Department of Cancer Biology

Division of Oncology
癌細胞シグナル研究分野

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

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

Tohru Tezuka, Takanobu Nakazawa, Kazumasa Yokoyama, Shin-ichiro Kina, Seiji Kawa, Jumhee Kim, June Goto, Hui Liu, Mina Delawary, Sachiko Taniguchi, Naosuke Hoshina, Miho Ohsugi, and Tadashi Yamamoto

The Src-family protein-tyrosine kinases (PTKs) are implicated in various neural functions such as synaptic plasticity, formation of neural network, and myelination. To analyze the roles of Src and Fyn, we have been focusing on the various substrates of these kinases. Our own studies have shown that NR2A and NR2B subunits of *N* -methyl-D-aspartate (NMDA) type of ionotropic glutamate receptors are the major substrates of Fyn in neuron. NMDA receptors play important roles in synaptic plasticity, synaptogenesis, and excitotoxity. To elucidate the biological significance of Fyn-mediated phosphorylation of NR2 A and NR2B at the molecular level, we have determined Fyn-mediated phosphorylation sites on NR2A and NR2B subunits. Phosphorylation at Tyr-1472 on NR2B, which is the major tyrosine phosphorylation site, is significantly enhanced after induction of LTP, a cellular basis for learning and memory, suggesting that Tyr-1472 phosphorylation is important for synaptic plasticity. To further establish the biological significance of Tyr-1472 phosphorylation, we generated mice with a knock-in mutation of the Tyr-1472 site to phenylalanine (Y1472F) and showed that Tyr-1472 phosphorylation is essential for amygdaloid functions. Electron-microscopic analyses reveal that the Y1472F mutant of the NR2B shows improper localization at synapses in the knock-in mice. The knock-in mice show impaired fear-related learning and reduced amygdaloid long-term potentiation. We further show that NMDA receptor-mediated CaMKII signaling is impaired in the mice. We thus identify Tyr-1472 phosphorylation as a key mediator of synaptic plasticity and fear-related learning in the amygdala. We have also found that Tyr-1325 on NR2A is a major tyrosine phosphorylation site. Tyr-1325 phosphorylation is important for modulating NMDA

receptor-mediated currents in recombinant NR1-NR2A channels. Roles of other tyrosine phosphorylation sites on NR2A and NR2B are going to be analyzed using the same strategy as that for Tyr-1472 of NR2B.

In parallel of these studies, to uncover the signaling pathways in which Src and Fyn are involved, we have been trying to identify binding partners, substrates and transcriptional target genes of these kinases in the brain. We have already identified a number of novel proteins that could be associated with or phosphorylated by the Src-family kinases. Characterization of these putative binding partners and substrates that include RhoGAPs, RhoGEFs, and proteins without known motifs is in progress. One of them is TCGAP, a brain-enriched GTPase-activating protein for TC10 and Cdc42. Fyn interacted with and phosphorylated TCGAP in cells that expressed these proteins exogenously. They also interacted with each other in the brain. The level of tyrosine phosphorylation of TCGAP was reduced in *fyn*-knockout mice, suggesting that TCGAP is a substrate for Fyn *in vivo.* The GAP activity of TCGAP toward Cdc42 was suppressed by Fyn-mediated phosphorylation of TCGAP. Overexpression of wild-type TCGAP in PC12 cells suppressed nerve growth factor (NGF)-induced neurite outgrowth, whereas a GAP defective mutant of TCGAP enhanced the neurite outgrowth. The level of tyrosine phosphorylation of TCGAP was enhanced by NGF stimulation in PC12 cells. These results suggest that Fyn critically regulates TCGAP function in neural cells.

Accumulating evidence shows that not only tyrosine phosphorylation but also tyrosine dephosphorylation regulates various brain functions. We have shown that protein-tyrosine phosphatase PTPMEG binds to glutamate receptors, NMDA receptor and GluRδ2, via its PDZ domain. PTPMEG is a family member of band 4.1 domain-containing protein-tyrosine phosphatases, and is expressed prominently in thalamus and cerebellum in the brain. To know the physiological roles of PTPMEG, we generated PTPMEG$^{-/-}$ mice. The mice showed impaired cerebellum-dependent learning and synaptic plasticity, suggesting that tyrosine dephosphorylation events regulated by PTPMEG are critical for functions of the cerebellum.

The Cbl-family ubiquitin ligases are also associated with and phosphorylated by the Src-family kinases. Among the family members, Cbl and Cbl-b are expressed in the brain with distinct expression profiles. We found that Cbl-family proteins suppress the signaling through various tyrosine kinases such as Src, ErbB-2, and ErbB-4 through ubiquitination of these kinases. In addition, we found that Cbl also ubiqutinates tyrosine-phosphorylated mDab1, an essential protein for brain architecture. Roles of Cbl and Cbl-b in neural functions are under investigation.

2. The biological role of Tob family proteins and Tob-interacting molecules

Toru Suzuki, Junko Tsuzuku, Takahisa Nakamura, Mitsuhiro Yoneda, Masahiro Morita, Takamitsu Nishikawa, and Tadashi Yamamoto

By screening a cDNA expression library with autophosphorylated c-erbB-2 protein, we isolated cDNA clones coding for its possible substrates. One of the genes thus identified, termed *tob,* encodes a 45kDa protein with homology to the growth suppressing proteins Btg1 and PC3. We also cloned novel genes homologous to *tob* that were termed *tob-2* and *ana.* Microinjection experiments showed that exogenously expressed Tob, Tob-2, and ANA proteins suppress growth of NIH3T3 cells. We also found that Tob is rapidly phosphorylated by Erk1 and Erk2 upon growth factor stimulation. Intriguingly, cyclin D 1 expression is elevated in serum-starved *tob*$^{-/-}$ cells. Thus, Tob inhibits cell growth by suppressing cyclin D1 expression, which is cancelled by Erk1- and Erk2-mediated Tob phoshorylation. We propose that Tob is critically involved in the control of early G1 progression.

To elucidate physiological function of Tob, we generated *tob*-deficient mice. *tob*-deficient mice had a greater bone mass resulting from increased number of osteoblasts. We also found that aged mice lacking Tob expression develop a variety of tumors. Levels of *tob* mRNA are often decreased in human cancers, implying antioncogenic role of *tob* in cancer development. Furthermore, embryonic fibroblasts deficient for *tob* displayed a marked increase in chromosomal aberration, including breakage, translocation, and aneuploidy after the treatment of DEN. To circumvent functional redundancy between Tob and Tob-2, disruption of the *tob*-2 gene and subsequent generation of double knockouts *(tob-/-tob2-/-)* were performed. Analysis of their phenotypes is underway.

Recently, we found that *tob* is involved in DNA damage response. Tob protein and mRNA is rapidly induced after DNA damage such as UV irradiation. Increase of Tob after DNA damage is independent of tumor suppressor p53, in contrast to *btg*2. Biological function of Tob in DNA damage response is to be investigated.

Among the Tob family proteins, Tob and Tob 2 proteins contain a putative nuclear localization signal (NLS) that is present near the amino-

terminus. We show that Tob is a nuclear protein that is imported into the nucleus through a nuclear localization signal (NLS)-mediated mechanism. Mutation in the NLS sequence of Tob affects its nuclear localization and impairs anti-proliferative activity. Additionally, Tob contains a nuclear export signal (NES). In oncogenic ErbB 2-transformed cells, nuclear export of Tob is facilitated by NES-mediated mechanism, resulting in decrease of its anti-proliferative activity. These results indicate that regulation of nuclear localization of Tob is important for its anti-proliferative activity.

Other studies to establish biological significance of the *tob* family members are as follows.

i) Analysis of *Cnot7*-deficient mice. We previously identified Cnot7 as a Tob-interacting molecule by yeast two-hybrid screening. *Cnot7*-deficient males are sterile owing to oligo-astheno-teratozoospermia, suggesting that Cnot 7, a CCR4-associated cofactor, is essential for spermatogenesis. Maturation of spermatids is unsynchronized and impaired in *Cnot7*$^{-/-}$ seminiferous tubules. Transplantation of spermatogonial stem cells from male *Cnot7*$^{-/-}$ mice to seminiferous tubules of c-*kit* mutant mice restores spermatogenesis, suggesting that the function of testicular somatic cells is damaged in the *Cnot7*$^{-/-}$ condition. Importantly, the testicular phenotypes of *Cnot7*$^{-/-}$ mice are similar to those of *Rxrb*$^{-/-}$ mice. We further show that Cnot7 binds the AF-1 domain of Rxrb and Rxrb malfunctions in the absence of Cnot7. These suggest that Cnot7 functions as a coregulator of Rxrb in testicular somatic cells to contribute to spermatogenesis.

