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Recent progress in oncogene research has revealed that oncogenes and antioncogenes play important roles in the genesis and/or development of cancer. Structural and functional analysis of the protooncogenes revealed that a large majority of them encode proteins with protein-tyrosine kinase activity 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. Furthermore, accumulating evidence suggests that protein-tyrosine kinases and transcription factors play important parts in signal transduction processes of various biological systems including immune system and central nervous system. Our current interest is to elucidate the function of protein tyrosine kinases, protein tyrosine phosphatases, and other molecules regulating transcription factors in signaling pathways of immune system, central nervous system, and cancer cells.

1. Role of the Cbl-family proteins and a novel tyrosine kinase substrate BANK in the signaling through tyrosine kinases

Tomoharu Yasuda, Minsoo Kim, Kazumasa Yokoyama, Yuji Yamanashi, Tohru Tezuka, and Tadashi Yamamoto

c-cbl is a cellular homologue of the *v-cbl* retroviral oncogene carried by the CasNS-1 retrovirus. In our initial studies, c-Cbl was idenfied as a protein associated with and prominently phosphorylated by a Src-family tyrosine kinase Lyn in the B-cell antigen receptor (BCR) signaling. To date, the mammalian Cbl-family is consisted of c-Cbl (Cbl hereafter), Cbl-b, and Cbl-c/Cbl3 that we have identified. The family proteins are substarates for various types of protein-tyrosine kinases (PTKs), such as EGF receptor, Lyn, and JAK. They contain a TKB (tyrosine kinase binding) domain and a RING finger motif at their amino-terminal region. Recent biochemical and genetical analyses of the Cbl-family have implicated that the family proteins are negative regulators of the signals downstream of various tyrosine kinases by acting as RING-type ubiquitin ligases. However, since Cbl also functions as a positive signal transducer in the integrin signaling, the roles of the Cbl-family proteins likely are more complicated.

To analyze the roles of Cbl and Cbl-b in the BCR signaling, we have established DT40 chicken B cell lines lacking Cbl and/or Cbl-b by homologous recombination. Unexpectedly, analyses of these DT40 cells revealed opposite roles of Cbl and Cbl-b in terms of BCR-mediated calcium signaling. BCR-mediated PLC-γ2 activation and calciun mobilization were enhanced by Cbl deficiency, whreas they were atttenuated by Cbl-b deficiency. Our biochemical data showed that Cbl negatively regulates the PLC-γ2 pathway by inhibiting the association of PLC-γ2 with BLNK, an adaptor protein critically important for PLC-γ2 recruitment to the membrane and subsequent activation of PLC-γ2 upon BCR stimulation. In contrast, Cbl-b played a positive role in regulation of PLC-γ2 by Btk tyrosine kinase, by acting as a scaffold protein for formation of Btk/BLNK/PLC-γ2 complex. Mouse lymphocytes deficient in Cbl-b are hyperproliferative, suggesting that Cbl-b negatively regulates other signaling pathways in antigen receptor signaling. The negative regulatory roles of Cbl-b in the BCR signaling are under investigation.

We have previously identified Cbl-c, the third member of the Cbl-family. The expression pattern of *cbl-c* mRNA is distinct from that of *cbl* or *cbl-b* mRNA, being high in the digestive organs, such as the colon and the small intestine. Since Src is up-regulated in some colon cancer cell lines, we examined whether the Cbl-family proteins suppress transforming activity of v-Src. All three Cbl-family proteins suppressed colony formation in soft agar of v-Src-transformed NIH3T3 cells, when they are exogenously expressed by retroviral systems. However, only Cbl-c, but Cbl and Cbl-b, reverted morphological transformation induced by v-Src. In addition, the protein level of v-Src was decreased only by Cbl-c among the Cbl-family. Consistently, wild-type, but not TKB domain or RING finger mutants, of Cbl-c induced ubiquitination of c-Src. Therefore, Cbl-c would suppress v-Src-induced transformation by ubiqitination and subsequent degradation of v-Src. The mechanism by which Cbl and Cbl-b suppress v-Src-induced transformation is clearly distinct from degradation of v-Src, and now under investigation.

