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 and chromosome dynamics that are involved in the regulation of cell cycle progression, particularly that of mitosis.

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

Toru Suzuki, Junko Tsuzuku, Mitsuhiro Yoneda, Masahiro Morita, Yan Zhang, Kentaro Ito, Shinichiro Ogawa, Akinori Takahashi, Yusuke Nakajima and Tadashi Yamamoto

By screening a cDNA expression library using ErbB-2 protein as a probe, we identified Tob, a 45kDa protein as an ErbB2-interacting protein. Tob was homologous to the growth suppressing proteins, Btg1 and Btg2/PC3. We also cloned novel genes, *tob2* and *ana* that are homologous to *tob*. These proteins compose a functionally related anti-proliferative protein family, called the Tob/Btg family.

To elucidate the physiological function of the Tob/Btg family proteins, we generated mice lacking *tob, tob2*, or *ana*. By characterizing them pathologically and molecularly, we found the following. (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. Furthermore, expression of *RANKL* mRNA in stromal cells was increased in the absence of Tob2 and decreased in the presence of Tob2. Tob2 interacted with vitamin D3 receptor (VDR), suggesting its involvement in VDR-mediated regulation of transcription. In conclusion, 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. Since lung adenocarcinoma is thought to be mainly derived from type II alveolar epithelial cells, Ana may be involved in development of lung tumor. Indeed, *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 the lung adenocarcinoma examined. These data suggested that downregulation of *ana* gene might be responsi-

ble for lung adenocarcinoma progression. Currently, we are investigating the molecular mechanism by which Ana regulates progression of lung tumor.

Recently, we found that Tob is involved 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 resulted in frequent induction of apoptosis irrespective of the presence of functional p53 even at UV doses that do not promote Tob degradation. Thus, proteosomal clearance of Tob provides a novel p53-independent pathway for UV-induced apoptosis.

Other studies to establish biological significance of the *tob* family members are following. First, we purified Tob-containing complexes from HeLa cells that stably express the Flag epitope-tagged Tob by immunoprecipitation with anti-Flag antibody. Mass spectrometric analysis showed that CNot1, CNot2, CNot3, CNot6/CCR4, CNot7 and CNot9 were included in the Tob complex. We also showed that Tob suppresses the deadenylase activity of CCR4/Not complex at least *in vitro*. Although the CCR4/Not complex possesses mRNA deadenylase activity, and thus regulates the stability of mRNAs, biological roles of the complex in mammals remain unknown. Second, we performed yeast two-hybrid screening using Tob as a bait, and identified a serine/threonine kinase Ndr. Interestingly, a yeast homolog of the kinase is reported to associate with the CCR4/Not complex. Third, we newly identified CCR4b /CNot6L, which is homologous to yeast CCR4 deadenylase. CCR4b forms a multi-subunit complex similar to the yeast CCR4/Not complex. Suppression of CCR4b by RNA interference (RNAi) results in growth retardation of NIH3T3 cells accompanied by elevation of both $p27^{Kip1}$ mRNA and $p27^{Kip1}$ protein. Reintroduction of wild-type CCR4b but not mutant CCR4b lacking deadenylase activity restores the growth of CCR4b-depleted NIH3T3 cells. The data suggest that CCR4b regulates the cell growth in a manner dependent on its deadenylase activity. Our findings suggest that CCR4b regulates the turnover rate of specific target mRNAs. It should be elucidated how CCR4/Not deadenylase complex recognize the specific mRNAs. Fourth, we examined the effect of RNAi-mediated knock down of the other components of the CCR4/Not complex on cell proliferation and differentiation. So far, we found that depletion of several components of the complex results in cell growth arrest or in extensive cell death. The cell death occurred in a manner dependent on the caspase activity. We are now examining the mechanisms by which cell growth or apoptosis was induced by down-regulation of the components of the CCR4/Not complex. Finally, to examine the biological functions of CCR4/Not complex, we generated *cnot3, cnot6, cnot6L, cnot8, cnot9* and *ndr*-deficient mice. Our data suggest that most of the components in the complex are involved in embryonic development and energy homeostasis. To understand the underlying mechanism by which each molecule in the complex regulates such biological events, we are in search of specific mRNAs targeted by the CCR4/Not complex. Several approaches such as microarray analysis, Real-time PCR, and Northern blot analysis have been undertaken.

• 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 characterizing brain specific substrates of these kinases, including *N*-methyl-D-aspartate (NMDA) type of ionotropic glutamate receptors. Our own studies have shown that GluN2A and GluN2B subunits of NMDA receptors, which play important roles in learning, memory formation, and emotional behaviors, are the major substrates of Fyn and Src. We identified Tyr-1472 phosphorylation on GluN2B and Tyr-1325 phosphorylation on GluN2A as the major tyrosine phosphorylation site of GluN2B and GluN2A, respectively. Using the knock-in mouse lines expressing mutant GluN2B with a Tyr-1472-Phe (Y1472F) mutation or expressing mutant GluN2A with a Tyr-1325-Phe (Y1325F) mutation, we have shown that Tyr-1472 phosphorylation is a key mediator of fear-related learning and emotional behavior in the amygdala and that Tyr-1325 regulates depression-related behavior. We are now characterizing molecular mechanisms underlying these behavioral abnormalities.

