Department of Cancer Biology

Division of Oncology
癌細胞シグナル研究分野

Professor	Tadashi Yamamoto, Ph.D.	教　授	理学博士	山　本　　雅
Research Associate	Jiro Fujimoto, Ph.D.*	助　手	医学博士	藤　元　次　郎*
Research Associate	Tohru Tezuka, Ph.D.	助　手	理学博士	手　塚　　徹
Research Associate	Miho Oosugi, Ph.D.	助　手	理学博士	大　杉　美　穂
Research Associate	Toru Suzuki, Ph.D.	助　手	理学博士	鈴　木　　亨
Faculty Member	Takanobu Nakazawa, Ph.D.	特任教員	医学博士	中　沢　敬　信

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

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

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

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

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

In parallel of these studies, to uncover the signaling pathways in which the Src-family and BREK are involved, we have been trying to identify binding partners, substrates and transcriptional target genes of these kinases in the brain. We have already identified a number of novel proteins that could be associated with or phosphorylated by the Src-family kinases. Characterization of these putative binding partners and substrates that include RhoGAPs, RhoGEFs, and proteins without known motifs is in progress. One of them is p250GAP, a novel brain-enriched GTPase-activating protein for Cdc42 and RhoA that interacts with both Fyn and the NR2B subunit of NMDAR. Within neurons, p 250GAP was highly concentrated in the postsynaptic density and colocalized with NR2B and PSD-95. When overexpressed in neuroblastoma cells, p250GAP suppresses the activities of Rho-family proteins, which results in alteration of neurite outgrowth. NMDAR stimulation leads to dephosphorylation and redistribution of p250 GAP in hippocampal slices. Taken together, p 250GAP is likely to be involved in NMDA receptor activity-dependent actin reorganization in dendritic spines. The Cbl-family ubiquitin ligases are also associated with and phosphorylated by the Src-family kinases. Among the family members, Cbl and Cbl-b are expressed in the brain with distinct expression profiles. We found that Cbl-family proteins suppress the signaling through various tyrosine kinases such as Src, ErbB-2, and ErbB-4 through ubiquitination of these kinases. In addition, we found that Cbl also ubiqutinates tyrosine-phosphorylated mDab 1, 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, Takashi Miyasaka, 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. Oncogenic Ras-induced transformation and growth factor-induced cell proliferation are efficiently suppressed by mutant Tob that carries alanines but not glutamates, mimicry of phospho-serines at the phosphorylation sites. Intriguingly, cyclin D 1 expression is elevated in serum-starved *tob*$^{-/-}$ cells. Thus, Tob inhibits cell growth by suppressing cyclin D1 expression, which is cancelled by Erk1- and Erk2-mediated Tob phoshorylation. We propose that Tob is critically in-

volved in the control of early G1 progression.

To elucidate physiological function of Tob, we generated *tob*-deficient mice. *tob*-deficient mice had a greater bone mass resulting from increased number of osteoblasts. We also found that aged mice lacking Tob expression develop a variety of tumors, such as hemangiosarcomas and hepatocarcinomas, at high frequency. Intraperitoneal injection, at two weeks after birth, of the carcinogen diethylnitrosamine (DEN) led to more frequent generation of liver tumors in *tob*-deficient mice than in wild-type mice. $tob^{-/-}$ $p53^{-/-}$ mice show accelerated tumor formation in comparison with single null mice. Levels of *tob* mRNA are often decreased in human cancers, implying *tob* in cancer development. Furthermore, embryonic fibroblasts deficient for *tob* displayed a marked increase in chromosomal aberration, including breakage, translocation, and aneuploidy after the treatment of DEN. To circumvent functional redundancy between Tob and Tob-2, disruption of the *tob*-2 gene and subsequent generation of double knockouts ($tob^{-/-}$ $tob2^{-/-}$) were performed. Analysis of their phenotypes is underway.

Among the Tob family proteins, Tob and Tob 2 proteins contain a putative nuclear localization signal (NLS) that is present near the amino-terminus. We 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 widely spread. (1) Generation of *caf1*-deficient mice. *caf1* -deficient males are sterile owing to oligo-astheno-teratozoospermia, suggesting that Caf1, a CCR4-associated transcriptional cofactor, is essential for spermatogenesis. Maturation of spermatids is unsynchronized and impaired in $caf1^{-/-}$ seminiferous tubules. Transplantation of spermatogonial stem cells from male $caf1^{-/-}$ mice to seminiferous tubules of *c-kit* mutant mice restores spermatogenesis, suggesting that the function of testicular somatic cells is damaged in the $caf1^{-/-}$ condition. Importantly, the testicular phenotypes of $caf1^{-/-}$ mice are similar to those of $RXRb^{-/-}$ mice. We further show that Caf1 binds the AF-1 domain of RXRb and RXRb malfunctions in the absence of Caf1. Therefore, Caf1 appears to function as a new coregulator of RXRb in testicular somatic cells and is thus involved in spermatogenesis. There are data suggesting that Caf1 is associated with deadenylase activity, suggesting that Tob-Caf1 complex may be involved in translational regulation. To elucidate functional relation between Tob and Caf1 in vivo, $tob^{-/-}$ $caf1^{-/-}$ mice are generated. (2) Search for other molecules that interact with Tob. We performed yeast two-hybrid screening using Tob as a bait, and identified novel serine/threonine kinase, a tyrosine-phosphorylated protein containing Zn-finger motif, and a molecule responsible for the stability of Tob protein. (3) As the other projects, generation of *ana*-deficient mice and *cnot3* (a component of CCR4/Caf1/NOT complex)-deficient mice and characterization of a large protein complex containing Tob are now in progress.

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, Jiro Fujimoto, 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 development of chromosomal and/or centrosomal abnormalities is often observed in tumor cells and a specific chromosome or centrosomal abnormality may often be one of the first events in the development of a cancer. It is well known that microtubule-based motor proteins are involved in spindle formation and chromosome movements in mitosis, and that orchestrated regulation by many mitotic kinases is important for the progression of each step of mitosis.

i) Chromokinesin Kid

Kid is a member of the chromosome-associated kinesin family, identified in our lab in 1996. The kinesin-like motor domain of Kid is present in its amino-terminal half and has the plus-end directed motor activity. Its carboxyl-terminal half contains a helix-hairpin-helix DNA binding motif. Kid is colocalized with spindles and chromosomes during prophase through metaphase and plays an essential role in the alignment of chromosome arms at the metaphase plate. Upon metaphase/anaphase transition, Kid dramatically alters its localization to the boundary between the chromosomes and MTs during anaphase. 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. To further investigate whether Kid has any other functions through mitosis, we depleted Kid from HeLa cells using RNA interference (RNAi). We found that depletion of Kid from HeLa cells resulted in 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 crosslink microtubules into bundles is required, but its DNA binding activity is 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. Moreover, we showed that both MT-binding sites and DNA-binding domain, but not phosphorylation on Thr463, were essential for the proper localization of Kid during anaphase. Together with our recent finding that Kid-depleted HeLa cells often exhibits defects in chromosome segregation, we speculate that strong MT binding activity of unphosphorylated Kid may contribute to stable association of anaphase chromosomes with MTs during anaphase chromosome segregation. In addition, we identified importin a as a Kid binding protein and defined two nuclear localization signals in the region between a motor and DNA-binding domain of Kid, suggesting that localization and functions of Kid are controlled by importin α/β complex.

ii) Mitotic kinases

We have previously identified genes encoding human serine/threonine protein kinases *hLATS1* and *hLATS2* 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 previously found that hLATS2 and Ajuba were co-localized with the centrosomes during mitosis and were required for the accumulation of γ-tubulin at the centrosomes and subsequent spindle organization at G2/M phase transition. In addition, our ongoing studies indicate that Ajuba interacts with beta-catenin and negatively regulates beta-catenin-TCF-induced transactivation, suggesting 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 adhesion and cell-cell contact, which are disassembled during mitosis.