To investigate the functional relationships between Cnot7 and Tob family proteins, we examined the biological significance of Cnot7 in bone formation because we had previously reported that *tob*-deficient mice have a greater bone mass due to an increased number of osteoblasts. Like *tob*-deficient mice, micro-CT analysis of the bone volume revealed enhancement of bone volume per tissue in *Cnot7*-deficient mice compared to control mice. In order to examine the osteoblastic properties of the cells, bone marrow cells were cultured in the presence or absence of beta -glycerophosphate and ascorbate. Cnot7 deficiency enhanced alizarin red positive nodule formation compared to control cells. In contrast, TRAP-positive osteoclast-like cell development in bone marrow cells was similar between Cnot 7$^{-/-}$ mice and wild-type mice, suggesting that Cnot7 deficiency mainly affects cells in the osteoblast lineage.

Intriguingly, we also observed that Cnot7, Tob and Tob2 are involved in the differentiation of mesenchymal cells into adipocytes. Protein levels of Tob or Tob2 increase rapidly after induction of adipogenesis. Retrovirally expressed Cnot7, Tob and Tob2 suppressed the adipogenic differentiation of C3H10T1/2 multipotential mesenchymal cells. These findings raise a possibility that Tob, Tob2 or Cnot7 are involved in the differentiation of mesenchymal cells.

ii) Search for other molecules that interact with Tob. We performed yeast two-hybrid screening using Tob as a bait, and identified novel serine/threonine kinase, and a molecule responsible for the stability of Tob protein. We also purified Tob-containing complexes from HeLa cells that stably express Flag-tagged Tob by immunoprecipitation with antibody against Flag. Mass spectrometric analysis showed that Tob is included in CCR4/NOT complex. The CCR4/NOT complex possesses mRNA deadenylase activity, suggesting that the complex regulates the stability of mRNAs. Currently, we are examining the biological functions of CCR4/NOT complex by generating *cnot3* and *cnot6*-deficient mice.

3. Role of chromokinesin Kid and mitotic kinases in execution of cell division

Miho Ohsugi, Noriko Tokai-Nishizumi, Keiko Haraguchi, Yasuomi Horiuchi, Chenyu Bai, Naoki Oshimori, Natsuko Masuda, and Tadashi Yamamoto

Mitosis is a process whereby a complete copy of the genetic information is distributed to each new cell during cell division. This process is critically important, with even small errors leading to aneuploidy or cell death. The 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) Chromokinesin Kid

The human chromokinesin Kid/kinesin-10 is a member of the chromosome-associated kinesin family identified in our lab in 1996. Kid is a plus end-directed microtubule-based motor with DNA-binding domains, and is required for the proper chromosome alignment at the metaphase plate. We found that depletion of Kid from HeLa cells using RNA interference (RNAi) causes multiple phenotypes, including shortening of the spindle pole-to-pole distance, misalignment of metaphase chromosomes, and defects in anaphase chromosome segregation. To rescue the

short spindle phenotype, Kid's ability to cross-link microtubules into bundles was required, but its DNA binding activity was not. Our findings indicate that independent of its role in chromosome movement, Kid contributes to spindle morphogenesis by inducing microtubule bundling to stabilize spindle microtubules. On the other hand, at anaphase, Kid shows unique localization in the interstices between adjacent chromosomes. We found that both MT- and DNA-binding domains were essential for the proper localization of Kid during anaphase. In Kid-depleted HeLa cells, anaphase chromosomes appeared to less tightly adhere to each other. Our data suggest that Kid may play a role in connecting adjacent chromosomes to hold individual anaphase chromosomes together by acting as a glue able to bind to both MTs and chromosomes. As for the regulatory mechanism of Kid, we previously reported that phosphorylation on Thr463 by Cdc2/cyclin is essential for the proper localization of Kid on chromosome arms and its function in aligning chromosome arms. In addition, we defined two functional nuclear localization signals in Kid and identified importin α as a Kid binding proteins. Our ongoing study suggests that importin α/β complex controls both localization and functions of Kid. Furthermore, we have generated *Kid*-deficient mice, analysis of which will facilitate our understanding of the physiological role and importance of Kid.

ii) Mitotic kinases

hLATS1 and *hLATS2* are mitotic serine/threonine protein kinases which share significant homology with a *Drosophila* tumor suppressor gene *lats*. FISH analysis revealed that the *hLATS1* and *hLATS2* genes are localized to chromosome 6q24-25.1 and 13q11-12, respectively. In these regions, loss of heterozygosity (LOH) is observed in various cancers including breast cancers, hepatocellular carcinomas, and renal carcinomas. To examine the role of hLATS2 in tumorigenesis and cell cycle regulation, we searched for hLATS2-binding proteins and identified several LIM domain-containing proteins including Ajuba and Zrp1/Trip-6. We showed that hLATS2 and Ajuba are co-localized at the centrosomes during mitosis. By RNAi experiments, we demonstrated that both proteins are required for the accumulation of γ-tubulin at the centrosomes and subsequent spindle organization. In addition, we found that Ajuba interacts with beta-catenin and negatively regulates beta-catenin-TCF-induced transactivation. We showed that Ajuba promotes GSK-3beta mediated phosphorylation of beta-catenin and subsequent degradation of beta-catenin. Our data suggest a novel function of Ajuba as a negative regulator of the Wnt signaling pathway. We also explore the role of Zrp1/Trip-6 in focal adhesions and cell-cell contact sites, which are disassembled during mitosis, and found that Zrp1 plays an important role in the signaling pathway that regulates activities of RhoGTPase families.

Plk1 is another highly conserved mitotic kinase that plays multiple pivotal roles in mitosis, meiosis, and also in oncogenesis. However, the exact mechanism of Plk1's action remains to be elucidated. To address this issue, the searches for novel substrates and binding partners of Plk-1 are in progress. To date, we have identified several substrates of Plk1, including a novel centrosomal protein, depletion of which causes centrosome fragmentation at prometaphase, resulting in multipolar spindles.

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Interaction of cell surface molecules and their ligand mediates a complex array of cell signaling. These cell surface events are profoundly affected by various types of post-translational modifications. Proteolysis is the one of such post-translational mechanisms regulating cellular functions in physiological and pathological situations. Most of the extracellular proteinases belong either to serine proteinase or metalloproteinase. We have studied a series of membrane-anchored type of matrix metalloproteinases called membrane-type matrix metalloproteinases (MT-MMPs). Our current issue is to understand how these MT-MMPs are regulated in physiological condition or what kinds of roles they play in the cellular events such as invasion or proliferation.

1. Membrane-type matrix metalloproteinase-1 (MT1-MMP) is a processing enzyme for human laminin gamma 2 chain

Koshikawa, N., Minegishi, T., Sharabi, A., Quaranta, V., Seiki, M.

Processing of the laminin-5 (Ln-5) gamma 2 chain by membrane-type-1 matrix metalloproteinases (MT1-MMP) promotes migration and invasion of epithelial and tumor cells. We previously demonstrated that MT1-MMP cleaves the rat gamma 2 chain at two sites, producing two major C-terminal fragments of 100 (gamma 2') and 80 (gamma 2 x) kDa and releasing a 30-kDa fragment containing epidermal growth factor (EGF)-like motifs (domain III (DIII) fragment). The DIII fragment bound the EGF receptor (EGF -R) and stimulated cell scattering and migration. However, it is not yet clear whether human Ln-5 is processed in a similar fashion to rat Ln-5 because one of the two MT1-MMP cleavage sites present in rat gamma 2 is not found in human gamma 2. To identify the exact cleavage site for MT1-MMP in human Ln-5, we purified both the whole molecule as well as a monomeric form of human gamma 2 that is frequently expressed by malignant tumor cells. Like rat Ln-5, both the monomer of gamma 2, as well as the gamma 2 derived from intact Ln-5, were cleaved by MT1-MMP in vitro, generating C-terminal gamma 2′ (100 kDa) and gamma 2 x (85 kDa) fragments and releasing DIII fragments (25 and 27k Da). In addition to the conserved first cleavage site used to generate gamma 2′, two adjacent cleavage sites (Gly(559)-Asp(560) and Gly(579)-Ser(580)) were found that could generate the gamma 2 x and DIII fragments. Two of the three EGF-like motifs present in the rat DIII fragment are present in the 27-kDa human fragment, and like the rat DIII, this fragment can promote breast carcinoma cell migration by engaging the EGF-R. These results suggest that MT1-MMP processing of Ln-5 in human tumors may stimulate the EGF-R, resulting in increased tumor cell scattering and migration that could possibly increase

their metastatic potential.