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 using solid-phase phosphorylation screening, yeast two-hybrid screening, and proteomic ap-

proaches. As a result, we have identified a number of putative mediators of Fyn- and Src-mediated signaling, including NYAP, FAK, p250GAP, TCGAP, Nogo-A, and RhoGEFs. Among these proteins, we demonstrated that NYAP family proteins are the most heavily tyrosine-phosphorylated proteins, accounting for about half of tyrosine phosphorylation reactions in the developing neuron. We generated NYAP family knockout mice and demonstrated that the NYAP family plays a pivotal role as the central scaffold in the PI3K signaling pathway. We are now studying the physiological roles of p250GAP, TCGAP, and FAK as well as NYAP in the neuronal functions.

In database search for novel protein-tyrosine kinases, we identified a kinase that is highly expressed in the brain and termed it as BREK (Brain-Enriched Kinase). It turned out that BREK is the same as AATYK2/LMTK2 found at the almost same time by others. BREK is a member of a family consisting of AATYK1/LMTK1, AATYK2/BREK/LMTK2, AATYK3/LMTK3. BREK has also been termed as KPI-2/CPRK. All the family members are predominantly expressed in the brain. We showed that BREK plays a role in NGF signaling in PC12 cells, suggesting that BREK is involved in neural development and function in early postnatal development. To investigate the physiological role of BREK, we generated mice with targeted disruption of *brek*. While *brek* is highly expressed in the brain, it is also expressed in the testis. We have found that $brek^{-/-}$ male mice are infertile with azoospermia. We are currently studying the roles of BREK in the central nervous system using the knockout mice.

• Roles of chromokinesin Kid and mitotic kinases in execution of cell division

Miho Ohsugi, Noriko Tokai-Nishizumi, Naoki Oshimori, Natsuko Masuda, Kenji Iemura, 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. In addition, Importin α directly associates with Kid via nuclear localization signals (NLSs) and Ran-GTP-mediated dissociation of importin α from Kid promotes the accumulation of Kid on chromosomes. During anaphase and telophase, Kid is localized in the interstices between adjacent chromosomes and contributes to the tight clustering of anaphase chromosomes (anaphase chromsome compaction). Kid deficiency 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 maternal factor specifically require Kid to prevent formation of multinucleated cells. We further analyzed the chromosome dynamics during anaphase II and pronuclear formation process in wild type and Kid-deficient oocytes. Our data suggest that Kid is important for both establishment and maintenance of anaphase/telophase chromosome cluster especially during female pronuclear formation process.

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 Plk1's actions remain to be elucidated. Through the solid-phase phosphorylation screening, we previously identified several Plk1 substrates including Kizuna (Kiz) which when depleted causes fragmentation and dissociation of the pericentriolar material (PCM) from centrioles at prometaphase, resulting in multipolar spindles. Using a yeast two-hybrid screen for Kiz-interacting proteins, we identified an uncharacterized centrosome protein Cep72. RNAi experiments demonstrate that Cep72 is essential for localization of CG-NAP, a large coiled-coil protein forming the structural framework of the PCM, as well as Kiz. Cep72 is also involved in γ-tubulin ring complexes (γTuRCs) recruitment to the centrosome and CG-NAP confers the microtubule nucleation activity on the γTuRCs. During mitosis, the Cep72-mediated centrosomal

MTOC activity helps connect spindle microtubules to the centrosome so that forces generated by chromosome movement along microtubules converge on the PCM. Cep72-recruited Kiz ensures structural integrity of the PCM to endure the microtubule-mediated forces. Our data further suggest that Cep72-mediated spindle pole formation is essential to align chromosomes at the metaphase plate and to generate tensions between kinetochores. 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.

iii) Chromosome dynamics in early mice embryo

In vertebrates, oocytes are arrested at metaphase of the second meiosis, and fertilization triggers the anaphase onset and emission of the second polar body. Then, nuclear envelopes are assembled around maternal and paternal chromosomes, forming separate haploid male and female pronuclei. In mouse embryo, this topological genome separation appears to be preserved up to the four-cell embryo stage and then gradually disappears. Maternal proteins and transcripts stored in oocytes control embryonic development, until zygotic gene activation begins. In mice, minor gene activation begins at the 1-cell stage that is followed by a major gene activation at the 2-cell stage. Therefore, completion of oocyte meiosis II and first couple of mitosis after fertilization are almost exclusively under maternal control. We are interested in how the structure and behavior of maternal and paternal chromosomes change around the transition point from maternal to embryonic control. We are addressing these issues by several approaches including time-lapse imaging of chromosomes and some nuclear proteins in early mouse embryos.