Plk-1 is another highly conserved mitotic kinase that plays multiple pivotal roles in mitosis. Furthermore, Plk-1 is found to be overexpressed in a variety of human tumors. However, the exact mechanism of action of Plk-1 in vivo remains to be elucidated. To address this issue, the searches for novel targets (substrates) and binding partners of Plk-1 are in progress.

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Department of Cancer Biology

Division of Cancer Cell Research
腫瘍細胞社会学分野

Professor	Motoharu Seiki, D.M.Sc.	教	授	医学博士	清木元治
Lecturer	Ikuo Yana M.D., D.M.Sc.	講	師	医学博士	梁　幾勇
Research Associate	Naohiko Koshikawa, Ph.D.	助	手	理学博士	越川直彦
Research Associate	Isamu Gotoh, Ph.D.	助	手	理学博士	後藤　勇

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 1 matrix metalloproteinase cytoplasmic tail binding protein-1 (MTCBP-1) is a new member of the Cupin superfamily: A possible multifunctional protein acting as an invasion suppressor down-regulated in tumors

Takamasa Uekita, Isamu Gotoh, Takeshi, Yoshifumi, Hiroshi Sato Takayuki, Shiomi, Yasunori Okada, and Motoharu Seiki

Membrane-type1matrix metalloproteinase (MT 1-MMP) is a member of the matrix metalloproteinase (MMP) family that collectively degrades most components of the extracellular matrix (ECM). By anchoring to the plasma membrane through a transmembrane domain, MT1-MMP acts in the pericellular space on the cell surface. This property is particularly suitable for the degradation of ECM required for cellular functions such as migration, invasion, proliferation, and the regulation of cell morphology. Since MT 1-MMP is frequently expressed in malignant tumors, it is believed to play a major role in tumor invasion by degrading the ECM in the direction of cell migration and by processing of cell surface molecules.

As a member of the MMP family, MT1-MMP has a propeptide, a catalytic domain, hinge, and a hemopexin-like (HPX) domain starting from the N-terminus and this extracellular portion is linked to the membrane through the transmembrane domain, which follows a short cytoplasmic tail composed of 20 amino acids. As an invasion-promoting enzyme, the activity, localization, and turnover of MT1-MMP are tightly regulated during cell locomotion. For example, MT1-MMP localizes at the leading edge of migrating cells and this localization is regulated through interaction between CD44 and the HPX domain. CD44 is a cell adhesion molecule that acts as a receptor for hyaluronan (HA) and mediates flexible adhesion to the provisional HA-rich matrix in the dynamic ruffling membrane area. Such adhesion is expected to be followed by a firmer adhesion through integrins to gener-

ate force for migration. At the migration edge, MT1-MMP forms oligomers through the HPX domain with possible participation of the cytoplasmic tail, the hinge region, and ECM that binds to the molecule. Activation of proMMP-2 by MT1-MMP is expected to be carried out efficiently within the oligomeric complex at the leading edge. MT1-MMP exposed on the cell surface is regulated negatively by TIMPs, autodegradation, and internalization. Internalization of MT1-MMP depends on the cytoplasmic tail and a LLY motif in the region was found to act as a binding site for AP-2 complex that mediates incorporation of target proteins into clathrin-coated pits. Deletion of the cytoplasmic tail inhibits not only the internalization of MT1-MMP but also the invasion-promoting activity against the reconstituted basement membrane (Matrigel) mediated by MT1-MMP. Thus, the proteolytic activity of MT1-MMP alone is not enough for the invasion-promoting activity and it has to be regulated further by the function of the cytoplasmic tail.

Although the cytoplasmic tail is short, containing 20 amino acids, it is reported to affect functions of MT1-MMP such as the formation of oligomers, localization of the enzyme to the proteolytically active protrusions (invadopodia), internalization, and cell migration and invasion. However, it has not been elucidated how the cytoplasmic tail affects these functions except for the mechanism of internalization. To obtain clues about the mechanism by which the cytoplasmic tail regulates MT1-MMP, we attempted to isolate genes whose products interact with this portion using the yeast two-hybrid screening system. Using a cDNA library established from human fibroblast WI-38 cells, we isolated a new gene, whose product shows homology to members of the Cupin superfamily, a new family composed of proteins with diverse functions and which has a conserved 3D structure. We named this protein MT1-MMP cytoplasmic tail binding protein-1 (MTCBP-1). MTCBP-1 is expressed as a 19 kDa protein and is co-localized with MT1-MMP at the adherent membrane edge. It was also found in the cytoplasm and nucleus. The formation of a complex between MTCBP-1 and MT1-MMP was confirmed by co-immunoprecipitation. Characteristics of MTCBP-1 and its possible role as an invasion-suppressor are discussed.

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

Naoko Suenaga, Hidetoshi Mori, Yoshifumi Itoh, and Motoharu Seiki

Expression of MT1-MMP in tumor cell lines promotes migration, invasion, and proliferation of cells through its proteolytic activity in the pericellular milieu. In migrating cells, MT1-MMP mostly localizes at the ruffling membrane areas that form the leading edge of cells. Such localization of the enzyme is appropriate to act as a "proteolytic drill" to allow cells to invade the surrounding tissue and matrix. Previously we reported that CD44 is a critical molecule in this process, directing MT1-MMP to the migratory front. CD44 is a cell adhesion molecule and its globular lectin-like domain acts as a binding site for hyaluronan, an abundant glycosaminoglycan that fills intercellular spaces in various tissues. The ligand-binding domain is linked to the plasma membrane through the stem that is followed by the transmembrane and the cytoplasmic domains. While CD44 has many splicing variants that have insertions in the stem region, the basic and most common form is referred to as the hematopoietic type (CD44H). The cytoplasimic domain of CD44 interacts with the actin cytoskeleton and is important for CD44 to localize at the ruffling edge of migrating cells. MT1-MMP binds to the extracellular portion of CD44H through the hemopexin-like (HPX) domain and co-localizes in the cells.

Co-expression of MT1-MMP and CD44H promotes cell migration accompanied with increased CD44H shedding. Shedding of CD44 is an event frequently observed in many types of cells and shed CD44 has been detected in culture supernatants. Most of the cell lines shed CD44H constitutively. Expression of MT1-MMP generated two new fragments. In the previous study, we identified the C-terminal sequences of these fragments and the sites were referred to as cleavage site (CS)-1, -2, and -3, respectively from the N-terminus. The cleavage for the constitutive shedding occurs at CS-3 and a disintegrin and metalloproteinase (ADAM)-like proteases were thought to be responsible. Indeed, ADAM-10 and ADAM-17 was recently identified as an enzyme responsible for shedding CD44H in glioblastoma cells. CS-1 was cleaved by MT1-MMP directly because the same sequence can be cleaved even in recombinant proteins, though CS-2 was not cleaved in the same condition. By using the cleavage site-specific antibodies, CD44 H fragments cleaved at CS-1 were detected in human tumors at higher levels compared to the surrounding normal tissues, while those cleaved at CS-3 did not show significant difference.

Like other members of MMPs, the extracellular portion of MT1-MMP has a catalytic (CAT) domain linked to the HPX domain through hinge region. While the HPX domain is important to associate with CD44H, the CAT domain

is responsible for the actual shedding activity. We speculate that this shedding is required to keep adhesion of the leading edge at appropriate level. However, it is still not clear whether the shedding of CD44H actually occurs in the complex at the migratory edge. If the CD44H binding via the HPX domain is prerequisite for the following cleavage event, it is most probable that CD44H shedding by MT1-MMP occurs in the complex. The aim of this study was to elucidate how both the CAT and the HPX domains contribute to the CD44H shedding. We also constructed domain-swapping chimeras between MT1-MMP and other members of MT-MMPs and carried out comparative studies examining their ability to bind and shed CD44H.