2. CD44 binding through the hemopexin-like domain is critical for its shedding by membrane-type 1 matrix metalloproteinase

Suenaga, N., Mori, H., Itoh, Y., Seiki, M.

Membrane-type 1 matrix metalloproteinase (MT1-MMP) is a potent modulator of pericellular environment through its proteolytic activity and promotes migration, invasion, and proliferation of tumor cells. During cell migration, MT1-MMP binds to CD44H, a major hyaluronan receptor, through the hemopexin-like (HPX) domain and localizes at the migration front. MT1-MMP is also responsible for shedding CD44H, which supports CD44H-mediated cell migration. In this study, we asked whether the binding of MT1-MMP to CD44H is a prerequisite step for the successive shedding. Deletion of the HPX domain deprived MT1-MMP of its shedding activity. Furthermore, disruption of the CD44H/MT1-MMP complex by overexpressing the HPX fragments resulted in inhibition of the shedding. Thus, the CD44H in the complex appears to be the direct substrate of MT1-MMP for shedding. Interestingly, other members of the MT-MMP family showed varied extents of CD44H shedding. Domain swapping between MT1-MMP and other MT-MMPs revealed that the ability of the HPX domains to bind CD44H is conserved among them. However, the shedding activity was different depending on the catalytic domains. The conserved binding ability of the HPX domains suggests that CD44H may act as a core molecule assembling multiple MT-MMPs on the cell surface.

3. Competitive disruption of the tumor-promoting function of membrane type 1 matrix metalloproteinase/matrix metalloproteinase-14 in vivo

Nonaka, T., Nishibashi, K., Itoh, Y., Yana, I., Seiki, M.

Membrane type 1 matrix metalloproteinase (MT1-MMP) is a potent modulator of the pericellular environment and promotes tumor cell invasion and proliferation in many types of tumor. The activation of proMMP-2 and processing of collagen I by MT1-MMP have been thought to be important for its tumor-promoting function. These activities can be inhibited by mutant forms of MT1-MMP lacking the catalytic domain. However, the effect of such dominant-negative mutants has never been evaluated in vivo. Various mutants lacking the catalytic domain (dCAT) were prepared and confirmed to inhibit MT1-MMP activity in human fibrosarcoma HT1080 cells, and tumor cells expressing these mutants were implanted s.c. into nude mice to monitor tumor formation. Only the membrane-anchored form of a dCAT construct through the transmembrane domain [dCAT(1)] showed potent antitumor activity not only in HT1080 cells but also in gastric carcinoma MKN 28 and MKN45 cells expressing MT1-MMP. A soluble form of dCAT lacking the transmembrane domain did not show such activity. The expression of dCAT(1) in MKN28 or MKN45 further prevented the metastatic spread of tumor cells into the peritoneal cavity; however, dCAT(1) showed no effect against TMK-1, another gastric carcinoma cell line expressing no MT1-MMP. It is of note that the tumorigenicity of TMK-1 cells enhanced by MT1-MMP overexpression was, in turn, canceled by the additional expression of dCAT(1). Thus, MT1-MMP expressed in tumor cells seems to play a pivotal role in tumor growth in mice. The results also suggest new possibilities to abrogate the tumor-promoting function of MT1-MMP other than the conventional protease inhibitor-based approach.

4. Membrane-type 1 matrix metalloproteinase cytoplasmic tail binding protein-1 (MTCBP-1) acts as an eukaryotic aci-reductone dioxygenase (ARD) in the methionine salvage pathway

Hirano, W., Gotoh, I., Uekita, T., Seiki, M.

MTCBP-1 was identified as a protein that binds the cytoplasmic tail of membrane-type 1 matrix metalloproteinase (MT1-MMP/MMP-14). Since MTCBP-1 has a putative beta-barrel structure, it is presumably a member of the recently proposed cupin superfamily that contains tremendously diverged functions of proteins in spite of their well-conserved beta-barrel structure. MTCBP-1 shows significant homology to the bacterial aci-reductone dioxygenase (ARD) in the cupin family, which is an enzyme in the methionine salvage pathway (MTA cycle). Since it is difficult to speculate the functions of cupin proteins simply based on their sequence homology, we examined whether the eukaryotic ARD homologs surely function in the methionine metabolism. Under sulfur-depleted conditions, yeast could grow when substrate of MTA cycle was provided. Disruption of the yeast ARD homolog, YMR009w gene, abolished ability of the cells to grow in this culture condition. Re-expression of either the YMR009w or MTCBP-1 gene restored the cell growth. Mutation analysis revealed that the glutamic acid residue in the

beta-barrel fold and the N-terminal extension from the beta-barrel fold were found to be important for the activity to restore the growth. Thus, MTCBP-1 isolated as a binding protein for MT1-MMP was demonstrated to function as an ARD-like enzyme in the MTA cycle in yeast.

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癌遺伝形質分野

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Cell motility is the basis of various physiological and pathological phenomena, such as inflammation, morphogenesis, and metastasis of cancer cells. In order for cells to move, cells dynamically reorganize their actin and microtubular cytoskeletons. We have been examining the regulatory mechanism of the cytoskeleton in response to extracellular stimuli. We are also investigating the Wnt and Hedgehog signals, which regulate morphogenesis and are involved in development of human cancers.

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

Takeshi Terabayashi, Hideki Yamaguchi, and Hiroaki Miki

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

2. Identification of p50 as binding partner and negative regulator of Dishevelled

Yosuke Funato, and Hiroaki Miki

As described above, Dishevelled is a critical signal transducer of the Wnt-signal. To search for novel binding partner(s) of Dishevelled under a physiological condition, we generated a cell line that stably and moderately expresses FLAG-tagged Dishevelled and performed an immunoprecipitation with anti-FLAG antibody. Among the co-precipitated proteins with FLAG-Dishevelled, the most major one that migrated at about the 50-kDa region (p50) was identified by mass spectrometry. We first confirmed that

endogenous proteins of Dishevelled and p50 form an in vivo complex by immunoprecipitation analysis. p50 was found to bind to the basic -PDZ domain in Dishevelled, where binding to Frat/GBP, an important regulator of the Wnt-signal, occurs. Indeed, the co-expression of p50 completely suppressed Dishevelled-induced beta -catenin accumulation, morphological change of cells, and phosphorylation of Dishevelled itself. These results implicate that p50 is a physiological binding partner of Dishevelled and inhibits its function. We are now investigating the functional mechanism of p50 to further characterize the importance of p50 in the Wnt-signal.

3. Cdc37 stabilizes mammalian Fused, a kinase involved in Hedgehog signaling, through HSP90

Yoshiaki Kise, Kei Takenaka, and Hiroaki Miki

Fused is a serine/threonine kinase that plays an essential role in Hedgehog signaling in Drosophila. However, it still remains unknown whether its mammalian ortholog plays a similar role and how it is regulated in mammalian cells. To elucidate its binding partner protein(s), we generated a cell line that stably expresses FLAG-tagged Fused proteins and performed immunoprecipitation with anti-FLAG antibody. Among the co-precipitated proteins, we identified Cdc37 as the most abundant one. Cdc37 is known to be a co-chaperone protein for several kinases by co-operating with HSP90, suggesting that stability of Fused may be regulated by Cdc37/HSP90. Indeed, treatment of cells with HSP90 inhibitor geldanamycin resulted in rapid degradation of both ectopically expressed and endogenous Fused. We also found that Fused is constitutively ubiquitinated and degraded at proteasome, which is stimulated by treatment with with geldanamycin. In addition, geldanamycin treatment suppressed not only the Hedgehog signaling but also proliferation of prostate cancer cells as did cyclopamine, a specific inhibitor of the Hedgehog signaling. These results implicate that stability of Fused is regulated by Cdc 37/HSP90 through inhibition of ubiquitination.