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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. High throughput analysis of proteins associating with a proinvasive MT1-MMP in human malignant melanoma A375 cells

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Membrane-type 1 matrix metalloproteinase (MT1-MMP), a powerful modulator of the pericellular environment, promotes migration, invasion, and proliferation of cells. To perform its potent proteolytic activity in a controlled manner, MT1-MMP has to be regulated precisely. However, our knowledge about substrates and regulatory proteins is still very limited. In this study we identify a catalog of proteins that directly or indirectly interact with MT1-MMP. We expressed a FLAG-tagged MT1-MMP stably in human malignant melanoma A375 cells. We prepared cell lysate using Brij98 and MT1-MMP was affinity purified together with associating proteins using an anti-FLAG antibody. A distinct set of membrane proteins was found to copurify with MT1-MMP when biotin-labeled proteins were monitored. The proteins were analyzed with an integrated system composed of nano-flow liquid chromatography and tandem mass spectrometry. We identified 158 proteins including several previously reported to bind MT1-MMP, although most had not previously been identified. Six of these membrane proteins, including one previously shown to interact with MT1-MMP, were coexpressed with MT1-MMP in HT1080 cells. Five of the latter were found to associate with MT1-MMP in an immunoprecipitation assay. Immunostaining of cells expressing each of these test proteins revealed that one colocalized with MT1-MMP at the ruffling membrane and the other at the perinuclear vesicles. In contrast, another protein which did not coprecipitate with MT1-MMP showed no colocalization. Recombinant MT1-MMP cleaved two of the tested pro-

teins at least *in vitro*. Thus, we provide a valuable resource to identify substrates and regulators of MT1-MMP in tumor cells.

2. Identification and characterization of Lutheran blood group glycoprotein as a new substrate of membrane-type 1 matrix metalloproteinase 1 (MT1-MMP): a systemic whole cell analysis of MT 1-MMP-associating proteins in A431 cells

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Membrane-type 1 matrix metalloproteinase 1 (MT1-MMP) is a potent modulator of the pericellular microenvironment and regulates cellular functions in physiological and pathological settings in mammals. MT1-MMP mediates its biological effects through cleavage of specific substrate proteins. However, our knowledge of MT1-MMP substrates remains limited. To identify new substrates of MT1-MMP, we purified proteins associating with MT1-MMP in human epidermoid carcinoma A431 cells and analyzed them by mass spectrometry. We identified 163 proteins including membrane proteins, cytoplasmic proteins, and functionally unknown proteins. Sixty-four membrane proteins were identified and they included known MT1-MMP substrates. Of these, eighteen membrane proteins were selected and we confirmed their association with MT1-MMP using an immunoprecipitation assay. Co-expression of each protein together with MT1-MMP revealed that nine proteins were cleaved by MT1-MMP. Lutheran blood group glycoprotein (Lu) is one of the proteins cleaved by MT1-MMP and we confirmed the cleavage of the endogenous Lu protein by endogenous MT1-MMP in A431 cells. Mutation of the cleavage site of Lu abrogated processing by MT1-MMP. Lu protein expressed in A431 cells bound to laminin-511 and knockdown of MT1-MMP in these cells increased both their binding to Laminin-511 and the amount of Lu protein on the cell surface. Thus, we have identified new physiological substrates of MT1-MMP by analysis of the associating proteins with MT1-MMP and demonstrated that the list of membrane proteins is an enriched source of MT1-MMP substrates.

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

Takeharu Sakamoto and Motoharu Seiki

MT1-MMP is a proinvasive protease that regulates various cellular functions as evidenced by myriad defects in different types of cells and tissues in MT1-MMP-deficient ($MT1^{-/-}$) mice. Here we demonstrate that $MT1^{-/-}$ mice exhibit fewer infiltrating macrophages into sites of inflammation. $MT1^{-/-}$ macrophages exhibited a reduced ability to invade reconstituted basement membrane (Matrigel) and invasion by wild type (WT) macrophages was inhibited by a synthetic MMP inhibitor (BB94) to a level similar to that of $MT1^{-/-}$ cells. The rate of migration of $MT1^{-/-}$ macrophages was also low compared to that of the WT cells and re-expression of MT1-MMP in $MT1^{-/-}$ macrophages reconstituted their migratory activity. Unexpectedly, however, BB94 did not inhibit the migration of WT macrophages. The migration-boosting activity of MT1-MMP is retained in a mutant that lacks most of the extracellular portion including the catalytic and hemopexin-like domains. In contrast, deletion of the cytoplasmic tail (CP) abolished the activity completely. Thus, we have demonstrated that MT1-MMP regulates macrophages via its invasion-promoting protease activity as well as its CP-dependent non-proteolytic activity to boost cell migration.

4. Mint3 enhances the activity of hypoxia-inducible factor-1 (HIF-1) in macrophages by suppressing the activity of factor inhibiting HIF-1

Takeharu Sakamoto and Motoharu Seiki

Hypoxia Inducible Factor-1 (HIF-1) is a key transcription factor regulating cellular responses to hypoxia and is composed of α and β subunits. During normoxia, Factor Inhibiting HIF-1 (FIH-1) inhibits the activity of HIF-1 by preventing HIF-1α binding to p300/CBP via modification of the Asn^{803} residue. However, it is not known whether FIH-1 activity can be regulated in an oxygen-independent manner. In this study, we survey possible binding proteins to FIH-1 and identify Mint3/APBA3, which has been reported to bind Alzheimer's beta-amyloid precursor protein (APP). Purified Mint3 binds FIH-1 and inhibits the ability of FIH-1 to modify HIF-1α in vitro. In a reporter assay, the activity of HIF-1α is suppressed due to endogenous FIH-1 in HEK293 cells and expression of Mint3 antagonizes this suppression. Macrophages are

known to depend on glycolysis for ATP production due to elevated HIF-1 activity. FIH-1 activity is suppressed in macrophages by Mint3 so as to maintain HIF-1 activity. FIH-1 forms a complex with Mint3 and these two factors co-localize within the perinuclear region. Knockdown of Mint3 expression in macrophages leads to redistribution of FIH-1 to the cytoplasm and decreases glycolysis and ATP production. Thus, Mint3 regulates the FIH-1-HIF-1 pathway, which controls ATP production in macrophages, and therefore represents a potential new therapeutic target to regulate macrophage-mediated inflammation.