In screening of substrates for the Src-family PTKs in the central nervous system, we identified a novel protein with two ankyrin repeats. The protein, termed BANK (a B-cell specific protein with ankyrin repeats), had significant structural homology with BCAP (B cell adaptor for phosphoinositide 3-kinase: PI3K) and *Drosophila* Dof (Downstream of FGF receptor). BANK was phosphorylated by Syk tyrosine kinase upon BCR stimulation. Although BCAP and Dof participate in the PI3K and Ras-MAPK pathway, respectively, our results suggested that BANK positively regulates the PLC-γ2-calcium pathway. Consistently, BANK bound to both PLC-γ2 and IP_3 receptors in heterologous cells. We are further analyzing the mechanism by which BANK contributes to the calcium signaling.

2. Role of protein-tyrosine kinases in the central nervous system

Tohru Tezuka, Takanobu Nakazawa, Kazumasa Yokoyama, Shin-ichiro Kina, Seiji Kawa, June Goto, Hisashi Umemori, Miho Ohsugi, Jiro Fujomoto, Yoichiro Iwakura[1], Toshiya Manabe[2], and Tadashi Yamamoto:[1]Laboratory of Cell Biology, Center for Experimental Medicine and [2]Division of Neuronal Network, Department of Basic Medical Sciences

The Src-family protein-tyrosine kinases (PTKs) are implicated in various neural functions. For an example, mice lacking a Src-family PTK Fyn show multiple neural defects, that include impaired long-term potentiation (LTP), defective spatial learning, hypomyelination, increased ethanol sensitivity, and uncoordinated hippocampal structure. Our principal purpose is to further uncover the physiological importance of the Src-family PTKs in the central nervous system (CNS), and to establish the molecular mechanisms by which they contribute to those neural functions.

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. LTP is one of the most prominent examples of synaptic plasticity underlying memory formation. Several lines of evidence suggest that protein-tyrosine kinases (PTKs) play regulatory roles in LTP induction. For examples, recent findings show that 1) the N-methyl-D-aspartate (NMDA) type of glutamate receptor subunits, NR2A/2B, are significantly tyrosine phosphorylated in the brain; 2) Src interacts both physically and functionally with NMDA receptors (NMDARs); 3) NMDAR-dependent LTP and spatial learning are impaired in mice lacking Fyn. Our own studies have shown that Fyn is involved in tyrosine phosphorylation of NR2A/2B subunits. Postsynaptic density protein PSD-95 that directly interacts with NMDAR facilitated the tyrosine phosphorylation events. To analyze the biological significance of tyrosine phosphorylation of NMDARs, we have determined tyrosine phosphorylation sites of NR2A/2B for Fyn and identified that Tyr-1472 was the major tyrosine phosphorylation site of NR2B. The Tyr-1472 phosphorylation was significantly enhanced after induction of LTP in the hippocampal CA1 region, suggesting that Tyr-1472 phosphorylation of NR2B is improtant for synaptic plasticity. To establish the roles of Fyn-mediated phosphorylation of NR2A/2B, tyrosine to phenylalanine mutation was introduced on the phosphorylation sites. Analyses of the electrophysiological activities of the mutated receptors and phenotypes of mice having these mutant receptors are underway.

We have also shown that protein-tyrosine phosphatase PTPMEG is associated with glutamate receptors, NMDAR and GluRδ2, via its PDZ domain. Our data suggest that PTPMEG is involved in Fyn-mediated tyrosine phosphorylation of NR2A/NR2B. The physiological and biochemical characterization of PTPMEG is now in progress.

Among the Src-family kinases, which are expressed in the brain, Lyn is specifically expressed in the granular layers of the cerebellum as well as in basal ganglia and cerebral cortex. Possible involvement of Lyn in LTP and LTD (long term depression) is also under investigation by using $lyn^{-/-}$ mice.