3. Membrane-type matrix metalloproteinase-1 (MT1-MMP) is a processing enzyme for human laminin γ2 chain

Naohiko Koshikawa, Tomoko Minegishi, Andrew Sharabi, Vito Quaranta and Motoharu Seiki

Laminin-5 (Ln-5), a major component of the basement membrane, is a heterotrimer composed of α3, β3, and γ2 subunits. Migration and scattering of epithelial and tumour cells is induced by proteolytic processing of the γ2 chain of Ln-5. The γ2 chain is a 140-kDa polypeptide and forms a triple helix with the other subunits at its C-terminal. Processing of the γ2 chain occurs at the N-terminal generating two major C-terminal fragments of 100 kDa (γ2′) and 80 kDa (γ2x) (Fig. 1A), and this processing has been observed in different species including humans and rodents. Because of the limited availability of purified Ln-5, most biochemical studies in this area have been carried out using the rat protein. MT1-MMP and MMP2 were identified as the proteases responsible for the 2nd cleavage of the N-terminus generating the γ2x fragment. In contrast, only MT1-MMP cleaved the 1st site to generate the γ2′ fragment. The two cleavage sites on the rat γ2 chain were identified as G^{434}-D^{435} for γ2′ and A^{586}-L^{587} for γ2x.

Processing of the rat γ2 chain at these two sites releases an internal fragment containing three of the four EGF-like motifs in domain III (DIII). Although Ln-5 does not stimulate the EGF-receptor (EGF-R), the DIII fragment released has the ability to bind EGF-R and induce its phosphorylation. Inhibition of the processing using MMP inhibitors or inhibition of EGF-R activity using specific kinase inhibitors abolished cell migration on Ln-5. Thus, the DIII fragment generated by this processing appears to play a major role in the observed biological effect of Ln-5 and MT1-MMP. This system seems to function in vivo as well because MT1-MMP-deficient mice showed significantly reduced processing of the γ2 chain in the kidney resulting in abnormalities terminal differentiation of the tubular epithelium.

Processing of the γ2 chain by MT1-MMP appears to play critical roles in tumor growth and progression, because the γ2 chain is frequently expressed as a monomer in malignant tumours that express MT1-MMP. In addition, aggressive melanoma cells are known to form vascular-like networks (vascular mimicry) requiring expression of MT1-MMP and the γ2 chain. Treatment of these cells with a neutralizing antibody against MT1-MMP or anti-sense oligonucleotide against the γ2 gene abrogated the mimicry. It has also been reported that the γ2′ and γ2x fragments in humans are similar in size to the rat, suggesting that the processing of the human γ2 chain by MT1-MMP is similar to that of the rat.

However, a comparison of rat and human γ2 sequences reveals that although the 1st site (G^{434}-D^{435}) is conserved in humans the 2nd cleavage site of rat γ2 (A^{586}-L^{587}) is not. Thus, it is not known whether MT1-MMP directly cleaves the 2nd site in addition to the 1st and generates γ2x and the DIII fragment. Further confusion has been raised by two contradictory reports on this issue. Veitch et al. reported that the human γ2 chain cannot be processed by MT1-MMP even at the 1st site which is conserved between rodents and humans. Instead of MT1-MMP, they reported that the astacin family of proteases, such as bone morphogenic protein-1 (BMP-1) and mammalian Tolloid-like metalloproteinases (mTLDs), cleave the γ2 chain. On the other hand, Gilles et al. reported that recombinant MT1-MMP induces processing of Ln-5 deposited in the extracellular matrix, though the cleavage sites were not identified and it was not clear whether a DIII-like fragment was generated as a result of the processing.

In this study, we have attempted to settle this controversy by identifying the cleavage sites on the human γ2 chain cut for MT1-MMP. To this end, we purified the γ2 chain either as a monomer or a heterotrimer (Ln-5) from human cancer cell lines. Incubation of the purified γ2 chain and Ln-5 with a recombinant catalytic fragment of MT1-MMP generated the two C-terminal fragments (γ2′and γ2x) and released DIII-like fragments functionally. By purifying the γ2x fragment, two adjacent cleavage sites were determined. In addition, the γ2 monomeric chain that is expressed in human malignant tumours show greater sensitivity to MT1-MMP than the heterotrimer form of Ln-5.

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Department of Cancer Biology

Division of Cancer Genomics
癌遺伝形質分野

Associate Professor	Hiroaki Miki, Ph.D.	助教授	理学博士　三木裕明
Research Associate	Hideki Yamaguchi, Ph.D.	助手	医学博士　山口英樹
Research Associate	Kei Takenaka, Ph.D.	助手	薬学博士　竹中圭

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. IRSp53/Eps8 complex is important for positive regulation of Rac and cancer cell motility/invasiveness

Yosuke Funato[1,2], Takeshi Terabayashi[1], Naoko Suenaga[3], Motoharu Seiki[3], Tadaomi Takenawa[2], and Hiroaki Miki[1]: Div. [1]Cancer Genomics, [2]Biochemistry, and [3]Cancer Cell Research, Inst. Med. Sci., Univ. Tokyo

IRSp53 has been characterized as an adaptor protein that links Rho-family small GTPases such as Cdc42 and Rac to reorganization of the actin cytoskeleton. IRSp53 possesses a SH3 domain at its C-terminus, through which it binds to WAVE2, a WASP family actin regulating protein, and induces actin polymerization through Arp2/3 complex. We then searched for other binding partners for the IRSp53 SH3 domain and identified Eps8 as the most major binding protein in NIH3T3 fibroblast cells. We confirmed that IRSp53 and Eps8 form an *in vivo* complex and that Eps8 binds directly to the IRSp53 SH3 domain through its N-terminal proline-rich sequence. Thus far, Eps8 has been shown to form a trimolecular complex with Abi-1 and Sos-1, which shows GEF activity specific for Rac. This led us to investigate whether IRSp 53 affects the Eps8-mediated Rac activation, and found that the interaction of IRSp53 with Eps8 augments the Rac activation by reinforcing the formation of the Eps8/Abi-1/Sos-1 Rac-GEF complex. As we characterized IRSp53 as the molecule that functions downstream of Rac in the previous study, these results suggest the possibility that Rac may be able to activate Rac itself through IRSp53 and Eps8. Indeed, we observed that the expression of activated mutant of Rac induced significant activation of wild-type Rac. Interestingly, the complex formation between IRSp53 and Eps8, as determined by fluorescent resonance energy transfer (FRET) analysis, occurred at the leading edge of motile cells, and the motility and invasiveness of HT 1080 fibrosarcoma cells was suppressed by inhibiting the complex formation. These findings implicate the importance of the IRSp53/Eps8 complex in Rac-induced Rac-activation and cell motility.

2. The pre-mRNA-splicing factor SF3a66 functions as a microtubule-binding and -bundling protein.

Kei Takenaka[1], Hiroyuki Nakagawa[2], Shigeaki Miyamoto[2], and Hiroaki Miki[1]: [1]Div. Cancer Genomics, Inst. Med. Sci., Univ. Tokyo, and [2] Dept. Biochem. Sci., Kyusyu Inst. Tech.