5. A novel protein associated with MT1-MMP binds $p27^{kip1}$, and regulates RhoA activation, actin remodelling and matrigel invasion

Daisuke Hoshino, Taizo Tomari, Makoto Nagano, Naohiko Koshikawa and Motoharu Seiki

Pericelllular proteolysis by membrane-type 1 matrix metalloproteinase (MT1-MMP) plays a pivotal role in tumour cell invasion. Localization of MT1-MMP at the invasion front of cells, e.g. on lamellipodia and invadopodia, has to be regulated in co-ordination with reorganization of the actin cytoskeleton. However, little is known about how such invasion-related actin structures are regulated at the sites where MT1-MMP localizes. During analysis of MT1-MMP associated proteins, we identified a heretofore uncharacterized protein. This protein, which we call p27RF-Rho, enhances activation of RhoA by releasing it from inhibition by $p27^{kip1}$ and thereby regulates actin structures, though MT1-MMP does not play a role in this process. $p27^{kip1}$ is a well-known cell cycle regulator in the nucleus. In contrast, cytoplasmic $p27^{kip1}$ has been demonstrated to bind GDP-RhoA and inhibit GDP-GTP exchange mediated by guanine nucleotide exchange factors (GEFs). p27RF-Rho binds $p27^{kip1}$ and prevents $p27^{kip1}$ from binding to RhoA, thereby freeing the latter for activation. Knockdown of p27RF-Rho expression renders cells resistant to RhoA activation stimuli, whereas overexpression of p27RF-Rho sensitizes cells to such stimulation. p27RF-Rho exhibits a punctate distribution in invasive human tumour cell lines. Stimulation of the cells with LPA induces activation of RhoA and induces the formation of punctate actin structures within foci of p27RF-Rho localization. Some of the punctate actin structures co-localize with MT1-MMP and cortactin. Down-regulation of p27RF-Rho prevents both redistribution of actin into the punctate structures and tumour cell invasion. Thus, p27RF-Rho is a new potential target for cancer therapy development.

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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 and pathological functions of CADM1/TSLC1 protein in epithelial structure

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CADM1/TSLC1 is an immunoglobulin superfamily cell adhesion molecule and primarily involved in epithelial cell adhesion. We have previously shown that CADM1 associates with an actin-binding protein, 4.1B/DAL-1, and a member of scaffold proteins, membrane protein palmitoylated 3 (MPP3). We have newly demonstrated that MPP1/p55 and MPP2/DLG2, as well as MPP3, interact with both CADM1 and 4.1B, forming a tripartite-complex. Among MPP 1-3, MPP2 is recruited to the CADM1-4.1B complex in the early process of adhesion in HEK293 cells. By suppressing CADM1 expression using siRNA, HEK293 cells lose epithelia-like structure and show flat morphology with immature cell adhesion. 4.1B and MPP2, as well as E-cadherin and ZO-1, are mislocalized from the membrane by depletion of CADM1 in HEK293. These findings suggest that CADM1 is involved in the formation of epithelia-like cell structure with 4.1B and MPP2, while loss of its function could cause morphological transformation of cancer cells. To further investigate the function of this cascade, additional binding proteins to CADM1 are being identified by proteomics approaches. Dynamic regulation of CADM1 protein on the cell membrane is also being analyzed using photobleaching assay. Furthermore, molecular mechanism of transcriptional regulation of the *CADM1* gene is investigated.

2. Patho-physiological function of the CADM1 protein in animal models

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

To understand the physiological role of CADM1 protein in animal models, *Cadm1*-deficient mice were generated. We have previously reported that *Cadm1*$^{-/-}$ mice showed male infertility due to disruption of adhesion of spermatocytes to Sertoli cells in the seminiferous tu-

bules. Genetic complementation study of the *Cadm1*$^{-/-}$ mice by crossing the transgenic mice expressing human CADM1 is being investigated. We have also found that both *Cadm1*$^{-/-}$ and *Cadm1*$^{+/-}$ mice show significant increase in the incidence of spontaneous development of lung adenomas and adenocarcinomas, indicating that the *CADM1* is a potent suppressor gene in lung tumor. Additional molecular alterations in lung tumors are being investigated to understand the molecular mechanisms of lung tumorigenesis.

3. Functional analyses of CADM1 overexpression in adult T-cell leukemia -lymphoma (ATL) and a subset of other human tumors.