To further analyze the roles of the Src-family PTKs in the CNS, we have been searching for their activators and substrates as well as genes whose expression is regulated by the Src-family PTKs. Several candidates were isolated, and their characterization is in progress.

To further study the roles of PTKs in neural function and development of CNS, we have been characterizing two PTKs, Fak and ALK, which we previously identified (see below). Analyses of a novel PTK that we have identified by in silico screening are also in progress.

3. Elucidation of the Tob-mediated signaling pathway

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By screening a cDNA expression library with autophosphorylated c-erbB-2 protein, we isolated cDNA clones coding for its possible substrates. One of the genes thus identified, termed *tob*, encodes a 45-kDa protein with homology to the growth suppressing protein Btg1. Elevated expression of the Tob protein suppressed growth of the NIH3T3 cells. We assume that antiproliferative property of Tob might be canceled by the signaling through membrane tyrosine kinases. We also cloned novel genes homologous to *tob* which were termed *tob-2* and *ana*. Microinjection experiments showed that both exogenously expressed Tob-2 and ANA proteins suppress growth of NIH3T3 cells. Thus, we propose a new family of antiproliferative genes that includes *tob*, *tob-2*, *btg1*, *pc3/tis21/btg2*, and *ana*. In our study to address the mechanisms by which Tob suppresses cell growth, we showed that overexpression of Tob family proteins resulted in hypophosphorylation of the Rb protein, suggesting that activity of cyclin-dependent kinases (CDKs) were affected. We have also found that Tob is rapidly phosphorylated at multiple sites upon growth factor stimulation, and that Ser152, Ser154, and Ser164 are phosphorylated by Erk1 and Erk2 MAPKs. Tob mutant with Ser152, 154, 164 to alanine substitution largely suppressed Ras/MAPK dependent cell proliferation and transformation, whereas wild-type and the mutant with glutamates at those three positions, which are negatively charged amino acids mimicking phosphoserines, had no significant effect. These results suggest that the antiproliferative activity of Tob is controlled by MAPK-mediated phosphorylation and Tob is involved in regulation of growth factor-induced cell cycle entry. Among the Tob family proteins, Tob and Tob2 proteins contain a putative nuclear localization signal (NLS) that is present near the amino-terminus. We showed that Tob is a nuclear protein by immunostaining. Nuclear localization of Tob is impaired by mutation of the putative NLS. Furthermore, Tob NLS sequence alone could drive nuclear import of the EGFP-LacZ fusion protein, indicating that the NLS of Tob is functional. Unlike wild-type Tob, Tob NLS mutant protein is poorly antiproliferative, suggesting that subcellular localization of Tob is related to the regulation of cell growth. Because the Caf1 protein that interacts with Tob family proteins is thought to be a transcription factor, Tob may regulate expression of genes responsible for cell cycle control, such as G1 cyclins. Since Caf1 interacts with CDKs, Tob may regulate CDK activities through Caf1. The relevance of the interaction between Tob family proteins and Caf1 remains to be addressed. To elucidate physiological function of Tob, we generated *tob*-deficient mice. *tob*-deficient mice had a greater bone mass resulting from increased number of osteoblasts. Orthotopic bone formation in response to BMP2 was elevated in *tob*-deficient mice. Overproduction of Tob repressed BMP2-induced, Smad-mediated transcriptional activation. Finally, Tob associated with receptor-regulated Smads (Smad1, 5, and 8), and colocalizes with these Smads in the nuclear bodies upon BMP2 stimulation. The results indicate that Tob negatively regulates osteoblast proliferation and differentiation by suppressing the activity of the receptor-regulated Smad proteins. Furthermore, aged mice lacking Tob expression were shown to develop a variety of tumors, such as hemangiosarcomas and hepatocarcinomas, at a very 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. Furthermore, embryonic fibroblasts deficient for *tob* displayed a marked increase in chromosomal aberration, including breakeage, translocation, and aneuploidy after the treatment of DEN. To circumvent functional redundancy between Tob and Tob-2, disruption of the *tob*-2 gene and subsequent generation of double knockouts (*tob*-/-*tob*-2-/-) are underway. Other studies to establish biological significance of the *tob* family members are widely in progress.