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Department of Cancer Biology

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Gene expression is largely regulated by signal transduction triggered by various stimulations. Several lines of evidence indicate that genetic defects of molecules involved in the signal transduction or the gene expression lead to abnormal cell differentiation or tumor formation. Thus, we are mainly focusing on three such proteins including Tumor necrosis factor receptor-associated factor (TRAF), E coli. Ras-like protein (ERA) and Developmentally Regulated GTP-binding protein (DRG). Our current studies indicate that these proteins play critical roles in cell proliferation and differentiation. Therefore, elucidation of how these proteins work is definitely required for understanding onset of diseases. We are also searching for novel oncogenes and analyzing signal transduction pathways more comprehensively by recent techniques of genomics and proteomics.

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

Jin Gohda, Takayuki Matsumura, Masato Irisawa, Kosuke Yamazaki, Yuko Hata[1], Kentaro Semba and Jun-ichiro Inoue: [1]Division of Advanced Clinical Proteomics, IMSUT

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. We are also trying to elucidate the mechanisms by

which Tax protein of Human T cell leukemia virus 1 activates NFκB by proteomic approaches.

2. Role of TRAF6 in lymphocyte development

Taishin Akiyama, Junwen Qin, Hidehiko Motegi and Jun-ichiro Inoue

Thymic microenvironments are essential for generation of a T cell repertoire28. Cortical thymic epithelial cells (cTECs) are involved in selection of thymocytes capable of recognizing self-MHC, whereas medullary TECs (mTECs) play a crucial role in self-tolerance by eliminating self-reactive T cells. In TRAF6-/- mice, size of the thymic medulla was reduced, and the cortico-medullary junction was ill-defined. Immunohistochemical analysis revealed an abnormal distribution and impaired maturation of mTECs in TRAF6-/- thymus. Aire protein promotes ectopic expression of peripheral tissue-specific antigens (TSAs), thereby establishing central tolerance to TSAs. Expression of aire and TSAs are reduced significantly in 14-day-old TRAF6-/- thymus. The altered thymic organization and reduced aire expression strongly suggest that TRAF6-/- mice may possess an autoimmune phenotype. This speculation was supported by observations of inflammatory infiltrates in lung, liver, pancreas, and kidney of TRAF6-/- mice. Furthermore, the number of regulatory T cells (regT) was dramatically reduced in 14-day-old TRAF6-/- mice. To determine whether the autoimmune-like phenotypes of TRAF6-/- mice are related to altered thymic stroma, fetal thymi isolated from embryonic day 14 TRAF6-/- and control mice were grafted under the renal capsules of nude mice. Eight weeks after grafting, normal generation of thymocytes and distribution of mature T cells in spleen and lymph nodes were observed in recipients grafted with TRAF6-/-thymus (KO/nu) or control thymus (WT/nu). KO/nu mice had inflammatory infiltrates in lung, liver, pancreas, and kidney similar to those in TRAF6-/- mice. Furthermore, sera from KO/nu mice contained autoantibodies against whole liver, islets in Langerhans of the pancreas, and blood vessel walls in the lung. Taken together, these data indicate that the altered thymic stroma in TRAF6-/- mice is sufficient to induce autoimmunity.

A similarly altered thymic structure and multi-organ inflammation were reported as abnormalities in RelB-deficient mice and in alymphoplasia (aly) mice, which carry a mutation in NIK. RelB and its transcripts were not detected in TRAF6-/- fetal thymic stroma, indicating that RelB expression requires TRAF6 signaling. RelB forms heterodimers with p52, a processed product of p100. Processing of p100 is triggered by phosphorylation pf p100 catalyzed by IKKα, which is activated by NIK. In aly mice, RelB induction and p100 processing are impaired, while the ratio of p52 to p100, which is indicative of p100 processing, was not affected by TRAF6-deficiency. Therefore, at least two critical NFκB-related events may be essential for the initial stage of mTEC differentiation: induction of RelB expression, which requires both TRAF6 and NIK, and optimal processing of p100 to p 52, which requires NIK.

Autoimmunity induced by TRAF6-/- thymic stroma may be due to a defect in aire gene expression or a defect in the production of regT. Reduced regT production is also observed in aly mice. These results suggests that normal development of regT requires thymic microenvironments whose formation is directed by both TRAF6 and NIK-mediated signals.

3. Role of TRAF6 in osteoclastogenesis

Jin Gohda, Takayuki, Matsumura, Yuu Taguchi and Jun-ichiro Inoue

Receptor activator of NFκB (RANK) and RANK ligand (RANKL) play crucial roles in maintaining the homeostasis of bone remodeling, which is regulated by a balance of osteoblast-mediated bone formation and osteoclast-mediated bone resorption; stimulation of RANK in osteoclast progenitor cells is an essential step for osteoclast formation. Excess formation or activity of osteoclasts in human leads to pathological bone resorption, as observed in postmenopausal osteoporosis, rheumatoid arthritis, Paget's disease and tumor bone metastases. Therefore, precise elucidation of the regulatory mechanisms of osteoclast formation, particularly the molecular mechanisms of RANK signaling, is essential for understanding the onset of skeletal diseases and for developing drugs to treat these diseases.

Intracellular signaling pathways of RANK are mediated by members of the TRAF family. TRAF2, TRAF3, TRAF5 and TRAF6 bind to the cytoplasmic tail of RANK in vitro. We showed previously that TRAF6-deficient ($TRAF6^{-/-}$) mice exhibit severe osteopetrosis and are defective in osteoclast formation due to defective signaling from RANK upon binding of RANKL. CD40, another member of the TNF receptor superfamily, transmits signals through TRAF family members, including TRAF2, TRAF3, TRAF5 and TRAF6. We have previously shown that TRAF6 plays a crucial role in CD40-mediated NF□B and MAPK activation in osteoclast progenitor cells. Despite such similarities between roles of TRAF6 in RANK signaling and those in

CD40 signaling, stimulation of CD40 in osteoclast progenitor cells does not result in osteoclast formation. Therefore, RANK, but not CD 40, is able to transmit specific signals leading to osteoclastogenesis. Elucidation of the molecular mechanisms of this RANK-specific function in osteoclastogenesis is essential for developing drugs for the treatment of pathological bone resorption. Thus, we generated and expressed chimeric receptors, h40/mRK in which the extracellular domain of human CD40 was fused to the transmembrane domain and cytoplasmic tail of mouse RANK, and we searched for specific structures in RANK critical for osteoclastogenesis. Our results strongly suggest that RANK may harbor a specific domain that amplifies TRAF6 signaling. We are currently narrowing down such domains.

4. Function of TIFA, a TRAF6-binding protein, and its relative, TIFAB.

Hiroyasu Konno, Daisuke Ohshima, Takayuki Matsumura, Sayaka Yamane, Taishin Akiyama and Jun-ichiro Inoue

Although TRAF6-mediated signal transduction is necessary for proper development, the molecular mechanism by which TRAF6 exerts its biological effects remains unknown. To address this question, we have screened for proteins that bind and regulate TRAF6. TRAF-interacting protein with a forkhead-associated (FHA) domain (TIFA) has been identified as such a protein. Our characterization of TIFA revealed that TIFA is likely to link IRAK-1 to TRAF6 upon stimulation as a signal-dependent activator of TRAF6. We also identified a TIFA-related protein, TIFAB, which inhibits TIFA-mediated activation of NFκ□. TIFAB does not associate with members of the TRAF family but does bind TIFA. Our results suggested that TIFAB inhibits the TIFA-mediated TRAF6 activation possibly by inducing a conformational change in TIFA. We have recently generated TIFA-deficient and TIFAB-deficient mice. We will identify physiological roles of TIFA and TIFAB by analyzing these mutant mice.