SF3a (splicing factor 3a) complex is an essential component of U2 snRNPs (small nuclear ribonucleoprotein particles), which are involved in pre-mRNA splicing. This complex consists of three subunits: SF3a60, SF3a66 and SF3a120. Here, we report a possible non-canonical function of a well-characterized RNA-splicing factor, SF3a66. Ectopic expression experiments using each SF3a subunit in N1E 115 neuroblastoma cells reveals that SF3a66 alone can induce neurite extension, suggesting that SF3a66 functions in the regulation of cell morphology. A screen for proteins that bind to SF3a66 clarifies that SF3 a66 binds to beta-tubulin, and also to microtubules, with high affinity, indicating that SF3a66 is a novel MAP (microtubule-associated protein). Electron microscopy experiments show that SF3 a66 can bundle microtubules, and that bundling of microtubules is due to cross-bridging of microtubules by high-molecular-mass complexes of oligomerized SF3a66. These results indicate that SF3a66 is likely to be a novel MAP, and can function as a microtubule-bundling protein independently of RNA splicing.

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

Takeshi Terabayashi, Hideki Yamaguchi, and Hiroaki Miki

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

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

Yosuke Funato, Kei Takenaka, and Hiroaki Miki

As described above, Dishevelled is a critical signal transducer of the Wnt-signal. To search for novel binding partner(s) of Dishevelled under a physiological condition, we generated a cell line that stably and moderately expresses FLAG-tagged Dishevelled and performed an immunoprecipitation with anti-FLAG antibody. Among the co-precipitated proteins with FLAG-Dishevelled, 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.

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Department of Cancer Biology

Division of Cellular and Molecular Biology
分子発癌分野

Professor	Jun-ichiro Inoue, Ph.D.	教　授	薬学博士　井　上　純一郎
Associate Professor	Kentaro Semba, Ph.D.	助教授	理学博士　仙　波　憲太郎
Lecturer	Taishin Akiyama, Ph.D.	講　師	薬学博士　秋　山　泰　身
Research Associate	Jin Gohda, Ph.D.	助　手	薬学博士　合　田　　仁

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 four such proteins including Tumor necrosis factor receptor-associated factor (TRAF), E coli. Ras-like protein (ERA), Developmentally Regulated GTP-binding protein (DRG), and WT-1. Our current studies indicate that these three proteins play critical roles in cell proliferation and differentiation. Therefore, elucidation of how these proteins work is definitely required for understanding onset of diseases.

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

Jin Gohda, Masato Irisawa, Taishin Akiyama, Sayaka Yamane and Jun-ichiro Inoue

Transcription factor Rel/NFκB binds specifically to a decameric motif of nucleotide, κB site, and activates transcription. The κB sites are present in the enhancers of many genes including the immunoglobulin κ light chain gene, Interleukin-2 receptor α-chain gene, β-interferon gene and LTR of human immunodeficiency virus, which are involved in the regulation of the immune system. Recent studies showed that the activation of Rel/NFκB links to the anti-apoptotic signal. Thus, it is essential to understand the molecular mechanisms by which Rel/NFκB transcription factor is regulated. The activation of Rel/NFκB has been demonstrated to be carried out post-translationally upon extracellular stimuli through membrane receptors such as interleukin-1 receptor, tumor necrosis factor (TNF) α receptor or CD40. Rel/NFκB forms a complex with regulatory protein, IκB, and is sequestered in the cytoplasm prior to stimulation. Upon stimulation, IκB is rapidly phosphorylated on two specific serine residues by IκB kinase (IKK) complex followed by 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. TRAF6 becomes ubiquitinated upon IL-1 stimulation. We also found that TRAF6 becomes conjugated by a Lys48 (K

48) polyubiquitin chain and degraded upon stimulation. Therefore, it is possible that poly ubiquitin chain regulates TRAF6 both positively and negatively. To elucidate mechanisms of the ubiquitination-mediated regulation of TRAF6, we are trying to identify ubiquitination sites of TRAF6. Several lines of evidence suggest that TRAF6 is able to activate some kinase other than TAK1. To identify such kinases, we 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 leulemia virus 1 activates NFκB.

2. Role of TRAF6 in osteoclastogenesis

Jin Gohda and Jun-ichiro Inoue

Interactions of ligands and receptors of the tumor necrosis factor (TNF) superfamily trigger critical intracellular signals that are essential for development, homeostasis and adaptive responses of the immune system. Among various sets of the TNF family ligand and its receptor, receptor activator of NFκB (RANK, also known as TRANCE receptor) and RANK ligand (RANKL, also known as ODF, OPGL and TRANCE) 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. RANKL- or RANK-deficient mice display osteopetrosis due to a lack of osteoclasts. 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. TRAF2, TRAF3 and TRAF5 bind to the membrane-distal domain of the cytoplasmic tail of RANK, whereas TRAF6 interacts with the membrane-proximal domain). 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. Furthermore, RANK-induced activation of NFκB and MAPKs in osteoclast progenitor cells is abrogated in the absence of TRAF6, consistent with previous findings that NFκB and MAPKs play crucial roles in osteoclastogenesis. CD40, another member of the TNF receptor superfamily, transmits signals through TRAF family members, including TRAF 2, TRAF3, TRAF5 and TRAF6. As with RANK, TRAF2, TRAF3 and TRAF5 bind to the membrane-distal domain of the cytoplasmic tail of CD40, whereas TRAF6 interacts with the membrane-proximal domain.

This year, we have shown that TRAF6 plays a crucial role in CD40-mediated NFκB 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 CD40, 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. By introducing mutations into three TRAF6-binding sites of RANK, we found that h40/mRK with a single TRAF6-binding site efficiently induced Ca^{2+} oscillation and expression of NFATc 1, a master switch in osteoclastogenesis, whereas CD40 carrying a single TRAF6-binding site did not. However, expression of CD40 that was approximately 100 times greater than that of h40/mRK resulted in osteoclast formation, indicating that the RANK-TRAF6 signal is more potent than the CD40-TRAF6 signal in terms of NFATc 1 activation and osteoclastogenesis. These results suggest that RANK may harbor a specific domain that amplifies TRAF6 signaling.

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

Takayuki Matsumura, Hiroyasu Konno, Jin Gohda, Taishin Akiyama, Kentaro Semba 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. When overexpressed, TIFA activates NFκB and JNK. Furthermore, introduction of a mutation into TIFA that abolishes binding of TIFA to TRAF6 leads to loss of ability of TIFA to activate NFκB and JNK, indicating that interaction with TRAF6 is essential for the activity of TIFA. FHA domains are conserved sequences of 60-100 amino acids found mainly in eukaryotic nuclear proteins. Some of the FHA domain-containing proteins bind directly to phosphoserine/phosphothreonine residues via the FHA domain in much the same way that SH 2 domains interact with phosphotyrosine residues. TIFA carrying mutations in the FHA domain that are known to abolish FHA domain binding to phosphopeptide (G50ES66A mutant) cannot activate NFκB and JNK, suggesting that TIFA may be regulated by an unidentified phosphoprotein. Furthermore, analysis of endogenous proteins indicated that TIFA associates with TRAF6 constitutively, whereas it associates with IRAK-1 in an IL-1 stimulation-dependent manner. Therefore, TIFA is likely to link IRAK-1 to TRAF6 upon stimulation as a signal-dependent activator of TRAF6.

This year, we identified a TIFA-related protein, TIFAB, that inhibits TIFA-mediated activation of NFκB. TIFAB does not associate with members of the TRAF family but does bind TIFA. We analyzed the effect of TIFAB expression on the TRAF6/TIFA interaction by immunoprecipitation of TRAF6 and found that TIFA coprecipitated with TRAF6 was not changed. However, when we analyzed this interaction by immunoprecipitation of TIFA, we found that TIFAB significantly increased the amount of TRAF6 coprecipitated with TIFA. These findings suggest that TIFAB inhibits the TIFA-mediated TRAF6 activation possibly by inducing a conformational change in TIFA.