4. Characterization of ALK (Anaplastic Lymphoma Kinase) signaling in lymphoma malignancy and neural function

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Ki-1 lymphoma is a subtype of human malignant lymphomas characterized by the expression of CD30 (Ki-1 antigen) and an anaplastic large cell morphology. It is occasionally accompanied by a unique reciprocal chromosome translocation t(2;5)(p23;q35). We previously showed that a gene encoding the Ltk-like kinase, termed ALK, is located at the breakpoint and is responsible for the tumor formation as fusion protein p80(NPM-ALK). We also have cloned full-length proto-*alk* cDNA and analyzed its expression. ALK is expressed in the brain and spinal cord of em-

bryonic and neonatal mice, especially in specific regions of the nervous system such as the thalamus, mid brain, olfactory bulb, and ganglia. Expression of ALK in adult mice is low, suggesting its role in the development of the brain.

To further characterize ALK-mediated signaling, we isolated monoclonal antibodies against the extracellular domain of ALK. Some of the antibodies could stimulate the kinase activity of ALK and induce neurite outgrowth of ALK-expressing neuroblastoma cell line and suppress apoptosis upon serum withdrawal. This result suggested that ALK could mediate neurotrophic signals. Though natural ligand of ALK receptor is still not identified, this antibody-mediated approach could help to investigate the biological and biochemical activities of ALK.

We previouly reported that IRS-1 and SHC adaptor proteins associate with phosphorylated ALK and identified the binding site as NPXY-like phosphotyrosine-containing sequence. However, the oncogenic activity of p80(NPM-ALK) was not eliminated when the association of these two adaptor molecules were suppressed by a point mutation of the binding sites. This result suggested that there is another downstream factor which is essential for the oncogenicity of activated ALK kinase. We have been searching for its novel interacting molecules by yeast two-hybrid screening and identified SNT2(FRS2-beta) as the possible downstream factor of ALK-mediated signaling. SNT2 could interact with kinase-negative form of ALK and strongly phosphorylated by p80(NPM-ALK). To elucidate the function of SNT2 in the ALK-signaling, we have been identifying the precise binding properties of SNT2 with ALK.

5. Role of a kinesin-family protein Kid (kinesin-like DNA binding protein) in cell cycle regulation and chromosome segregation

Miho Ohsugi, Noriko Tokai-Nishizumi, Hirohisa Umemoto, and Tadashi Yamamoto

Microtubule-associated motor proteins are involved in spindle formation and chromosome movements in mitosis and meiosis. We previously cloned cDNAs for a gene termed *kid* that encodes a novel member of the kinesin family of proteins. The *kid* gene product is a 73-kDa protein and related to the Drosophila *nod* gene product, which is involved in chromosomal segregation during meiosis and mitosis. The microtubule-associated motor domain of Kid is present in its amino-terminal half and has the plus-end directed motor activity. Its carboxy-terminal half contains a helix-hairpin-helix DNA binding motif and is able to bind to specific DNA sequences similar to a part of α-satellite DNA. Overexpression of Kid, the motor domain, or the DNA binding domain abrogated chromosomal segregation. We also showed that Kid was phosphorylated at multiple sites during mitosis. We identified a Cdc2 kinase phosphorylation site on Kid and showed that Kid with a point mutation at the site failed to localize to chromosome, although the mutant Kid retains the ability to bind to mitotic chromosomes. These results suggest that Cdc2 kinase regulates the localization of Kid and the mutation on the phosphorylation site results in selective localization of Kid on microtubules by an unknown mechanism thereby preventing Kid from moving toward chromosome. Identification of other mitotic kinases that phosphorylate Kid and study of biological role of the phosphorylation are in progress. To further study the mechanisms for regulating localization, function and degradation of Kid, we are searching for Kid binding proteins by yeast two-hybrid system.