Mari Masuda[1], Miwako Iwai, Mika Sakurai-Yageta, Shigefumi Murakami, Tomoko Masuda, Akihiko Ito, and Yoshinori Murakami: [1]Chemotherapy Division, National Cancer Center Research Institute

We have previously reported in collaboration with others that CADM1 is overexpressed in adult T-cell leukemia-lymphoma (ATL) cells. It has been suggested that the expression of CADM1 protein promotes infiltration of leukemic cells into various organs which is one of the frequent clinical manifestations of ATL. We have newly demonstrated that the cytoplasmic domain of CADM1 directly interacts with T-lymphoma invasion and metastasis 1 (Tiam1), a Rac-specific guanine nucleotide exchange factor (RacGEF), through its PDZ domain. This interaction induces lamellipodia formation through Rac activation in HTLV-I transformed cell lines as well as ATL cell lines. These results indicate that Tiam1 integrates signals from CADM1 to regulate the actin cytoskeleton through Rac activation. It is probable, therefore, that the CADM1-Tiam1 interaction promotes cell motility leading to tissue infiltration of leukemic cells in ATL patients. Since overexpression of CADM1 is also observed in a subset of human tumors with high metastatic activity, its pathological significance is also being analyzed. Furthermore, possible cross-talk of CADM1 cascade with other known signal transduction pathways is examined by screening the inhibitors of CADM1-mediated cell adhesion and motility.

4. Analyses of genetic and epigenetic abnormalities in human tumors

Yasuhiro Ebihara, Mika Takahashi, Masayoshi Nagata, Taketo Kawai, Miwako Iwai, and Yoshinori Murakami

To understand the molecular features of multistage carcinogenesis in human, aberrations of CADM1 and the molecules in its cascade, as well as other key molecules in human tumorigenesis, were examined in various cancers. In 40 renal clear cell carcinoma (RCCC), loss of CADM4 expression was observed in 28 (70%) tumors and associated with vascular infiltration. Moreover, introduction of CADM4 into RCCC cells, 786-O, suppressed tumor formation in nude mice. These results suggest that CADM4 functions as a tumor suppressor in RCCC. Aberrations of the CADM1 cascade in other human tumors, including those from the lung, head and neck, breast, and bladder are being investigated.

5. CADM1 is a key regulator of nerve-mast cell interaction: possible mechanism involving CADM1 mRNA splicing

Akihiko Ito, Mitsuru Hagiyama, Naoki Ichiyanagi, Yusuke Nagara, Yuki Ikeda, 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 nerve and mast cells. Close apposition of nerve and mast cells *in vivo* is viewed as a functional unit of neuro-immune mechanisms, and is sustained mainly by *trans*-homophilic binding of CADM1. Cerebral nerve-mast cell interaction may be developmentally modulated, because neonatal mouse cerebrum contained abundant transcripts for two splicing isoforms, CADM1b and CADM1c, while adult cerebrum contained mainly CADM1d transcripts. To probe how individual isoforms are involved in nerve-mast cell interaction, Neuro2a, a neuroblastoma cell line expressing CADM1c endogenously, was modified to express additionally either GFP-tagged CADM1b or CADM1 d, and was cocultured on their neurite networks with wild-type mouse bone marrow-derived mast cells (BMMCs), which expressed CADM1c exclusively. Crosslinking experiments revealed that CADM1d preferred to cluster as a homodimer along Neuro2a neurites. Interestingly, when intracellular Ca^{2+} concentrations were monitored in neurite-attendant BMMCs after Neuro2a cells were specifically activated by histamine, the CADM1d assembly site was found to be more effective in evoking mast cell response against neurite activation. These results indicated that CADM1d is a specific neuronal isoform that enhances nerve-mast cell interaction, and sug-

gested that nerve-mast cell interaction might be reinforced in adult brains due to greater CADM 1d expression.

6. Physical aspects of cellular adhesion: establishment of a non-contact measurement system assisted by femtosecond laser

Akihiko Ito, Mitsuru Hagiyama, Naoki Ichiyanagi, Takahiro Mimae, Keiko Kimura

Although our understandings on adhesion molecules have lately made remarkable advances at genetic and molecular levels, physical aspects of cellular adhesion have not been fully examined yet. Currently, we developed a new method to estimate intercellular adhesive strength by focusing an intense femtosecond laser pulse in cell culture medium through an objective lens. Since this laser shot generates a transient stress, which propagates like "Tsunami" from the laser focal point with a few tens μm diameter, cell-cell detachment is induced as a function of the local magnitude of the "Tsunami", which depends on the laser intensity and the distance between the laser focal point and the cell-cell contact. For example, in monolayer culture of polarized epithelial MDCK cells, lateral membrane-lateral membrane dissociation between neighboring cells progressed gradually as intense laser shots were repeated. We estimated the force of the "Tsunami" by measuring the bending movement of an atomic force microscope cantilever placed instead of target cells in culture medium, and found that the force loaded on the detached cells was in a μN order, indicating that MDCK cells adhered to each other in a μN order of force.

Publications

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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 molecular mechanisms underlying TRAF6-mediated activation of signal transduction pathways and 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. Molecular mechanism of the regulation of Rel/NF-κB transcription factor