5. Role of TRAF6 in TLR signaling

Jin Ghoda, Takayuki Matsumura and Junichiro Inoue

Toll-like receptors (TLRs) signaling pathways are mediated by the TIR domain containing adopter molecules, MyD88, TIRAP, and TRIF. TRAF6 was shown to activate NFκB and MAPKs downstream of these TIR domain proteins to induce inflammatory cytokines expression. However, the precise role of TRAF6 in individual TLR signaling has never been analyzed genetically. We analyzed macrophages from TRAF6-deficient mice and made the following observations. In the absence of TRAF6, i) ligands for TLR2, TLR5, TLR7, and TLR9 failed to induce activation of NFκB and MAPKs or production of inflammatory cytokines, ii) TLR4 ligand-induced cytokine production was remarkably reduced, and activation of NFκB and MAPKs was observed, albeit with delayed kinetics, iii) in contrast with previously reported findings, TLR 3 signaling was not affected. These results indicate that TRAF6 is essential for myeloid differentiation factor (MyD) 88-dependent signaling but is not required for TIR domain-containing adaptor inducing IFN-b (TRIF)-dependent signaling.

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

Kosuke Ishikawa, Sakura Azuma, Jin Gohda, Taishin Akiyama, Kentaro Semba and Junichiro 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, although only GTP-binding activity has been shown for DRG. On the basis of structural features, DRG is classified as a small branch of the Obg family, which forms one large branch of the GTPase superfamily. Although other large branches such as the classic translation factor family (EF-Tu/EF-α subfamily, etc.) and the extended Ras-like family (Ras/Rab subfamily, etc.) have been well characterized, the physiological roles of the Obg family members remain to be elucidated. Genes homologous to the mouse *drg* were subsequently identified in a wide variety of species in eucaryotes and archaea, suggesting that DRG may play a fundamental cellular role. 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. This year, we idetified novel proteins, DRG family regulatory protein (DFRP) 1 and DFRP2, which regulate expression of DRG proteins through specific binding. In transient transfection experiments, DFRP1 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* tran-

scripts in *Xenopus* embryos and tissues are similar, indicating that these genes work cooperatively in various types of eucaryotic cells. Immunofluorescence experiments have revealed that the interaction between DRG1 and DFRP1 may occur in the cytoplasm. We generated *dfrp1* -knockout cells and found that endogenous expression of DRG1 is regulated by DFRP1, confirming that DFRP1 is a specific up-regulator of DRG1 *in vivo*. On the basis of these results, we propose that DRG1 and DRG2 are regulated differently despite their structural similarities.

7. Synthesis of compounds which specifically regulates the activity of Rel/NFκB transcription factors

Masami Ohtsuka[1] and Jun-ichiro Inoue: [1]Department of Bioorganic Medicinal Chemistry, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University.

Chemical compounds are designed and synthesized to test their ability to modulate the signal transduction pathways linked to the physiological response of cells. One of the research targets is the signaling linked to Rel/NFκB activation. Recently it was reported that polyhydroxycarboxylates derived from phenolic compounds have been found to inhibit the cytopathicity of HIV-1 and HIV-2 in MT-4 cells at concentrations that are not toxic to the host cells. The coumarin derivatives are also reported to be useful in the development of therapies for the treatment of viral infections and diseases, including AIDS. Considering the non-toxic nature of these compounds, we have investigated the inhibitory effect of the polyhydroxycarboxylates; 7,8-dihydroxy-4-methylcoumarin (7,8-DHMC), 5,7-dihydroxy-4-methylcoumarin (5,7-DHMC) and gallic acid on recombinant p50 protein-DNA binding with the objective of further designing an inhibitor and also to see how the structures of these compounds relate to their activities. Prasad *et al*. have shown that zinc plays an important role in the activation of NFκB. Therefore, considering the possible zinc-binding of polyphenolic carboxylate, we have also studied the effect of Zn^{2+} ions on the inhibitory activity of gallic acid and have also determined the stability constant of the zinc complexes of the three compounds; 7,8-DHMC, 5,7-DHMC and gallic acid.

EMSA studies show that gallic acid is more potent inhibitor of p50-DNA binding compared with the two coumarins. The models obtained from docking and optimization showed several interesting results, correlating the structure of these compounds with their respective activities. Biochemical studies have hinted at a relationship between the structural conformation of p50 with the DNA binding ability and function. Hence, it is plausible that if the conformation of the p50 is affected, its DNA binding ability may also get affected. From the modeling studies, it seems that among the three compounds, although all of them are making a general steric hindrance (due to van der Waals interactions) for the p50 to bind to its target DNA, gallic acid could also affect the conformation of the protein by making a network of hydrogen bonds with the aid of its uniformly spread hydroxyl group. On the other hand coumarins though active, have less possibilities of making hydrogen-bonding interactions with the protein. The structure of these compounds could be thus correlated with their activities. We found that the addition of zinc ion (1 equivalent) partially restored the DNA-binding property of p50. Chemical speciation and formation constant studies show that gallic acid forms a stronger 1: 1 complex with zinc at physiological pH in comparison to the dihydroxycoumarins. This strong complexation behavior of gallic acid towards zinc may be correlated with its inhibitory activity towards DNA- NFκB binding.

8. Gene expression profiling of NFκB-activated tumor cell lines

Kentaro Semba, Shinya Watanabe*, Sakura Azuma and Jun-ichiro Inoue: *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 superrepressor) which lacks phosphorylation sites by IKK shows that NFκB activity contributes to the survival or growth of tumor cells, tumor invasion and metastasis and angiogenesis. Tumor cells which 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 understand gene expression network regulated by NFκB for cancer research.

For this purpose, we are analyzing gene expression profiles and NFκB status of a large number of human tumor cell lines including colon, lung, pancreatic, esophagus, stomach, breast and ovary tumor cell lines. Clustering analysis of 44 colon, lung and pancreatic cell lines 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. Unexpectedly, genes correlated with NFκB status seem to be distinct among tumor types. Further functional analysis of those genes in each tumor type will enable us to develop more specific therapeutic drugs against tumors.

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Department of Cancer Biology

Division of Biochemistry (1)
腫瘍分子医学分野(1)

Professor	Tadaomi Takenawa Ph.D.	教　授	薬学博士　竹　縄　忠　臣
Research Associate	Toshiki Itoh. Ph.D.*	助　手	理学博士　伊　藤　俊　樹*
Research Associate	Shiro Suetsugu, Ph.D.	助　手	理学博士　末　次　志　郎
Research Associate	Takesi Ijuin, Ph.D.	助　手	理学博士　伊集院　　壮

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. PtdIns(3,4,5)P3 binding is necessary for WAVE2-induced formation of lamellipodia.

Tukasa Oikawa, Hideki Yamaguchi, Toshiki Itoh, Masayoshi Kato, Takeshi Ijuin, Daisuke Yamazaki, Shiro Suetsugu, and Tadaomi Takenawa

Polarized cell movement is triggered by the development of a PtdIns(3,4,5)P3 gradient at the membrane, which is followed by rearrangement of the actin cytoskeleton. The WASP family verprolin homologous protein (WAVE) is essential for lamellipodium formation at the leading edge by activating the Arp2/3 complex downstream of Rac GTPase. Here, we report that WAVE2 binds to PtdIns(3,4,5)P3 through its basic domain. The amino-terminal portion of WAVE2, which includes the PtdIns(3,4,5)P3-binding sequence, was localized at the leading edge of lamellipodia induced by an active form of Rac (RacDA) or by treatment with platelet-derived growth factor (PDGF). Production of PtdIns (3,4,5)P3 at the cell membrane by myristoylated phosphatidylinositol-3-OH kinase (PI(3)K) is sufficient to recruit WAVE2 in the presence of dominant-negative Rac and latrunculin, demonstrating that PtdIns(3,4,5)P3 alone is able to recruit WAVE2. Expression of a full-length mutant of WAVE2 that lacks the lipid-binding activity inhibited proper formation of lamellipodia induced by RacDA. These results suggest that one of the products of PI(3)K, PtdIns(3,4,5)P3, recruits WAVE2 to the polarized membrane and that this recruitment is essential for lamellipodium formation at the leading edge.