6. Characterization of protein-tyrosine phosphatases

Miho Ohsugi, Shin-ichiro Kina, and Tadashi Yamamoto

Protein-tyrosine phosphorylation is associated with various biological phenomena and is regulated by two types of enzymes with opposing activities, protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). Recent studies show that PTPs do not simply reverse the action of PTKs, but rather regulate signal transduction. Similar to PTKs, PTPs can be divided into two families, receptor PTPs and non-receptor PTPs, based on the presence or absence of extracellular and transmembrane domains.

We identified and cloned a gene termed *typ* that encodes a novel non-receptor PTP. Expression of *typ* was limited to the adult testis in both human and mouse. *in situ* hybridization and immunofluorescence studies revealed that the *typ* mRNA was specifically expressed in testicular germ cells that underwent meiosis (pachytene stage). Generation of *typ-/-* mice by gene targeting revealed that they were fertile despite of specific expression of *typ* during meiosis. Detailed analyses of the phenotype of *typ-/-* mice are in progress. To understand the role of Typ, we are searching for Typ binding proteins by yeast two-hybrid system.

In the course of studies of molecular mechanisms underlying GluR δ2-dependent motor learning, we identified protein-tyrosine phosphatase PTPMEG as a GluR δ2-interacting molecule. PTPMEG is a family member of band 4.1 domain-containing protein-tyrosine phosphatases and is expressed prominently in brain. PTPMEG bound to the putative C-terminal PDZ target sequence of GluR δ2 and GluR ε1 via its PDZ domain. Examination of the effect of PTPMEG on tyrosine phosphorylation of GluR ε1 unexpectedly revealed that PTPMEG enhanced Fyn-mediated tyrosine phosphorylation of GluR ε1 in its PTP activ-

ity-dependent manner. To know the physiological role of PTPMEG, especially in the tyrosine phosphorylation events of NMDA receptors, we are generating PTPMEG-/- mice.

7. Identification and analysis of LATS kinases, human homologs of a Drosophila tumor suppressor

Jiro Fujimoto, Yoshinori Abe, and Tadashi Yamamoto

We have previously identified genes encoding human serine/hreonine protein kinases with significant homology to a Drosophila tumor suppressor gene LATS. We named these two genes hLATS1 and hLATS2, and analyzed the function of the gene products. FISH analysis revealed that the hLATS1 and hLATS2 genes are localized to chromosome 6q and 13q, respectively. In these regions, loss of heterozygosity (LOH) is observed in various cancers including breast cancers, hepatocellular carcinomas, and renal carcinomas. We searched for mutations of the hLATS genes in various cancer cell lines and identified two cell lines that had alteration of the hLATS2 gene. One is a renal carcinoma cell line that had a large deletion in the hLATS2 gene, resulting in suppression of the hLATS2 protein expression. The other is a colon cancer cell line and had a point mutation in the coding region of the hLATS2 gene. This mutation caused the substitution of a single amino acid that was highly conserved among the protein kinases. In these two cell lines, phosphorylation of the proteins such as Bcl-2 and Raf-1, which are reported to be involved in Taxol (a microtubule inhibitor)-induced apoptosis, was not observed in the presence of Taxol. Ectopic expression of wild-type LATS-2 protein in these cells caused the phosphorylation of these proteins and enhanced the apoptiosis upon Taxol treatment. These results suggest that LATS-2 transmits an apoptotic signal caused by microtuble damage.

To further examine the role of LATS-2 in tumorigenesis and cell-cycle regulation, we have searched for molecules that interact with LATS-2 and identified some centrosome-localized proteins. Interestingly, our immunofluorescence study showed that LATS-2 was localized in centrosome area. Proper centrosome duplication is important for cell division and centrosomal abnormality is often observed in tumor cells. The role of LATS-2 in centrosome regulation is under investigation.