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Transcription factor Rel/NF-κB binds specifically to a decameric motif of nucleotide, κB site, and activates transcription. The activation of Rel/NF-κB has been demonstrated to be carried out post-translationally upon extracellular stimuli through membrane receptors such as members of the TLR/IL-1R family and of TNFR superfamily. Rel/NF-κB 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 of IκB. Rel/NF-κB subsequently translocates to the nucleus to activate transcription of target genes. This project is to identify a molecule that transduces signal from membrane receptors to Rel/NF-κB/IκB complex. We have previously identi-

fied upstream activators of Rel/NF-κB, 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 TRAF6, together with Ubc13/Uev1A complex, catalyses the formation of Lys63 (K63)-linked polyubiquitin chains that conjugate TRAF6 and NEMO, 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. Although TAK1 and mitogen-activated protein kinase kinase kinase 3 (MEKK3) are both crucial for IL-1-dependent activation of NF-κB, their potential functional and physical interactions remain unclear. This year, we demonstrated that TAK1-mediated activation of NF-κB required the transient formation of a signaling complex that included TRAF6, MEKK3, and TAK1. Site-specific, K63-linked polyubiquitination of TAK1 at Lys-209, likely catalyzed by TRAF6 and Ubc13, was required for the formation of this complex. After TAK1-mediated activation of NF-κB, TRAF6 subsequently activated NF-κB through MEKK3 independently of TAK1, thereby establishing continuous activation of NF-κB, which was required for the production of sufficient cytokines. Therefore, we propose that the cooperative activation of NF-κB by two mechanistically and temporally distinct MEKK3-dependent pathways that diverge at TRAF6 critically contributes to immune and inflammatory systems.

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

Taishin Akiyama, Yusuke Shimo, Daisuke Ohshima, Hiromi Yanai, 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. We previously found that TNF receptor family members RANK and CD40 cooperatively regulate the development of mTECs expressing Aire and TSAs. Ligation of RANK or CD40 is sufficient for inducing mature mTECs expressing Aire and TSAs in fetal thymic stroma in TRAF6 and NF-κB inducing kinase (NIK) dependent manner.

Another TNF receptor family member OPG, which is a decoy receptor of RANKL, is involved in controlling the mTEC development. OPG-deficient mice showed enhanced development of mTECs expressing Aire and several TSAs. We also have found that ligation of RANK but not CD40 induces OPG in fetal thymic stroma, suggesting that the expression of OPG is not directly coupled with final maturation of mTECs. Consistently, the expression of OPG is significantly reduced in RANKL-deficient mice. Deficiency of OPG resulted in slight but statistically significant increment in the ratio of regulatory T cells in CD4 single positive T cells in thymus. Overall, these data suggested that a negative feedback loop consisiting of RANKL-RANK-OPG in TECs control the number of mTECs and mTEC-dependent immunological tolerance induction.

3. Involvement of a novel TRAF6-interacting protein in anti-viral signaling pathways

Hirohisa Shiraishi, Taishin Akiyama, and Jun-ichiro Inoue

TRAF6 regulates innate and acquired immune responses by activating NF-κB and MAP kinases. To elucidate molecular mechanisms underlying the activation of TRAF6-dependent signal, we screened for proteins that bind TRAF6, by *in vitro* virus (IVV) method and subsequent co-immunoprecipitation assays. As a result, we identified a novel TRAF6-binding protein. In order to address the role of this protein in the TRAF6-mediated activation of NF-κB, we performed a reporter analysis using NF-κB promoter conjugated with luciferase gene. Although enforced expression of this protein did not activate NF-κB by itself, NF-κB activation induced by TRAF6-overexpression was enhanced when this protein was co-expressed. We have recently reported that TRAF6 plays a critical role in RIG-I like helicase (RLH) signaling, which con-

trols the expression of type-I interferon and inflammatory cytokines by responding against RNA virus infection. Moreover, this protein also binds TRAF3, but not TRAF2 and TRAF5. Given that both TRAF3 and TRAF6 function downstream of RIG-I, these results led us to check whether the identified protein is involved in the RIG-I pathway. The NF-κB reporter analysis revealed that RIG-I-induced NF-κB activation was inhibited by co-expression of this protein, suggesting that this protein could be a negative regulator for the RIG-I pathway. Taken together, our data leads to a hypothesis that the novel TRAF6-binding protein regulates the RLH pathway through modulating the function of TRAF6 and TRAF3.

4. Hunting for NF-κB-inducing kinase (NIK) associated proteins regulating immune response

Miho Shinzawa, Hiromi Yanai, Taishin Akiyama, and Jun-ichiro Inoue

Transcription factor NF-κB family plays an important role in regulating immune developments and immune responses. The activation of NF-κB is mainly divided into classical and alternative pathways. The alternative pathway requires NIK for the activation and regulates the development and organization of some lymphoid organs. The alymphoplasia (aly) mice, which carry a dysfunctional mutation in NIK gene, and NIK-deficient mice completely lack lymph nodes and Payer's patches, and exhibit disturbed thymus architecture. In addition to lymphoid organogenesis, T cells from aly mice show impaired proliferation and IL-2 production in response to anti-CD3 stimulation, indicating that a NIK-dependent signal regulates T cell response. In order to clarify the molecular mechanism underlying the activation of the NIK-dependent signal in immune response, we exploit proteins interacting with NIK by "*in vitro* virus" screening and subsequent immunoprecipitation assays. As a result, we identified a novel NIK-associated protein, which is able to suppress NF-κB activation induced by NIK overexpression. We are currently investigating the physiological importance of the interaction between NIK and this identified NIK-associated protein in T cells or other immune competent cells.

5. 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 crucial role in bone homeostasis in concert with osteoblasts, which mediate bone formation. Excess formation or activity of osteoclasts results in pathological bone resorption, such as postmenopausal osteoporosis and rheumatoid arthritis. Therefore, precise elucidation of the regulatory mechanisms of osteoclastogenesis is essential for understanding the onset of skeletal diseases and for developing drugs to treat such diseases.