2. FBP11 regulates nuclear localization of N-WASP and inhibits N-WASP-dependent microspike formation.

Kiyoto Mizutani, Shiro Suetsugu, and Tadaomi Takenawa

WASP family proteins are involved in cortical actin cytoskeleton reorganization. Neural Wiskott -Aldrich syndrome protein (N-WASP), a ubiquitously expressed WASP homologous protein, directly binds with Cdc42, activating Arp2/3 complex. In this study, we show that N-WASP-dependent microspike formation is inhibited by formin binding protein 11 (FBP11). Endogenous

FBP11 localizes with nuclear-speckles, and co-localization of N-WASP and FBP11 was observed when they were co-expressed. Epidermal growth factor (EGF) induced actin-microspike formation in COS7 cells. However, transient expression of FBP11 suppressed N-WASP-dependent actin-microspike formation by trapping N-WASP in the nucleus. These results indicate that FBP11 regulates localization of N-WASP, thus negatively regulating the function of N-WASP in the cytoplasm.

3. Myotubularin regulates the function of the late endosome through the gram domain-phosphatidylinositol 3,5-bisphosphate interaction.

[1]Kazuya Tsujita, [1]Toshiki Itoh, [1]Takeshi Ijuin, Yamamoto, [2]Assia Shisheva, [3]Jocelyn Laporte, and [1]Tadaomi Takenawa: [1]Department of Biochemistry, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Mianato-ku, Tokyo 108-8639, [2]Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan 48201, and the [3]Equipe Genetique Humaine Institute de Genetique et de Biologie Moleculaire et Cellulaire 1, rue Laurent Fries B.P., 10142-67404 Illkirch, France

Myotubularin and related proteins constitute a large and highly conserved family possessing phosphoinositide 3-phosphatase activity, although not all members possess this activity. This family contains a conserved region called the GRAM domain that is found in a variety of proteins associated with membrane-coupled processes and signal transduction. Mutations of myotubularin are found in X-linked myotubular myopathy, a severe muscle disease. Mutations in the GRAM domain are responsible for this condition, suggesting crucial roles for this region. Here, we show that the GRAM domain of myotubularin binds to phosphoinositide with the highest affinity to phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P2). In patients with myotubular myopathy, mutations in the myotubularin GRAM domain eliminate this binding, indicating that the PtdIns(3,5)P2 binding ability of the GRAM (glucosyltransferases, Rablike GTPase activators and myotubularin) domain is crucial for the functions of myotubularin in vivo. Stimulation of epidermal growth factor recruits myotubularin to the late endosomal compartment in a manner dependent on the phosphoinositide binding. Overexpression of myotubularin inhibits epidermal growth factor receptor trafficking from late endosome to lysosome and induces the large endosomal vacuoles. Thus, our data suggest that myotubularin phosphatase physiologically functions in late endosomal trafficking and vacuolar morphology through interaction with PtdIns(3,5)P2.

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

Shusaku Kurisu, Shiro Suetsugu, Daisuke Yamazaki, Hideki Yamaguchi, and 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.

5. N-WASP and WAVE2 Acting Downstream of Phosphatidylinositol 3-Kinase Are Required for Myogenic Cell Migration Induced by Hepatocyte Growth Factor.

Kazuhiro Kawamura[2], Kazunori Takano[2], Shiro Suetsugu[1], Shusaku Kurisu[1], Daisuke Yamazaki[1], Hiroaki Miki[3], Takeshi Endo[2], and Tadaomi Takenawa[1]: [1]Division of Biochemistry, and [3]Division of Cancer Genomics The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan [2]Department of Biology, Faculty of Science, and Graduate School of Science and Technology, Chiba University, Yayoicho, Inageku, Chiba, Chiba 263-

8522, Japan.

During skeletal muscle regeneration caused by injury, muscle satellite cells proliferate and migrate toward the site of muscle injury. This migration is mainly induced by hepatocyte growth factor (HGF) secreted by intact myofibers and also released from injured muscle. However, the intracellular machinery for the satellite cell migration has not been elucidated. To examine the mechanisms of satellite cell migration, we utilized satellite cell-derived mouse C2C12 skeletal muscle cells. HGF induced reorganization of actin cytoskeleton to form lamellipodia in C2C12 myoblasts. HGF treatment facilitated both nondirectional migration of the myoblasts in phagokinetic track assay and directional chemotactic migration toward HGF in a three-dimensional migration chamber assay. Endogenous N-WASP and WAVE2 were concentrated in the lamellipodia at the leading edge of the migrating cells. Moreover, exogenous expression of wild-type N-WASP or WAVE2 promoted lamellipodial formation and migration. By contrast, expression of the dominant-negative mutant of N-WASP or WAVE2 and knockdown of N-WASP or WAVE2 expression by the RNA interference prevented the HGF-induced lamellipodial formation and migration. When the cells were treated with LY294002, an inhibitor of phosphatidylinositol 3-kinase, the HGF-induced lamellipodial formation and migration were abrogated. These results imply that both N-WASP and WAVE2, which are activated downstream of phosphati-dylinositol 3-kinase, are required for the migration through the lamellipodial formation of C2C12 cells induced by HGF.

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Department of Cancer Biology

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

Associate Professor Seiichi Takasaki, Ph. D.

助教授 医学博士 高 崎 誠 一

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. Analysis of physiological selectin ligands expressed on the cell surface of cancer cells

Chi Chih Lin, Etsuko Mori, Seiichi Takasaki

The structures of physiological ligands that are recognized by the selectins are still not known in detail. The well-known candidate structures are based on the carbohydrate analysis of PSGI-1 and GlyCAM-1. The major capping structures of GlyCAM-1 were found to be 6-sulfo-sLe^x and 6'-sulfo-sLe^x, and in a complete structural analysis, two sulfated O-glycans were found representing these structures in core-2-branched side chains. 6-Sulfo-sLe^x was found to block the binding of L-selectin to GlyCAM-1. Analysis of the O-glycans of human PSGL-1, the ligand for P-selectin, revealed that only a minority of the O-glycans is fucosylated, and they occur as two major species both based on core-2-branchcd chains. Interestingly, PSGL-1 needs to be sulfated at one of the three tyrosine residues located at its N-terminus for binding to P-selectin. In contrast, the structural requirements that are necessary for the recognition of a ligand by E-selectin are different from those necessary for P- and L-selectin. Sulfation, which was found to be essential for P- and L-selectin ligands, is dispensable for E-sclectin ligands. This could either argue for more simple recognition structures on E-selectin ligands that are easily expressed on many different scaffold glycoproteins, or for discrete, but broadly expressed scaffold proteins in many different cells. Two such glycoprotein ligands were identified on mouse myeloid cells, ESL-1 and PSGL-1. However, E-selectin ligands on cancer cells except for myeloid cells have not been studied. Therefore, we examined several types of cancer cells, and found that a human hepatoma cell line, HepG2, shows strong adhesion to E-selectin. After treated with 1-deoxymannojirimycin which modulates glycosylation of N-glycans, HepG2 cells showed drastically reduced adhesion to a E-selectin/Fc chimera protein and IL-1βinduced HUVEC. Interestingly, we also observed that the adhesion of HepG2 cells to E-selectin is slightly affected after culture in the presence of benzyl-GalNAc, the O-glycosylation inhibitor. The result indicates that the adherence considerably depends on N-glycans of HepG2 cells. The normal phase HPLC analysis of N-glycans released from plasma membrane glycoproteins showed that HepG2 cells are enriched with high molecular weight glycans. MALDI-TOF/MS analysis showed that the N-glycans have α1,6-fucosylated bi-, tri-, and tetra-

antennary structures with and without N-acetyllactosamine repeats called poly-N-acetyllactosamines. Moreover, the TOF/MS spectra of the fragments released from the glycans by endo-β-galactosidase digestion showed that poly-N-acetyllactosamines of HepG2 N-glycans are decorated with a variety of Le^x and sialyl Le^x relating structures. This is the first study demonstrating an outline structures of poly-N-acetyllactosamines on N-glycans which are expressed on human cancer cells and might contribute to E -selectin binding.

