biallelic mutations in *DOK7* underlie a major subgroup of CMS with predominantly proximal muscle weakness that did not show tubular aggregates on muscle biopsy but were found to have normal AChR function but abnormally small and simplified NMJs. This new disease entity is termed as *DOK7* myasthenia.

The overwhelming majority of patients with *DOK7* myasthenia have at least one allele with a frameshift mutation that causes a truncation in the COOH-terminal region of Dok-7 and affects MuSK activation. Unlike NH_2-terminal moiety, which has the PH and PTB domains as essential domains for MuSK activation, little had been known about additional functional elements in the COOH-terminal moiety. In the current study, we identified a chromosome region maintenance 1 (CRM1)-dependent nuclear export signal (NES) in the COOH-terminal moiety and demonstrated that the NES-mediated cytoplasmic location of Dok-7 is essential for regulating the interaction with MuSK in myotubes. The NH_2-terminal PH domain is responsible for the nuclear import of Dok-7. We also showed that the src homology 2 (SH2) target motifs in the COOH-terminal moiety of Dok-7 are active, and crucial for MuSK activation in myotubes. In addition, CMS-associated missense mutations found in the PH or PTB domain inactivated Dok -7. Together, these findings demonstrated that, in addition to the NH_2-terminal PH and PTB domains, the COOH-terminal NES and SH2 target motifs play key roles in Dok-7-MuSK signaling for neuromuscular synaptogenesis. Ablation or disruption of these functional elements in Dok-7 probably underlies the neuromuscular junction synaptopathy observed in *DOK7* myasthenia.

We are investigating other types of mutations associated with *DOK7* myasthenia that do not appear to affect these known elements.

3. Negative regulation of PTK-mediated signaling by the Dok-family proteins in hematopoietic cells.

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The Dok-family proteins can be classified into three subgroups based on their structural similarities and expression patterns; namely, 1) Dok-1, -2, and -3, which are preferentially expressed in hematopoietic cells, 2) Dok-4, -5, and -6, which are preferentially expressed in non-hematopoietic cells, 3) Dok-7, which is preferentially expressed in muscle cells. As mentioned above, Dok-1 and its closest family Dok-2 recruit p120 rasGAP upon tyrosine phosphorylation to suppress Ras-Erk signaling. Indeed we had demonstrated that Dok-1 and Dok-2 are key negative regulators of the hematopoietic growth and survival signaling as well as the TLR4-mediated innate immune signaling. In the current study, we found that the Dok-family proteins that are preferentially expressed in hematopoietic cells negatively regulate PTK-mediated signaling and inhibit neoplastic transformation. We are investigating roles of these Dok-family proteins using genetically manipulated animals.

4. Dok-1 promotes adipocyte hypertrophy by inhibiting Erk.

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Although Dok-1 is preferentially expressed in hematopoietic cells, Dr. Kasuga's group in Kobe University found that its expression in mouse adipocytes is induced by consumption of a high-fat diet. In collaboration with his group, we demonstrated that mice lacking Dok-1 were leaner and showed improved glucose tolerance and insulin sensitivity compared with the wild-type controls. Embryonic fibroblasts from Dok-1 -deficient mice showed impaired adipogenic differentiation together with an increased activity of Erk and a consequent increase in the inhibitory phosphorylation of PPAR-γ. These findings suggested that Dok-1 promotes adipocyte hypertrophy by suppressing inhibitory effect of Erk on PPAR-γ and may thus confer predisposition to diet-induced obesity.

5. Negative regulation of PTK-mediated signaling by the Cbl-family proteins.

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The signaling pathways mediated by PTKs are critically involved in various aspects of the ontogenesis and exertion of physiological functions as well as the pathogenesis of many human diseases. Since we have identified Cbl (Casitas B-lineage lymphoma) as a protein that interacts with Lyn, a member of the Src-family PTKs, we have been investigating physiological roles of the Cbl-family proteins. In general, through their RING-type ubiquitin ligase activity, these proteins facilitate ubiquitination, thereby degradation of various tyrosine-phosphorylated proteins to negatively regulate the signaling mediated by PTKs.

The mammalian Cbl-family is comprised of Cbl, Cbl-b, and Cbl-c/Cbl-3. Cbl-c is the most recently discovered mammalian Cbl-family member, identified by our and other groups in humans and mice. Like other members, Cbl-c contains the conserved NH_2-terminal TKB (tyrosine kinase-binding) domain, linker and RING finger motif. However, Cbl-c lacks an extensive proline-rich sequence and a ubiquitin-associated leucine zipper motif that are present in Cbl and Cbl-b. This structural feature of Cbl-c is fairly similar to that of Sli-1, a *C. elegans* homologue of Cbl. The conserved region (TKB-linker-RING finger) of Cbl-c has only 50% identity to those of Cbl and Cbl-b, although Cbl and Cbl-b share 84% identity in this region. These characteristics as well as the phylogenetic studies show that Cbl-c is a distinct member among the mammalian Cbl-family proteins. The functional and structural difference of Cbl-c in comparison with Cbl is under investigation.

The physiological roles of the Cbl-family proteins are well analyzed in the immune system. They negatively regulate the signaling pathways evoked by lymphocyte antigen receptors and co-stimulatory receptors, thereby ensuring proper lymphocyte development and activation. How-

ever, the roles of the Cbl-family in other tissues and cell types are largely unknown. We found that the Cbl-family proteins properly suppressed EGF receptor-mediated signaling and cell proliferation in the epidermis, suggesting their regulatory role in the epidermal development and maintenance.