Osteoclastogenesis is tightly regulated by the RANK/RANKL-signaling in progenitor cells. Intracellular signaling pathways of RANK are mediated by an adaptor molecule, TRAF6. The RANK/RANKL-signaling activates NF-κB and AP-1, and induces PLCγ2-mediated Ca^{2+} oscillation, which is required for induction of NFATc1, a master transcriptional factor in osteoclastogenesis. However, the molecular mechanisms by which the RANK/RANKL-signaling mediates osteoclastogenic signals remains to be elucidated. This year, we identified a novel domain, HCR (highly conserved domain in RANK). HCR-deficient RANK could not induce osteoclast differentiation even in the presence of TRAF6-binding site. This is because lack of HCR resulted in impaired activation of NF-κB and PLCγ2 at 24 h after stimulation although their activation was not affected during first 1 h after stimulation. Addition of the HCR to the cytoplasmic tail of CD40, which itself is unable to induce osteoclastogenesis, resulted in long-term activation of RANK-signaling and activation of NFATc1 to induce osteoclast formation. These results suggest that the HCR is capable of inducing osteoclastogenesis in concert with the TRAF6-binding site. Furthermore, we found that the HCR binds Gab2, which then associates with PLCγ2 in a stimulation dependent manner. Moreover, our experiment revealed that Gab2 bound TRAF6. These results strongly suggest that HCR provides a platform for forming a novel signal complex containing Gab2, PLCγ2, and TRAF6 upon RANKL stimulation, and continuously activate RANK signaling. In addition we showed that expression of HCR-peptides itself severely inhibited formation of osteoclasts. This result indicates that HCR peptides itself is a potent inhibitor of osteoclastogenesis presumably by squelching signal complexes. These results suggest that, HCR-signaling may be a novel therapeutic target for pathologic bone resorption.

6. 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 NF-κB activation may lead to development of drugs to treat ATL. Constitutive NF-κB activation in HTLV-1-infected cells 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 NF-κB activation. However, little is known about how Tax induces activation of the IKK complex after it binds to NEMO.

We have previously established a cell-free system to analyze the Tax mediated activation of the IKK complex. Addition of recombinant Tax protein, which was produced by a vaculovirus protein expression system, to cell extracts resulted in the phosphorylation of IκBα, indicating that Tax protein can activate endogenous IKK complex in cell lysates. Using this cell-free system, We demonstrated that Tax-induced IKK activation requires unidentified factors on addition to the components of IKK complex. To identify the factors involved in the Tax-induced IKK activation, we purified the Tax-IKK protein complex from cell extracts. Proteomics analysis of the purified Tax-IKK complex showed that the complex includes ten novel components of the IKK complex. We are currently clarifying the involvement and functions of these protein factors in the Tax-induced IKK activation.

7. Role of TRAF6 in T cell proliferation

Hidehiko Motegi, Taishin Akiyama and Jun-ichiro Inoue

Recently, we found that CD4-positive T cells from TRAF6-deficient mice proliferated more rapidly than 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-deficient T cells. We are currently focusing on molecular mechanisms of such an abnormality in the absence of TRAF6.

8. Analysis of the molecular mechanism and the functional significance of constitutive NF-κB activation in basal-like subtype breast cancer cells

Noritaka Yamaguchi, Taku Ito, Mizuki Yamamoto, Hiroyuki Takayama, Mami Yokota, Kentaro Semba[1] and Jun-ichiro Inoue

Recent studies of gene expression profiles have revealed that breast cancer is categorized into four subtypes: luminal, basal-like, ERBB2-positive, and normal breast-like. Among these subtypes, the basal-like subtype is the most malignant form of breast cancer and resistant to currently available targeted therapeutic strategies, such as hormone therapy and Herceptin. Our past study has shown that NF-κB is constitutively activated and crucial for proliferation of basal-like subtype breast cancer cells. The protein levels of NF-κB-inducing kinase (NIK) were up-regulated, and knockdown of NIK reduced constitutive NF-κB activation in these cells. These results suggested that up-regulation of NIK is one of the major causes of constitutive NF-κB activation and that the NIK-NF-κB pathway may be an attractive therapeutic target for the basal-like subtype breast cancer. This year, we have analyzed a molecular mechanism leading to up-regulation of NIK in the basal-like subtype breast cancer cells and found that *NIK* mRNA expression levels were elevated in them. We have also found that *NIK* expression may be epigenetically regulated in breast cancer cells and are currently analyzing details of this epigenetic mechanism regulating *NIK* expression. To understand the molecular function of NF-κB in the basal-like subtype cells, we have analyzed NF-κB target genes in these cells by expression of the non-degradable IκBα super-repressor, which can specifically inhibit NF-κB activation. Several promising candidates, which are thought to be involved in cancer stem cells, bone metastasis, and immunosuppression, have been identified, and their functional significance is currently analyzed in breast cancer xenocraft models.