2. Analysis of apotosis inducing nucleosides secreted from natural suppressor cell line

Aishun jin, Seiichi Takasaki, and Etsuko Mori

The therapy of choice for primary cancer is surgery and, for advanced case, mainly chemotherapy. Several nucleoside derivatives have been used for chemotherapy. For example, the administration of 5-fluorouracil has been used as the fundamental chemotherapy. In the previous studies, it was found that apoptosis inducing nucleosides (AINs) are secreted from CD57-HLA-DR^{bright} natural suppressor (CD57.DR-NS) cell line. To provide scientific evidence before clinical application, the anti-tumor effects of AINs on human gastric carcinoma (GCIY)-bearing severe combined immunodeficiency (SCID) mice were examined by monitoring tumor cell growth and change of body weight of mice. The results obtained evidenced that AINs strongly induces apoptosis in the tumor tissues in SCID mice with decrease of tumor size and without loss of body weight. We found that peak 5 and peak 6 (P5 and P6) components among the six components of AINs isolated by high performance liquid chromatography are the most effective. The anti-tumor effects of P5, P6 and their mixture were dose-dependent. Thus, the effective method of administration of AINS for tumor regression without exhaustion was established in the present study. AINs are a novel anti-tumor reagent derived from 57.DR-NS cell line, which was cloned from human first trimester decidual tissue. Therefore, AINS are natural substances but not synthetic material and this is the outstanding advantage of AINs differentiating from other chemical anti-cancer reagents. Thus, AINs are unique substances that can generate apoptosis in malignant cells while lacking toxicity in normal cells, suggesting possible refrainment from side-effects in clinical trials.

3. Cell adhesion molecules involved in mammalian fertilization

Etsuko Mori, Dong Cun Jin, Naoei Yoshitani, Seiichi Takasaki

Fertilization is mediated by complex molecular interactions between sperm and egg. An extracellular matrix called the zona pellucida (ZP) which consists of a few glycoproteins surrounds mammalian eggs. The ZP plays important roles in sperm-egg binding, induction of sperm acrosome reaction, and block to polyspermy. On the basis of oligosaccharide structures of porcine ZP glycoproteins so far elucidated, we prepared various multivalent oligosaccharide probes, and applied them to detection of the putative sperm carbohydrate recognition molecules. The results indicated that two kinds of molecules, one recognizing the N-acetyllactosamine structure and the other recognizing the Le^x structure, are expressed on boar sperm heads. Now, we are trying to identify the putative carbohydrate recognition molecules. We prepared plasma membranes of boar sperm, and solubilized with detergents. When the supernatants of the triton X-100 solubilized membranes after centrifugation (100,000g) were applied on the plastic well coated with the multivalent probe carrying N-acetyllactosamine oligosaccahrides, several proteins were shown to bind the plate by SDS-PAGE. The N-acetyllactosamine binding protein was partially purified by dextran sulfate-Sepharose column chromatography, and analyzed by gel filtration. The sugar binding activities were eluted in the high molecular weight region. SDS-PAGE analysis indicated the active fraction still contains multiple components. Similar situation was also observed in the analyses by ion-exchange chromatography and affinity chromatography, suggesting that the putative N-acetyllactosamine binding protein forms a complex with other proteins even in the detergent solubilized state. When the solubilized membrane proteins were coated on the ELISA plate, their carbohydrate binding activity could not be detected. However, addition of phopholipids to the detergent free proteins to form liposome followed by coating on the plate enabled us to detect the carbohydrate binding activity. Thus, it is likely that the putative carbohydrate binding protein requires the membranous environment for the expression of its strong binding activity. As an alternative approach to identify sperm carbohydrate binding proteins, we have been introducing a recombinant technology. Based on the partial peptide sequence data of proteins that bound to the oligosaccharide probe containing N-acetyllactosamine structure, we cloned several cDNAs of candidate carbohydrate binding proteins. We are currently producing recombinant proteins

and analyzing their adhesion activities.

Publications

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Department of Cancer Biology

Division of Genetics
腫瘍抑制分野

Professor	Masabumi Shibuya, M.D., D.M.Sc.	教　授	医学博士	渋　谷　正　史
Lecturer	Noriko Gotoh, M.D., D.M.Sc.	講　師	医学博士	後　藤　典　子
Research Associate	Naoyuki Yabana, Ph.D.	助　手	理学博士	矢　花　直　幸
Research Associate	Yoshiko Sakurai, D.M.Sc.	助　手	医学博士	櫻　井　佳　子

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 purified a novel VEGF-related protein from a snake venom, and partly clarified the signals toward vascular permeability. Furthermore, we identified a single tyrosine residue on VEGFR-2 which is essential for angiogenesis in vivo. We are also examining the physiological and pathological roles of FRS2. This year we have found that FRS2 plays critical roles for multiple developmental processes including eye development. Furthermore, FRS2 appears to be involved in maintenance of several stem cells including trophoblast stem (TS) cells, retinal stem cells and neural stem cells.

1. A Novel Snake Venom Vascular Endothelial Growth Factor (VEGF) Predominantly Induces Vascular Permeability through Preferential Signaling via VEGF Receptor-1.

Hiroyuki Takahashi, [1]Shosaku Hattori, [2]Akihiro Iwamatsu, [3]Hajime Takizawa, and Masabumi Shibuya: [1]Amami Laboratory of Injurious Animals, Institute of Medical Science, University of Tokyo, [2]Central Laboratories for Key Technology, KIRIN BREWERY Company Ltd., Yokohama, [3]Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo

Vascular endothelial growth factor-A/vascular permeability factor (VEGF-A/VPF) induces both angiogenesis and vascular permeability mainly through VEGFR-2 activation. VEGF-A binds VEGFR-1 as well, but the importance of VEGFR-1 signaling in vascular permeability has been largely neglected. This year we at the first time purified and characterized a novel VEGF-like protein from Habu (*Trimeresurus flavoviridis; T.f.*) snake venom. *T.f.* snake has a venom-specific VEGF-like molecule, *T.f.* snake venom VEGF (svVEGF), in addition to VEGF-A. *T.f.* svVEGF has almost 10-fold less mitotic activity than VEGF-A_{165}, a predominant isoform of human VEGF-A, but a similar effect on vascular permeability. *T.f.* svVEGF binds VEGFR-1 and induces its autophosphorylation to almost the same extent as $VEGF_{165}$, but binds VEGFR-2 weakly and induces its autophosphorylation almost 10-fold less effectively than VEGF-A_{165}. This unique binding affinity for VEGFR-1 and -2 leads to the vascular-permeability-dominant activity of *T.f.* svVEGF. These results suggest that Habu have acquired a highly purposive molecule for a to-

xin, which enhances the toxicity in envenomation without inducing an effective angiogenesis and the following regeneration of damaged tissues, taking advantage of the difference in signaling properties involving VEGFR-1 and VEGFR-2 between vascular permeability and angiogenesis. *T.f.* svVEGF is thus a potent inducing factor selectively for vascular permeability through preferential signaling via VEGFR-1. These data strongly indicate the importance of VEGFR-1 signaling in vascular permeability.