5. Role of TRAF6 in TLR signaling

Jin Gohda, Kosuke Yamazaki, Takayuki Matsumura and Jun-ichiro Inoue

Toll-like receptors (TLRs) signaling pathways are mediated by the TIR domain containing adopter molecules, MyD88, TIRAP, and TRIF. TRAF6 was shown to activate NFκB and MAPKs downstream of these TIR domain proteins to induce inflammatory cytokines expression. We have previously demonstrated that TRAF6 is essential for myeloid differentiation factor (MyD) 88-dependent signaling but is not required for TIR domain-containing adaptor inducing IFN-□ (TRIF)-dependent signaling. To understand the molecular mechanisms by which TRAF6 transduces MyD88-dependent signal, we are trying to purify protein complexes that contain TRAF6 and identify components of the complexes.

6. Functional characterization of Developmentally Regulated GTP-binding protein (DRG)

Kosuke Ishikawa, Taishin Akiyama and Jun-ichiro Inoue

drg gene was identified by subtractive cDNA cloning as a gene highly expressed in mouse embryonic brain. The DRG protein has G-motifs that are known to constitute the core of the GTPase domain. Genes homologous to the mouse *drg* were subsequently identified in a wide variety of species in eucaryotes and archaea, suggesting that DRG may play a fundamental cellular role. Li et al. first identified the second *drg*-related gene from both human and mouse, and demonstrated that two distinct members, DRG2 and DRG1, comprise DRG family in eucaryotes. However, no clear difference has been reported in their functions. Our aim is to clarify their physiological functions and regulatory mechanisms. We have previously identified novel proteins, DRG family regulatory protein (DFRP) 1 and DFRP2, which regulate expression of DRG proteins through specific binding. In transient transfection experiments, DFRP 1 specifically binds DRG1, and DFRP2 preferentially binds DRG2. DFRPs provide stability to the target DRG proteins through physical association, possibly by blocking the poly ubiquitination that would precede proteolysis of DRG proteins. DFRPs are highly conserved in eucaryotes, and the expression patterns of *dfrp1* and *drg1* transcripts in *Xenopus* embryos and tissues are similar, indicating that these genes work cooperatively in various types of eucaryotic cells. This year, we are able to demonstrate that DRG 1- or DRG2-deficiency results in reduction of proliferation rate of Hela cells. We are currently trying to understand how DRGs regulate cell proliferation.

7. Identification of NFκB-target genes in tumor cells

Noritaka Yamaguchi[2], Takashi Nishina, Sakura

Azuma, Shinya Watanabe[3], Kentaro Semba and Jun-ichiro Inoue: [2]Institute for Biomedical Engineering, Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, [3]Department of Clinical Informatics, Tokyo Medical Dental University School of Medicine

NFκB plays an important role in tumor development. In contrast to normal cells in which NFκB activity is transiently induced by several extracellular stimuli, many types of tumors and tumor cell lines show constitutive activation of NFκB. Overexpression of an IκBα mutant (IκB super repressor) which lacks phosphorylation sites by IKK revealed that NFκB activity contributed to the survival or growth of tumor cells, tumor invasion and metastasis and angiogenesis. Tumor cells that show constitutive NFκB activity are also highly resistant to anticancer drugs or ionizing radiation and inhibition of NFκB activity increases their sensitivity to such treatments. Therefore, it is important to elucidate gene expression network regulated by NFκB for anticancer therapy.

For this purpose, we first made gene expression profiles of one hundred and twenty-five human tumor cell lines from various tissue origins including colon, lung, pancreatic, esophagus, stomach, breast and ovary. Clustering analysis of the cell lines by NFκB status showed that there exist genes whose expression is correlated with NFκB status. As predicted, some genes are known NFκB target genes and others are known to activate NFκB. Interestingly, ERBB 2 status and NFκB status looked inversely correlated in breast cancer cell lines, implying that ERBB2-amplification and NFκB activation may contribute to tumor progression of each group of breast cancer cells, respectively.

Microarray-based expression analysis of NFκB target genes will enable us to develop more specific therapeutic drugs against tumors. For this purpose, we are establishing breast tumor cell lines which express IκB super repressor by tetracycline-inducible system and also establishing cell type-independent protein transduction system using protein transduction domain (PTD)-fused IκB super repressor.

8. Analysis of EGFR-family signal network by proteomic approach

Takashi Miyasaka[1], Yuko Hata[1], Masaaki Oyama[4], Jun-ichiro Inoue, Kentaro Semba and Tadashi Yamamoto[1]: [4]Laboratory of Functional Genomics, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo

Lung cancer is the leading cause of cancer mortality all over the world including Japan. Recent finding of EGFR mutation in non-small cell lung cancer (NSCLC) has dramatic impact on lung cancer therapy with EGFR inhibitor, gefitinib: Retrospective studies of NSCLC patients with gefitinib treatment showed close association between EGFR mutations and clinical response. Mutant EGFR-mediated signal transduction pathways in NSCLC are, however, just beginning to be understood. Recent proteomics techniques have enabled us to analyze signal transduction pathways more comprehensively and more quantitatively. We are currently analyzing phosphotyrosine-containing protein complexes in mutated EGFR-dependent NSCLC cell lines by LC-MS/MS. We are also establishing a proteomics technique called "SILAC" (stable isotope labeling with amino acids in cell culture) to analyze dynamics of phosphotyrosine-mediated signal transduction pathways.

9. Clustering analysis on the basis of gene expression profiling of ERBB2-amplified breast cancer cells

Kentaro Semba, Shinya Watanabe[3], Sakura Azuma and Jun-ichiro Inoue:

ErbB2/Her2/Neu is a receptor-type protein tyrosine kinase, whose amplification and increased expression is closely correlated with prognosis of breast cancer. Aberrant signal transduction mediated by overexpressed ErbB2 is thought to be involved in growth and metastatic potential of breast cancer cells. Although a humanized monoclonal anti-ErbB2 antibody (trastuzumab) has shown benefits in metastatic patients, the molecular mechanism of breast cancer development, especially what genes contribute to breast tumorigenesis cooperatively with ErbB2 has not been studied extensively. To elucidate the characteristics of breast cancer cells with overexpression of ErbB2, we analyzed expression profiles of 32,000 genes in thirty-five human breast cancer cell lines. Clustering analysis based on ErbB2 mRNA level showed that a set of sixty-four genes classified breast cancer cell lines into two major groups and that tumor cells with overexpression of ErbB2 might have arisen from one of the two groups. Interestingly, at least two of the sixty-four genes were amplified in a part of breast cancer cell lines, suggesting that they may contribute to tumor progression.

10. Identification of novel oncogenes at the amplified gene loci

Kentaro Semba, Shinya Watanabe[3], Sakura Azuma and Jun-ichiro Inoue:

Aberrant gene expression due to genomic imbalances are thought to underlie tumor progression. Recent genome-wide analysis of copy number and expression have identified causative genes in various types of tumors. Alignment of the expression profiles of one hundred and twenty-five human tumor cell lines according to the chromosomal localization has enabled us to identify candidates of amplified loci (amplicons). Subsequently we confirmed at least seven novel amplicons in breast cancer cell lines by Southern blot analysis, some of which were found to be amplified also in breast tumor tissues. Genes located in those amplicons may contribute to a more aggressive phenotype of breast cancers and may be ideal targets for anticancer therapies. We are searching for novel amplicons in other tumor cell lines.

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

1. Rac-WAVE2 signaling is involved in the invasive and metastatic phenotypes of murine melanoma cells.

Shusaku Kurisu, Shiro Suetsugu, Daisuke Yamazaki, Hideki Yamaguchi, Tadaomi Takenawa.

WAVEs (WASP-family verprolin-homologous proteins) regulate the actin cytoskeleton through activation of Arp2/3 complex. As cell motility is regulated by actin cytoskeleton rearrangement and is required for tumor invasion and metastasis, blocking actin polymerization may be an effective strategy to prevent tumor dissemination. We show that WAVEs, especially WAVE2, are essential for invasion and metastasis of melanoma cells. Malignant B16F10 mouse melanoma cells expressed more WAVE1 and WAVE2 proteins and showed higher Rac activity than B16 parental cells, which are neither invasive nor metastatic. The effect of WAVE2 silencing by RNA interference (RNAi) on the highly invasive nature of B16F10 cells was more dramatic than that of WAVE1 RNAi. Membrane ruffling, cell motility, invasion into the extracellular matrix, and pulmonary metastasis of B16F10 cells were suppressed by WAVE2 RNAi. WAVE2 RNAi also had a profound effect on invasion induced by a constitutively active form of Rac (RacCA). In addition, ectopic expression of both RacCA and WAVE2 in B16 cells resulted in further increase in the invasiveness than that observed in B16 cells expressing only RacCA. Thus, WAVE2 acts as the primary effector downstream of Rac to achieve invasion and metastasis, suggesting that suppression of WAVE2 activity holds a promise for preventing cancer invasion and metastasis.