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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. Cell adhesion molecules involved in mammalian fertilization

Etsuko Mori, Dong Cun Jin and Seiichi Takasaki

Fertilization is the sum of the cellular mechanisms that pass the genome from one generation to the next and initiate development of a new organism. An ovulated mammalian egg is enclosed by two layers: an outer layer of cumulus cells and an inner, thick extracellular matrix, the zona pellucida. Sperm penetrate both layers to reach the egg plasma membrane. Sperm also bind transiently to the egg zona pellucida and the egg plasma membrane and then fuse. Recognition of the zona pellucida by the sperm cell can be subdivided in two phases: the primary binding in which proteins that are situated only at the apical plasma membrane of the acrosome-intact sperm head interact with the zona pellucida and the secondary binding in which the molecules of acrosome-reacted sperm cell interact with the zona pellucida. Sperm-zona binding results almost instantaneously in the acrosome reaction. To solve the mechanism underlying these processes, identification of functional molecules on the surface of both gametes is essential. Its importance is also pointed out by clinical studies of infertility. Male-factor infertility is primarily diagnosed by abnormal semen variables (concentration, motility, and morphology). However, it has been shown by clinical examination that more than 50% of couples with no fertilization during in vitro fertilization were normal on semen analysis. Remarkably, 75% of these men had sperm that poorly bound to the zona pellucida and 47% had no sperm that penetrate the zona pellucida. In addition, 25% of infertile men with normal semen variables and normal sperm binding to the zona pellucida had a defective zona-induced acrosome reaction. Thus, defects in sperm binding and/or induction of the acrosome reaction are important causes of sperm dysfunction in men with apparently normal semen. We are now focusing on the adhesion mechanism working in fertilization, especially at the stage of sperm-zona pellucida binding. An effort has been made to define the sperm surface protein(s) that binds to the zona. Many candidates have been proposed, but none has found wide acceptance. For example, male mice obtained with gene knockout for the sperm surface proteins fertilin β, cyritestin or the spermatogenesis-specific chaperone calmegin are infertile and produce sperm that cannot bind to the zona. pellucida. However, the calmegin-null sperm have been shown to lack fertilin β.

Both fertilin β and cyritestin knockout sperm, through an unknown mechanism, lose not only the deleted gene product but other membrane proteins as well. Thus, the sperm protein(s) responsible for the zona pellucida binding has not been identified. We have so far analyzed oligosaccharides of boar zona pellucida glycoproteins and found out that oligosaccharides containing N-acetyllactosamine structure and Lewis X structure are important for sperm binding. Based on the structural information about the functional ligands on the zona pellucida, we have been investigating sperm molecules recognizing the oligosaccharides of zona pellucida glycoproteins. Two kinds of affinity technology were applied to detect or isolate carbohydrate binding proteins in detergent-solubilized boar sperm plasma membrane. The first was to detect proteins that bind to the plastic well coated with dextran-based multiple oligosaccharide with N-acetyllactosamine structure. The second was to isolate the proteins using the Sepharose beads coupled with desialylated α1-acid glycoprotein that contains N-acetyllactosamine -type oligosaccharides. Then, the two proteins with apparent molecular masses of 70 kDa and 40 kDa were detected as candidates for sperm carbohydrate binding proteins. Proteomics analysis suggested that the two proteins are members of ADAM (a disintegrin and metalloprotease). Both of the recombinant proteins produced by yeast were shown to have adhesion activity to cell surface integrin molecules, and we are currently analyzing the carbohydrate binding activity of the proteins produce by mammalian cells. We also analyzed recombinant ADAMs of mice that were produced by yeast, and found that ADAM4, ADAM5 and ADAM6 also bind to integrins. Their role in sperm-oviduct epithelial cell interaction is being studied.

2. Analysis of cancer-related sialic acid and sialylated glycans

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Sialylated glycoconjugates seem to be involved in tumour biology, since aberrant glycosylation patterns are very common in human and animal neoplasias. These tumor-associated carbohydrate antigens are of prognostic value, since their expression frequently correlates with invasiveness, metastasis and the tumor grade. The majority of sialylated tumor markers in man involve changes in the presentation of N-acetylneuraminic acid (NeuAc). However, evidence is accumulating to suggest that N-glycolylneuraminic acid (NeuGc) can appear in certain human cancers. Actually, we found that NeuGc-containing glycoproteins are expressed in human hepatocarcinoma tissue and other tumor cell lines. In addition, we found that sera from carcinoma patients contain antibodies recognizing NeuGc-containing glycoconjugates. Their content was much higher than that of normal humans. Thus, the quantification of NeuGc and antibody recognizing NeuGc may be of clinical value. However, expression of NeuGc in man is a paradox in glycobiology, because CMP-NeuAc hydroxylase that is involved in the de novo synthesis of NeuGc is non-functional in man. The mechanism by which human cancer cells synthesize NeuGc has to be elucidated.

Sialic acid is usually linked to terminal galactose residues, internal N-acetylglucosamine, or N-acetylgalactosamine residues of glycans in α-configulation. No β-linked sialic acids have so far been found in glycoproteins and glycolipids. However, it is possible that β-linked sialic acids are synthesized by the cells under pathological condition. To address this issue, an antibody raised against a synthetic β-linked sialic acid containing glycolipid was used. Interestingly, gangliosides from human cancer cell lines were stained with the antibody by the immunochemical method, but those from normal cells were not. The result suggests that β-linked sialic acids are expressed on the glycoconjugates of cancer cells. However, there is no chemical evidence that the epitose is really sialylated by β-linkage. Structural analysis of the epitope glycans is now in progress.