8. Segregation of TRAF6-mediated signaling pathways

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Signals emanating from the receptor for interleukin-1 (IL-1), lipopolysaccharide (LPS) or osteoclast differentiation factor/receptor activator of NFκB ligand (ODF/RANKL) stimulate transcription factors AP-1 through mitogen-activated protein kinase (MAPK) activation and NFκB through IκB kinase (IKK) activation. We have previously identified upstream activators of these kinases, tumor necrosis factor receptor-associated factor 6 (TRAF6). However, molecular mechanisms by which TRAF6 activates various downstream kinases remain to be elucidated. Moreover, results of earlier studies designed to investigate the role of TRAF zinc-binding regions, which are required for TRAF signal transduction, were often contradictory, most likely because TRAF proteins were grossly overexpressed in cell lines that endogenously express TRAF. This year, we identified functional domains of TRAF6 under physiological conditions established by appropriate expression of TRAF6 mutants in TRAF6-deficient cells. In IL-1 and LPS signaling pathways of mouse embryonic fibroblasts (MEFs), the RING finger and first zinc finger domains are dispensable for NFκB activation but are required for full activation of MAPK. However, IL-1 and LPS signals utilize distinct regions within the zinc finger domains of TRAF6 to activate NFκB. Furthermore, the RING finger domain is dispensable for differentiation of splenocytes to multinuclear osteoclasts, but is essential for osteoclast maturation. Thus, TRAF6 plays essential roles in both the differentiation and maturation of osteoclasts by activating various kinases via its multiple domains.

9. Physiological role of TRAF6

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We have previously identified TRAF6 as a putative signal transducer of CD40 by yeast two-hybrid system. However, it is not known whether TRAF6 plays pivotal roles in immune system *in vivo*, and whether it has additional functions yet to be identified. Thus, we have generated TRAF6-deficient mice

by homologous recombination-based gene knockout. TRAF6$^{-/-}$ mice exhibit severe osteopetrosis due to the lack of osteoclasts. *ex vivo* co-culture experiments revealed the inability of osteoclast precursor cells derived from TRAF6$^{-/-}$ mice to differentiate into functional osteoclasts in response to osteoclast differentiation factor (ODF), indicating that RANK, a receptor of ODF, utilizes TRAF6 for signal transduction. On the other hand, the number of sIgM^{+}B220^{+} B cells is markedly reduced in both spleen and bone marrow, whereas T cell development is normal in the absence of TRAF6. However, thymocytes from TRAF6 deficient mice do not proliferate in response to IL-1. IL-1 does not activate JNK and NFκB of TRAF6 deficient embryonic fibroblast, while TNFα does. These results indicate that TRAF6 is essential for IL-1 signaling. Furthermore, TRAF6$^{-/-}$ mice are defective in lymph-node, but Peyer's patch organogenesis. These results identify TRAF6 as an essential component of ODF signaling pathway, and also show that TRAF6 plays pivotal roles in immune and inflammatory systems *in vivo*.

10. Role of Dok family proteins in negative regulation of cell proliferation and transformation

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Dok was initially identified as a tyrosine-phosphorylated 62 kDa protein, p62, coimmunoprecipitated with p120 rasGAP. p62 was also known as a common substrate of wide spread protein-tyrosine kinases (PTKs) in a variety of signaling situations. We and others first purified p62 and cloned its cDNA. The sequence of the cDNA revealed that p62 has PH (pleckstrin homology) and PTB (phosphotyrosine binding) domains in its N-terminal half followed by many SH2 target sites. This suggests that p62 is a docking protein like IRS-1 (insulin receptor substrate-1), and it was designated Dok or p62^{dok}.

To know the physiological role of Dok, we established the *dok* gene-deficient mouse. Up to one year after birth, the dok KO mice showed no obvious lesion and were fertile. However, splenic B cells from Dok KO mice showed elevated MAP kinase activation upon B cell receptor (BCR) or BCR/FcγRIIB stimulation as compared to wild-type splenic B cells. In addition, those Dok-deficient B cells grew upon BCR and FcγRIIB cocrosslinking, which did not induce proliferation of wild-type splenic B cells. These data indicate that Dok is a negative regulator of MAP kinase and cell proliferation, at least, in BCR/FcγRIIB-mediated signaling events.