2. Molecular mechanisms of invadopodium formation: the role of the N-WASP-Arp2/3 complex pathway and cofilin.

Hideki Yamaguchi[3], Mike Lorenz[3], Stephan Kempiak[3], Corina Sarmiento[3], Salvatore Coniglio[3], Marc Symons[3], Jeffrey Segall[3], Robert Eddy[3], Hiroaki Miki[2], Tadaomi Takenawa[1], and John Condeelis[3]: [1]Department of Biochemistry and [2]Division of Cancer Genomics Institute of Medical Science, University of Tokyo, Shirokanedai, Mianato-ku, Tokyo 108-

8639, Department of Anatomy and Structural Biology, [3]Albert Einstein College of Medicine, Bronx, NY 10461

Invadopodia are actin-rich membrane protrusions with a matrix degradation activity formed by invasive cancer cells. We have studied the molecular mechanisms of invadopodium formation in metastatic carcinoma cells. Epidermal growth factor (EGF) receptor kinase inhibitors blocked invadopodium formation in the presence of serum, and EGF stimulation of serum-starved cells induced invadopodium formation. RNA interference and dominant-negative mutant expression analyses revealed that neural WASP (N-WASP), Arp2/3 complex, and their upstream regulators, Nck1, Cdc42, and WIP, are necessary for invadopodium formation. Time-lapse analysis revealed that invadopodia are formed de novo at the cell periphery and their lifetime varies from minutes to several hours. Invadopodia with short lifetimes are motile, whereas long-lived invadopodia tend to be stationary. Interestingly, suppression of cofilin expression by RNA interference inhibited the formation of long-lived invadopodia, resulting in formation of only short-lived invadopodia with less matrix degradation activity. These results indicate that EGF receptor signaling regulates invadopodium formation through the N-WASP-Arp2/3 pathway and cofilin is necessary for the stabilization and maturation of invadopodia.

3. WICH, a member of WASP-interacting protein family, cross-links actin filaments.

Masayoshi Kato, and Tadaomi Takenawa

In yeast, Verprolin plays an important role in rearrangement of the actin cytoskeleton. There are three mammalian homologues of Verprolin, WIP, CR16, and WICH, and all of them bind actin and Wiskott-Aldrich syndrome protein (WASP) and/or neural-WASP. Here, we describe a novel function of WICH. In vitro cosedimentation analysis revealed that WICH not only binds to actin filaments but also cross-links them. Fluorescence and electron microscopy detected that this cross-linking results in straight bundled actin filaments. Overexpression of WICH alone in cultured fibroblast caused the formation of thick actin fibers. This ability of WICH depended on its own actin cross-linking activity. Importantly, the actin cross-linking activity of WICH was modified through a direct association with N-WASP. Taken together, these data suggest that WICH induces a bundled form of actin filament with actin cross-linking activity and the association with N-WASP suppresses that activity. WICH thus appears to be a novel actin bundling protein.

4. WASP-related proteins, Abi1 and Ena/VASP are required for Listeria invasion induced by the Met receptor.

Hélene Bierne1[3], Hiroaki Miki[2], Metello Innocenti3[3], Giorgio Scita, Frank B. Gertler[3], Tadaomi Takenawa5[1] and Pascale Cossart1[3],*: [1]Department of Biochemistry and [2]Division of Cancer Genomics Institute of Medical Science, University of Tokyo, Shirokanedai, Mianato-ku, Tokyo 108-8639, [3]Unité des Interactions Bactéries-Cellules, Institut Pasteur, INSERM U 604, INRA USC2020, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France, The European Institute of Oncology (IEO) and the FIRC Institute for Molecular Oncology (IFOM), Via Adamello 16, 20139 Milan, Italy

Internalisation of the pathogenic bacterium Listeria monocytogenes involves interactions between the invasion protein InlB and the hepatocyte growth factor receptor, Met. Using colocalisation studies, dominant-negative constructs and small interfering RNA (siRNA), we demonstrate a cell-type-dependent requirement for various WASP-related proteins in Listeria entry and InlB -induced membrane ruffling. The WAVE2 isoform is essential for InlB-induced cytoskeletal rearrangements in Vero cells. In HeLa cells, WAVE1, WAVE2 and N-WASP cooperate to promote these processes. Abi1, a key component of WAVE complexes, is recruited at the entry site in both cell types and its inactivation by RNA interference impairs InlB-mediated processes. Ena/VASP proteins also play a role in Listeria internalization, and their deregulation by sequestration or overexpression, modifies actin cups beneath entering particles. Taken together, these results identify the WAVE complex, N-WASP and Ena/VASP as key effectors of the Met signalling pathway and of Listeria entry and highlight the existence of redundant and/or cooperative functions among WASP-family members.

5. Interaction of HSP90 to N-WASP leads to activation and protection from proteasome -dependent degradation.

Sun Joo Park, Shiro Suetsugu and Tadaomi Takenawa

Neural Wiskott-Aldrich syndrome protein (N-WASP) regulates reorganization of the actin cytoskeleton through activation of the Arp2/3 complex. Here, we show that heat shock protein

90 (HSP90) regulates N-WASP-induced actin polymerization in cooperation with phosphorylation of N-WASP. HSP90 binds directly to N-WASP, but binding alone does not affect the rate of N-WASP/Arp2/3 complex-induced in vitro actin polymerization. An Src family tyrosine kinase, v-Src, phosphorylates and activates N-WASP. HSP90 increases the phosphorylation of N-WASP by v-Src, leading to enhanced N-WASP-dependent actin polymerization. In addition, HSP90 protects phosphorylated and activated N-WASP from proteasome-dependent degradation, resulting in amplification of N-WASP-dependent actin polymerization. Association between HSP90 and N-WASP is increased in proportion to activation of N-WASP by phosphorylation. HSP90 is colocalized and associated with active N-WASP at podosomes in 3Y1/v-Src cells and at growing neurites in PC12 cells, whose actin structures are clearly inhibited by blocking the binding of HSP90 to N-WASP. These findings suggest that HSP90 induces efficient activation of N-WASP downstream of phosphorylation signal by Src family kinases and is critical for N-WASP-dependent podosome formation and neurite extension.

6. A novel function of WAVE in lamellipodia: WAVE1 is required for stabilization of lamellipodial protrusions during cell spreading

Daisuke Yamazaki, Takashi Fujiwara, Shiro Suetsugu and Tadaomi Takenawa,

When a cell spreads and moves, reorganization of the actin cytoskeleton pushes the cell membrane, and the resulting membrane protrusions create new points of contact with the substrate and generate the locomotive force. Membrane extension and adhesion to a substrate must be tightly coordinated for effective cell movement, but little is known about the mechanisms underlying these processes. WAVEs are critical regulators of Rac-induced actin reorganization. WAVE2 is essential for formation of lamellipodial structures at the cell periphery stimulated by growth factors, but it is thought that WAVE1 is dispensable for such processes in mouse embryonic fibroblasts (MEFs). Here we show a novel function of WAVE in lamellipodial protrusions during cell spreading. During spreading on fibronectin (FN), MEFs with knockouts (KOs) of WAVE1 and WAVE2 showed different membrane dynamics, suggesting that these molecules have distinct roles in lamellipodium formation. Formation of lamellipodial structures on FN was inhibited in WAVE2 KO MEFs. In contrast, WAVE1 is not essential for extension of lamellipodial protrusions but is required for stabilization of such structures. WAVE1-deficiency decreased the density of actin filaments and increased the speed of membrane extension, causing deformation of focal complex at the tip of spreading edges. Thus, at the tip of the lamellipodial protrusion, WAVE2 generates the membrane protrusive structures containing actin filaments, and modification by WAVE1 stabilizes these structures through cell-substrate adhesion. Coordination of WAVE1 and WAVE2 activities appears to be necessary for formation of proper actin structures in stable lamellipodia.