2. Essential role of Flk-1/VEGFR-2 tyrosine residue-1173 in vasculogenesis in mice.

Yoshiko Sakurai, Kaori Ohgimoto, Yuki Kataoka[4], Nobuaki Yoshida[4] and Masabumi Shibuya. [4]Division of Gene Expression and Regulation, Institute of Medical Science, University of Tokyo

Flk-1/VEGFR-2 (human counterpart, KDR) tyrosine kinase, which is one of the two VEGF-A receptors, is crucial for vascular development. Recently, we showed that among tyrosine residues of VEGFR-2, tyrosine residue-1175 (Y1175, corresponding to Y1173 in murine Flk-1) and Y 1214 (Y1212 in Flk-1) are autophosphorylated in response to VEGF-A, and Y1175 is important for VEGF-dependent phospholipase Cγ/protein kinase C/mitogen-activated protein kinase activation leading to DNA synthesis in cultured endothelial cells. However, the importance of these tyrosine residues in VEGFR-2 *in vivo* is not known. To examine the role of these VEGFR-2 tyrosine residues *in vivo*, we generated knock-in mice substituting Y1173 and Y1212 of the *Flk-1/VEGFR-2* gene with phenylalanine, respectively. As a result, *Flk-1*^{1173F} homozygous mice died between embryonic days 8.5 (E8.5) and E9.5 without any organized blood vessels and yolk sac blood islands, and hematopoietic progenitors were severely reduced similar to the case of *Flk-1* null mice. In contrast, *Flk-1*^{1212F} homozygous mice were viable and fertile. These results suggest that the signaling via Y1173 of Flk-1/VEGFR-2 is essential for endothelial and hematopoietic development during embryogenesis.

3. Membrane-fixation of VEGFR1 ligand-binding domain is important for vasculogenesis/angiogenesis in mice.

Sachie Hiratsuka[5], Kazuki Nakao[6], Kenji Nakamura[7], Motoya Katsuki[8], Yoshiro Maru[5] and Masabumi Shibuya. [5]Department of Pharmacology, Tokyo Women's Medical University School of Medicine, Tokyo, [6]Laboratory for Animal Resource and Genetic Engineering RIKEN, Center for Development biology, Hyogo, [7]Reproductive Engineering Section Mouse Genome Technology Center, Mitsubishi Kagaku Institute of Life Science, Tokyo, [8]National Institute for Basic Biology, Aich.

VEGF-A regulates vasculogenesis and angiogenesis using two tyrosine kinase receptors, VEGFR-1 and VEGFR-2. VEGFR-1 null mutant mice die at E8.5-E9.0 due to an overgrowth of endothelial cells and vascular disorganization, suggesting that VEGFR-1 plays a negative role in angiogenesis. We previously showed that the tyrosine kinase (TK) domain of VEGFR1 is dispensable at embryogenesis, since VEGFR-1 TK-deficient mice survived and were basically healthy. However, the molecular basis is not yet clearly understood. To test the hypothesis that the specific role of VEGFR-1 at early embryogenesis is to recruit its ligand to the cell membrane, we deleted transmembrane (TM) domain in TK-deficient VEGFR-1 mice. Surprisingly, about half of the VEGFR-1(TM-TK)-deficient mice were embryonic lethal due to a poor development of blood vessels, whereas other mice were healthy. In the VEGFR-1(TM-TK)−/− mice having growth arrest, membrane-targeted VEGF-A was reduced, resulting in suppression of VEGFR-2 phosphorylation. Furthermore, the embryonic lethality in VEGFR-1(TM-TK)−/− mice was significantly increased to 80-90% when genotype of VEGFR-2 was changed from (+/+) to heterozygote (+/−) in 129/C57BL6 mice. These results strongly suggest that the membrane-fixed ligand-binding region of VEGFR-1 traps VEGF-A for the appropriate regulation of VEGF signaling in vascular endothelial cells in early embryogenesis.

4. Signaling through FRS2 docking proteins

A. FRS2 family docking proteins with overlapping roles in activation of MAP kinase have distinct spatial-temporal patterns of expression of their transcripts.

Noriko Gotoh, Shaked Laks[9], Misako Nakashima[10], Irit Lax[9] and Joseph Schlessinger[9]: [9]Department of Pharmacology, Yale University School of Medicine, New Haven, USA, [10]Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University.

FRS2α and FRS2β, two members of the FRS2 family of docking proteins, become tyrosine phosphorylated in response to fibroblast growth factor (FGF) or nerve growth factor (NGF) stimulation. Tyrosine phosphorylated FRS2α serves as a platform for the recruitment of multiple signaling proteins for activation of the Ras-MAP

kinase signaling cascade. We report that *Frs2*α and *Frs2*β have distinct spatio-temporal expression patterns in mouse embryos. We further show that FRS2β can compensate for the loss of FRS2α for activation of MAP kinase when expressed in fibroblasts from *Frs2*$\alpha^{-/-}$ mouse embryos. We propose that the FRS2 family proteins have distinct roles in vivo through activation of common signaling proteins including MAP kinase.

B. Tyrosine phosphorylation sites on FRS2α responsible for Shp2 recruitment are critical for induction of lens and retina.

N. Gotoh, M. Ito[11], S. Yamamoto, I. Yoshino, N. Song[12], Y. Wang[12], I. Lax[9], J. Schlessinger[9], M. Shibuya, and R.A. Lang[12]: [11]Department of Anatomy, National Defense Med. College, Tokorozawa, [12]Division of Developmental Biology, Department of Ophthalmology, The Children's Hospital Research Foundation, Cincinnati, USA.

Early development of the lens and retina is dependent upon reciprocal inductive interactions between the embryonic surface ectoderm and the underlying neuroepithelium of the optic vesicle. FGF signaling has been implicated in this signal exchange. The docking protein FRS2α is a major mediator of FGF signaling by providing a link between FGF-receptors (FGFRs) and a variety of intracellular signaling pathways. Following FGF stimulation, tyrosine phosphorylated FRS2α recruits four molecules of the adaptor protein Grb2 and two molecules of the protein tyrosine phosphatase Shp2 resulting in activation of the Ras/ERK and PI-3 kinase/Akt signaling pathways. In this report we explore the role of signaling pathways downstream of FRS2 α in eye development by analyzing the phenotypes of mice that carry point mutations in either the Grb2 binding sites (*Frs2*α^{4F}) or the Shp 2 binding sites (*Frs2*α^{2F}) of FRS2α. While *Frs2* $\alpha^{4F/4F}$ mice exhibited normal early eye development, all *Frs2*$\alpha^{2F/2F}$ embryos were defective in eye development and showed anophthalmia or microphthalmia. Consistent with the critical role of FRS2α in FGF signaling, the level of activated ERK in *Frs2*$\alpha^{2F/2F}$ embryos was significantly lower than that observed in wild type embryos. Furthermore, expression of Pax6 and *Six3*, molecular markers for lens induction, were decreased in the *Frs2*$\alpha^{2F/2F}$ presumptive lens ectoderm. Similarly, the expression of Chx10 and *Bmp4*; genes required for retinal precursor proliferation and for lens development, respectively, was also decreased in the optic vesicles of *Frs2*$\alpha^{2F/2F}$ mice. These experiments demonstrate that intracellular signals that depend on specific tyrosine residues in FRS2α lie upstream of gene products critical for induction of lens and retina.

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