Because level of Dok tyrosine phosphorylation correlates with oncogenic potential of upstream oncoproteins with PTK activity, it was postulated that Dok promotes cell transformation. However, this does not fit well to our conclusion described above. Thus, we studied Dok function in Src-mediated transformation of NIH-3T3 cells. Dok co-expression with Src resulted in about 75% reduction of the transformation. However, Dok with dysfunctional mutation of its PTB did not inhibit Src transformation. Because this Dok mutant was barely tyrosine phosphorylated by Src, it is suggested that tyrosine phosphorylation of Dok is important for its inhibitory effect to cell transformation. Consistently, Dok did not inhibit Ras-mediated transformation of NIH-3T3 cells. Finally, a set of C-terminal deletion mutants of Dok revealed that 27 amino acid region of Dok (337-363) is important for the inhibitory function. Notably, this region contains rasGAP SH2 biding motif and the inhibitory effects of the Dok mutants correlated with their rasGAP binding activity so far tested.

From these data, we propose that Dok is a negative regulator of cell growth and transformation downstream of variety of PTKs. Given that Dok and its relative Dok-2 are heavily tyrosine phosphorylated in hematopoietic stem cells from patients of chronic myelogenous leukemia caused by Bcr-Abl oncoprotein having high PTK activity, Dok might protect us from the leukemia and/or its blast crisis.

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山梨裕司：BCR シグナルと rasGAP 結合蛋白質 p62dok．免疫 2000-20001（岸本忠三編）．中山書店．2000

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Department of Cancer Biology
Division of Cancer Cell Research

Cells are held in tissue through interaction with extracellular matrices (ECM). Such interaction is important not only to maintain tissue architecture but also to regulate various functions of the cells there. Regulation of cell growth, differentiation, apoptosis, migration and morphology is known to depend on the ECM environment. Malignant cancer cells have defect to interact with ECM and fail to maintain normal tissue architecture. For example, many cancer cells have defect in cell-cell adhesion and acquire ability to degrade surrounding ECM by the action of matrix-degrading enzymes such as matrix metalloproteinases (MMPs). These events collectively enables invasive growth of cancer cells and metastasis at the final stage. Our major goal is to understand the mechanism of cancer cell invasion especially focusing on the roles of the cell surface proteases.

1. Membrane-Type 1 Matrix Metalloproteinase Cleaves CD44 And Promotes Cell Migration

Masahiro Kajita, Yoshifumi Itoh, Tadashige Chiba, Hidetoshi Mori, Akiko Okada, Hiroaki Kinoh and Motoharu Seiki

Migratory cells including invasive tumor cells frequently express CD44, a major receptor for hyaluronan, and membrane-type 1 matrix metalloproteinase (MT1-MMP) that degrades pericellular ECM. In this study, we demonstrate that MT1-MMP acts as a processing enzyme for CD44H, releasing it into the medium as a soluble 70 kDa fragment which subsequently stimulates cell motility. Co-expression of MT1-MMP and mutant CD44H lacking the MT1-MMP-processing site could not promote cell migration, suggesting that the processing of CD44H by MT1-MMP is critical in the migratory stimulation. Moreover, expression of the mutant CD44H inhibited the cell migration promoted by CD44H and MT1-MMP in a dominant negative manner. The pancreatic tumor cell line, MIA PaCa-2, was found to shed the 70-kDa CD44H fragment in a MT1-MMP dependent manner. Expression of the mutant CD44H in the cells as well as MMP inhibitor treatment effectively inhibited the migration, suggesting that MIA PaCa-2 cells indeed use the CD44H and MT1-MMP as migratory devices. These findings revealed a novel interaction of the two molecules that have each been implicated in tumor cell migration and invasion.

2. CD44 is a molecular link that connects MT1-MMP to the actin cytoskelton

Hidetoshi Mori, Yoshifumi Itoh, Masahiro Kajita, Hiroshi Sato[1], Ikuo Yana and Motoharu Seiki: [1]Dept. Mol. Onc., Cancer Res. Inst., Kanazawa Univ.