9. NIK is involved in constitutive activation of the alternative NF-κB pathway and proliferation of pancreatic cancer cells

Takashi Nishina, Noritaka Yamaguchi, Jin Gohda, Kentaro Semba[1] and Jun-ichiro Inoue:

Pancreatic cancer has one of the poorest prognoses among human neoplasms. Constitutive activation of NF-κB is frequently observed in pancreatic cancer cells and is involved in their ma-

lignancy. However, little is known about the molecular mechanism of this constitutive NF-κB activation. This year, we showed that the alternative pathway is constitutively activated and NIK is significantly expressed in pancreatic cancer cells. SiRNA-mediated silencing of NIK expression followed by subcellular fractionation revealed that NIK is constitutively involved in the processing of p100 and nuclear transport of p52 and RelB in pancreatic cancer cells. In addition, NIK silencing significantly suppressed proliferation of pancreatic cancer cells. These results clearly indicate that NIK is involved in the constitutive activation of the alternative pathway and controls cell proliferation in pancreatic cancer cells. Therefore, NIK might be a novel target for the treatment of pancreatic cancer.

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Department of Cancer Biology

Division of Genetics
腫瘍抑制分野

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Assistant Professor	Tohru Tezuka, Ph.D.	助　教　理学博士	手　塚　　徹

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., Tezuka, T., Hamuro, J., Ikegami, T., Higuchi, O., and Yamanashi, Y.:

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 Src homology 2 (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 correct, central region of muscle upon embryogenesis, which is known as prepatterning of the receptors, 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, including prepatterning of AChRs, 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 LDL receptor-related protein 4 (LRP4), which forms a complex with MuSK and acts as an Agrin-binding module of the complex. In particular, we are interested in why LRP4, a receptor of Agrin, appears to be essential for Agrin-independent, but Dok-7- and MuSK-dependent prepatterning of AChRs during embryogenesis.

2. Pathophysiological mechanisms underlying *DOK7* myasthenia.

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As mentioned above, impaired clustering of AChRs could 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. We previously 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 cultured myotubes. The NH_2-terminal PH domain is responsible for the nuclear import of Dok-7. We also demonstrated that the SH2 target motifs in the COOH-terminal moiety of Dok-7 are active, and crucial for MuSK activation in cultured myotubes. To further investigate pathophysiological mechanisms underlying *DOK7* myasthenia, we have established mice ectopically expressing Dok-7 proteins that have mutations in the COOH-terminal moiety. Since some of these mutants lack major phosphorylation sites and are indeed hardly tyrosine phosphorylated by MuSK, this study may also address if tyrosine phosphorylation, thereby an adaptor function, of Dok-7 is required for NMJ formation in vivo. Effects of the mutations in vivo are under investigation.

3. Preparation of an Adeno-associated virus vector for Dok-7 expression.

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ence.

As mentioned above, *DOK7* myasthenia is associated with impaired neuromuscular formation due to decreased ability of Dok-7 to activate MuSK in myotubes. Interestingly, in vivo overexpression of Dok-7 increased MuSK activation and promoted NMJ formation in the correct, central region of the skeletal muscle. Because these genetically manipulated mice did not show any sign of disease, overexpression of Dok-7 in the skeletal muscle of patients with *DOK7* myasthenia might ameliorate NMJ formation and muscle weakness. As an initial step toward a potential therapy, we are generating an Adeno-associated virus-based vector that could strongly express Dok-7 in the skeletal muscle for a long period.

4. 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 into aggressive tumor cells in vivo. We are investigating roles of these Dok-family proteins using genetically manipulated animals.

5. Proteomic analyses.

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To gain insights into signaling mechanisms underlying a variety of physiological and pathophysiological events, including NMJ formation and tumorigenesis, we have performed mass spectrometry-based proteomic analyses. We are investigating roles of candidate proteins that appear to be involved in each biological event.

6. Development of a method for evaluation of serum autoantibodies in patients.

Higuchi, O., and Yamanashi, Y.

Autoimmune disorders are caused by antibodies generated in patients against self-antigens. However, pathogenic antibodies remain to be identified in many cases, hampering their diagnosis and clinical management. To overcome this roadblock, we have developed a luminescence-based novel method to efficiently detect serum autoantibodies in patients. We are investigating pathogenic involvement of patient autoantibodies that have been identified by this method.

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

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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 and 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 was identified by our and other groups as the latest member in humans and mice. Like other members, Cbl-c contains the conserved NH_2-terminal tyrosine kinase-binding (TKB) domain, linker, and RING finger motif. However, Cbl-c lacks an extensive proline-rich sequence and an ubiquitin-associated leucine zipper motif that are present in the COOH-terminal moieties of Cbl and Cbl-b. In addition, the conserved region (TKB-linker-RING finger) of Cbl-c has only 50% identity to those of Cbl and Cbl-b, but Cbl and Cbl-b have 84% identity to each other in this region. These characteristics as well as the phylogenetic stud-

ies show that Cbl-c is a distinct member among the mammalian Cbl-family proteins. In the current study, we identified a single amino acid residue that is essential for a characteristic function and structure of Cbl-c. The structure-function relationship of the other members of the Cbl-family is also under investigation.

Publications

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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 fertilinβ.

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 an 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. The previous search for materials which are reactive to the antibody suggested that the commercial pig pepsin preparation contains the epitope.Therefore, we tried to isolate epitope-containing materials in the pepsin preparation in combination of ethanol extraction of the material with reversed phase- and normal phase-HPLC, and found that several fractions are reactive to the antibody, MS/MS analysis supported that glycoconjugates containing β-linked sialic acids exists in nature.