7. Regulation of cancer cell motility through actin reorganization

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Cell migration is a critical step in tumor invasion and metastasis, and regulation of this process will lead to appropriate therapies for treating cancer. Cancer cells migrate in various ways, according to cell type and degree of differentiation. The different types of cell migration are regulated by different mechanisms. Reorganization of the actin cytoskeleton is the primary mechanism of cell motility and is essential for most types of cell migration. Actin reorganization is regulated by Rho family small GTPases such as Rho, Rac, and Cdc42. These small GTPases transmit extracellular chemotactic signals to downstream effectors. Of these downstream effectors, Wiskott-Aldrich syndrome protein (WASP) family proteins are key regulators of cell migration. Activated WASP family proteins induce the formation of protrusive membrane structures involved in cell migration and degradation of the extracellular matrix. Inhibition of Rho family small GTPase signaling suppresses the migration and invasion of cancer cells. Thus, control of cell migration via the actin cytoskeleton provides the possibility of regulating cancer cell invasion and metastasis.

8. A novel membrane tubulation and PI(4,5)P 2-binding domain involved in endocytosis

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A variety of endocytotic and actin regulatory proteins, such as, CIP4, FBP17, syndapin/pacsins and cdc15 have FCH domain which is highly conserved from yeast to mammal. However, alignment of these proteins further showed that there is a wider conserved area than FCH

domain including FCH domain and predicted coiled coil region. We named this region as extended FC (EFC) domain. Here we found that EFC domain of FBP17, CIP4, FER, PSTPIP1 and PSTPIP2 strongly binds to phosphatidiylinositol 4,5-bisphosphate [PI(4,5)P2] among phosphoinositides. These EFC domain alone tubulated membranes in vivo and deformed PI(4,5)P2-containing liposomes into narrow tubules in vitro. Mutagenesis analysis showed that evolutionally conserved basic amino acid residues were critical for lipid binding and tubulation of membranes in vivo and in vitro. In addition, overexpression of FBP17 inhibited endocytosis of EGF receptor, though its effect was induced through EFC domain. Knock down of FBP17 also inhibited EGF uptake. FBP17 could recruite N-WASP to plasma membranes where they activate actin polymerization. Furthermore, when invaginating tubules were induced by FBP17 expression, EGF receptor, dynamin and N-WASP were co-localized at membrane tubules, suggesting that these proteins form the functional complex in membrane invagination steps.

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Department of Cancer Biology

Division of Biochemistry (2)
腫瘍分子医学分野(2)

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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 normal and pathological processes

Etsuko Mori, Dong Cun Jin and Seiichi Takasaki

Cell-cell and cell-matrix adhesion, as well as proteolysis of the extracellular matrix, are vital for normal processes such as tissue morphogenesis and wound healing, as well as for pathologies such as tumor cell invasion and metastasis. A variety of cell surface adhesion proteins and proteases are important players in these events. We are now focusing on the adhesion mechanism between sperm and egg. Several cell adhesion mechanisms are working in fertilization. First, sperm adhere and bind to the extra cellular matrix of egg called the zona pellucida (ZP). The bound sperm are induced to undergo acrosome reaction, and then penetrate the ZP. Finally, the acrosome-reacted sperm adhere and fuse to egg plasma membrane. To solve the mechanism underlying these processes, identification of functional molecules on the surface of both gametes is essential. Therefore, we have so far analyzed oligosaccharides of boar ZP glycoproteins and found out the structural feature of oligosaccharides which are important for sperm binding. Based on the structural information about the ligands on egg, we are investigating sperm molecules recognizing the oligosaccharides of ZP glycoproteins. Two kinds of affinity technology were applied to the study. First, we prepared a dextran-based probe containing multiple oligosaccharides with N-acetyllactosamine structure which is a potential ligand for boar sperm. The oligosaccharide probe was used to coat the surface of a 96-well plastic dish, and detergent-solubilized plasma membrane of boar sperm was added to the dish. After extensive washing, the bound proteins were biotinylated and analyzed by SDS-PAGE, resulting in the detection of two proteins with apparent molecular masses of 70 kDa and 40 kDa. Affinity column chromatography was also used for the study. We prepared Sepharose beads coupled with desialylated α1-acid glycoprotein which contains N-acetyllactosamine-type oligosaccharides. The detergent-solubilized sperm plasma membrane was then applied to the bead column, and proteins eluted from the column were analyzed by SDS-PAGE. Then, it was shown that The two proteins with apparent molecular masses of 70 kDa and 40 kDa are recovered in the retarded fractions. The results suggest that these two proteins are candidates for sperm carbohydrate binding proteins recognizing the oligosaccahrides on the egg ZP-glycoproteins. In-

terestingly, analysis of tryptic peptides from the 70 and 40 kDa proteins by the MS/Ms method and homology search of determined partial amino acid sequences indicated that both of the proteins show sequence similarity to members of ADAM, a gene family encoding membrane proteins with a disintegrin and metalloprotease domain. This protein family are unique in possessing both a potential cell adhesion activity as well as a potential protease activity. We cloned cDNA encoding a large parts of 70kDa protein, one of the potential carbohydrate binding proteins of boar sperm, and produced its recombinant protein. The protein was shown to have adhesion activity to cell surface integrin molecules. We are currently analyzing its carbohydrate binding activity. Analysis of the 40 kDa protein is also in progress.

2. Analysis of cancer-related sialylation of ferritin

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It has been demonstrated that the total ferritin content increases in the serum of patients with various malignancies. However, it is known that inflammation also causes the increase of serum ferritin. Another cancer-associated change is a shift to acidic ferritins enriched with H subunits. The ratio of H to L subunits in ferritin varies depending on the tissue type and physiological status of the cells. For example, H-subunit is predominant in heart and kidney and L-subunit is in liver. Serum ferritins are composed of two forms; one actively secreted from cells like cancer cells and the other passively released from damaged cells in various tissues. We can not discriminate both forms in serum. It is necessary to approach the problem from a different aspect. In this study, we compared ferritins from hepatocarcinoma and normal liver and found that hepatocarcinoma tissue ferritin contains quite larger amounts of sialic acids as compared with normal liver tissue ferritin. The present quantitative analysis supports the previous observation that incubation of human liver ferritin with neuraminidase did not change the isoelectric focusing pattern. Interestingly, ferritins from spleen and heart have been shown to be also resistant to neuraminidase. Thus, it is worthy of notice that the status of sialylation is clearly different between normal and cancer ferritins. In addition, we could show the clear difference in sialic acid species between normal and hepatoma ferritins. The Western blot analysis and chemical analysis based on HPLC and mass spectrometry indicated that hepatocarcinoma tissue ferritin expresses considerable amounts of NeuGc as well as NeuAc, while normal liver ferritin exclusively expresses NeuAc. This difference is of clinically great importance. It will be interesting to analyze sialylation of ferritins from various tissues in physiological and pathological conditions. A gene encoding CMP-NeuAc hydroxygenase that converts CMP-NeuAc to CMP-NeuGc resulting in incorporation of NeuGc to glycans has an exon deletion/frame shift mutation in humans. Therefore, it has been believed that human cells can not synthesize and express NeuGc. However, we could present the existence of NeuGc in human hepatocarcinoma tissue and an erythroleukemic cell line K562 in this study as well as in other studies. The present consensus is that the expression of NeuGc in human cancer cells is due to uptake of non-human NeuGc from dietary sources and its incorporation into cells. Therefore, we examined if the expression of NeuGc by K562 cells is affected by the culture media. Two serum-free media, ASF104 and ASF104N, were first tested. When the media were subjected to the HPLC analysis of sialic acids, no clear peaks corresponding to NeuGc-DMB and NeuAc-DMB were detected. Furthermore, the spectra of MALDI-TOF/MS analysis did not show any ions corresponding to NeuGc-DMB and NeuAc-DMB. Then, K562 cells were grown in the serum-free media, and cells were harvested after three passages. Both HPLC analysis and MALDI-TOF/MS analysis of sialic acids in ferritins indicated that NeuGc is still expressed in ferritin of K562 cells cultured in the serum-free media. The result is not explained by uptake and utilization of exogenous NeuGc. There might be an alternative mechanism by which human cancer cells synthesize and express NeuGc. Further study will solve the issue.

Department of Cancer Biology

Division of Genetics
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Our major research interest is to elucidate the molecular mechanisms involved in signal transduction for malignant transformation and for tumor angiogenesis. We have been mainly focusing on the functions of tyrosine kinases [VEGF receptors-1(Flt-1), VEGFR-2 (Flt-1/KDR) and FGF receptor], an adaptor/docking protein FRS2, and VEGF-independent regulation of angiogenesis. This year we found that VEGF-A is important not only for endothelial cell proliferation/differentiation but also for a guidance of VEGFR-positive progenitor cells in early embryogenesis. Furthermore, we showed that VEGFR-1 is essential for the bone marrow formation in M-CSF-deficient mice. We also found that FRS2α plays critical roles for corticogenesis and for FGF2-dependent proliferation of neural progenitor cells. Lastly, we showed a unique role of FRS2β/SNT2 for negative regulation in EGF receptor tyrosine kinase signaling pathways.

1. VEGF-A is involved in guidance of VEGFR-positive cells to anterior portion in early embryogenesis

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The hemangioblast in the mesoderm gives rise to both angioblasts and hematopoietic stem cells. The movement of hemangioblast precursor cells in the fetal trunk is a critical event in early embryogenesis. VEGF signaling appears to be involved in this migration given the partial disturbance of VEGF receptor(R)-positive cell accumulation/migration in VEGFR2-null or VEGFR1 -truncated mice. However, it is not clear how the VEGF system regulates this migration or its direction. This year we showed that the expression of VEGF-A is dominant in the anterior portion, whereas VEGFR1 and VEGFR2 are expressed in the posterior portion of the embryo. An inhibitor for VEGFR kinase blocked the migration of VEGFR-positive cells in a whole-embryo culture system. In addition, VEGFR-positive cells migrated toward a VEGFR1 or VEGFR2-specific ligand in vitro. Furthermore, VEGFR-positi-

ve cells derived from wild-type or VEGFR2+/− mice rapidly moved anteriorly, whereas the cells derived from VEGFR2+/− mice carrying a truncated VEGFR1 [VEGFR1(TM-TK)−/−] migrated little when injected into wild-type mice. Based on these results, we suggest that VEGF-A protein concentrated in the anterior region plays an important role in the guidance of VEGFR-positive cells from the posterior portion to the head region by interacting with VEGFR in the mouse embryo.

2. Vascular endothelial growth factor receptor-1 signaling is essential for osteoclast development and bone-marrow formation in CSF-1-deficient mice.

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VEGF receptor-1 (VEGFR-1/Flt-1) is a high affinity tyrosine kinase receptor for VEGF, and regulates angiogenesis as well as monocyte/macrophage functions such as cell migration. We previously showed that the osteoclast-deficiency in osteopetrotic *Csf1*^{op}/*Csf1*^{op} (*op/op*) mice is gradually restored in an endogenous VEGF-dependent manner. However, the molecular basis of the recovery is still not fully understood. To examine which VEGFR is important and to clarify how CSF-1 and VEGF signals interact in osteoclastogenesis, we introduced a VEGFR-1-signaling deficiency (*Flt1*$^{TK-/-}$) into *op/op* mice. The original *Flt1*$^{TK-/-}$ mice showed mild osteoclast reduction without bone marrow suppression. The double mutant (*op/opFlt1*$^{TK-/-}$) mice, however, exhibited very severe osteoclast-deficiency, and did not have numbers of osteoclasts sufficient to form the bone marrow cavity. The narrow bone marrow cavity in the *op/opFlt1*$^{TK-/-}$ mice was gradually replaced with fibrous tissue, resulting in severe marrow hypoplasia and extramedullary hematopoiesis. These results strongly suggest that the interaction of signals via VEGFR-1 and via CSF-1R plays a predominant role not only in osteoclastogenesis but also in the maintenance of bone marrow functions.

3. Various endothelial cells are differently regulated by Inhibitory Smad system.

Mari Kiyono and Masabumi Shibuya

We have recently shown that vascular endothelial cells (EC) in the rat pupillary membrane, a temporary capillary network in the anterior chamber of the lens, are postnatally regressed mostly via apoptosis dependent on BMP4. This critical cytokine is transiently secreted from matured lens epithelial cells. However, it is still open question whether the suppressive effect of BMP4 is universal to all the type of ECs in the body. This year, we examined this point and found that BMP4 efficiently induces apoptosis only in limited types of EC such as human umbilical vein EC (HUVEC). Other types of EC including coronary arterial EC are highly resistant to BMP4. Furthermore, the resistance to BMP4 well correlates with the gene expression of several members of Smad family, an intracellular signaling molecules of TGFβ pathway. These results strongly suggest that BMP4/Smads are a novel regulator for the stability of vascular EC.

4. Signaling through FRS2 docking proteins

A. Essential role of Shp2-binding sites on the docking protein FRS2α for mammalian corticogenesis and for FGF2-dependent proliferation of cultured neural progenitor cells.

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Mammalian corticogenesis occurs through a complex process that includes neurogenesis in which neural progenitor cells proliferate, differentiate, and migrate. It has been recently reported that neurogenesis occurs in the subventricular zone (SVZ), a region previously thought to be the primary site of gliogenesis. It has been recognized that in the SVZ intermediate progenitor cells, derived from radial glial cells that are multipotent neural stem cells, produce only

neurons. However, the molecular mechanisms underlying the regulation of neural stem cells and intermediate progenitor cells as well as their contribution to overall corticogenesis remain unknown. The docking protein FRS2α is a major mediator of signaling via fibroblast growth factors (FGFs) and neurotrophins. FRS2α mediates many on its pleiotropic cellular responses by recruiting the adaptor protein Grb2 and the protein tyrosine phosphatase Shp2 upon ligand stimulation. Here, we report that targeted disruption of Shp2 binding sites in FRS2α leads to severe impairment in cerebral cortices development in mutant mice. The defect in corticogenesis appears to be due at least in part to abnormalities in intermediate progenitor cells. Genetic evidence is provided that FRS2α plays critical roles in the maintenance of intermediate progenitor cells and in neurogenesis in cerebral cortex. Moreover, FGF2-responsive neurospheres, which are cell aggregates derived from neural stem/progenitor cells (NSPCs), from FRS2α mutant mice were smaller than those of wild-type mice. However mutant NSPCs were able to self-renew, demonstrating that Shp2-binding sites on FRS2α play an important role in NSPC proliferation but are dispensable for NSPC self-renewing capacity in response to FGF2 stimulation.

B. Unique role of SNT-2/FRS2β/FRS3 docking/adaptor protein for negative regulation in EGF receptor tyrosine kinase signaling pathways.

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The membrane-linked docking protein SNT-2/FRS2β/FRS3 becomes tyrosine phosphorylated in response to fibroblast growth factors (FGFs) and neurotrophins and serves as a platform for recruitment of multiple signaling proteins, including Grb2 and Shp2, to FGF receptors or neurotrophin receptors. We previously reported that SNT-2 is not tyrosine phosphorylated significantly in response to epidermal growth factor (EGF) but that it inhibits ERK activation via EGF stimulation by forming a complex with ERK2. In the present report, we show that expression of SNT-2 suppressed EGF-induced cell transformation and proliferation. The activities of the major signaling molecules in EGF receptor (EGFR) signal transduction pathways, including autophosphorylation of EGFR, were attenuated in cells expressing SNT-2 but not in cells expressing SNT-2 mutants lacking the ERK2-binding domain. Furthermore, SNT-2 constitutively bound to EGFR through the phosphotyrosine binding (PTB) domain both with and without EGF stimulation. Treatment of cells with MEK inhibitor U0126 partially restored the phosphorylation levels of MEK and EGFR in cells expressing SNT-2. On the basis of these findings, we propose a novel mechanism of negative control of EGFR tyrosine kinase activity with SNT2 by recruiting ERK2, which is the site of negative-feedback loop from ERK, ultimately leading to inhibition of EGF-induced cell transformation and proliferation.

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