Membrane-type 1 matrix metalloproteinase (MT1-MMP) promotes pericellular ECM degradation during tumor cell invasion. ECM is not only physical barrier but also important foothold during cell movement. Therefore cell surface localization of MT1-MMP has to be regulated coordinately during the process. Here, we demonstrate that MT1-MMP associates with F-actin indicating that localization of the enzyme is regulated accordingly to the reorganization of cytoskelton. Its association was dependent on the ecto-domain called hemopexin like domain

(PEX) rather than the cytoplasmic domain. We found that the PEX domain of MT1-MMP directly binds to the ecto-domain of standard form of CD44 (CD44H) that interacts with F-actin through the cytoplasmic domain. Overexpression of a mutant CD44H (CD44ΔCP) that lacks the cytoplasmic domain in the cells expressing MT1-MMP diminished the association of the enzyme to F-actin. These results indicate that CD44H is a molecular link that connects MT1-MMP to actin cytoskelton. Inhibition of the actin-association of MT1-MMP by CD44ΔCP disrupted localization of the enzyme to lamellipodium structure and also suppressed the Matrigel invasion activity of HT-1080 cells. These findings reveal a novel molecular interaction that is critical for cellular invasiveness in the tissue.

3. Membrane-type 6 matrix metalloproteinase (MT6-MMP, MMP-25) is the second glycosyl-phosphatidyl inositol (GPI)-anchored MMP

Shin-ichi Kojima[2], Yoshifumi Itoh, Shun-ichiro Matsumoto[2], Yasuhiko Masuho[1] and Motoharu Seiki:[2]Helix Research Institute, Inc.

Recently identified membrane-type 6 matrix metalloproteinase (MT6-MMP) has a hydophobic stretch of 24 amino acids at the very C-terminal end. This hydrophobisity pattern is similar to GPI-anchored MMP, MT4-MMP, and other GPI-anchored proteins. Thus, we tested the possibility that MT6-MMP is a GPI-anchored proteinase. Our result showed that [^{3}H]ethanolamine, that can be incorporated into the GPI unit, labeled the MT6-MMP as well as MT4-MMP. In addition, phosphatidylinositol-specific phospholipase C (PI-PLC) treatment released MT6-MMP from the surface of transfected cells. These results strongly indicate that MT6-MMP is in fact a GPI-anchored protein. Since two members of MT-MMPs are now assigned as GPI-anchored proteinase, MT-MMPs can be subgrouped into GPI-type and transmembrane-type.

4. Membrane-type 5 matrix metalloproteinase is expressed in differentiated neurons and regulates axonal growth

Hiromi Hayashita-Kinoh, Hiroaki Kinoh, Akiko Okada, Kiyoshi Komori, Yoshifumi Itoh, Tadashige Chiba, Masahiro Kajita, and Motoharu Seiki

Expression of membrane–type 5 matrix metalloproteinase (MT5-MMP) in the mouse brain was examined by immunostaining and in situ hybridization. MT5-MMP was expressed in peripheral and central nervous systems, especially in post-mitotic neurons. After birth, though expression of MT5-MMP was declined in the cerebrum and observed only in restricted areas, such as olfactory bulb and dentate gyrus, MT5-MMP expression was increased in the cerebellum. Coincide with MT5-MMP expression, gelatinolytic activity was detected by in situ zymography. Using mouse embryonal carcinoma P19 cells, we could demonstrate that expression of MT5-MMP was specifically induced in differentiated neurons. MT5-MMP was detected at the edge of growth corn of the isolated neurons from the dorsal root ganglia. Proteoglycans showed inhibitory effect on the neurite extension and it was abolished by MT5-MMP. These results indicate that MT5-MMP expressed in neuronal cells may play a role in axonal growth, and eventually regulates neural network formation.

5. Identification of *cis*-acting promoter elements that support expression of membrane-type 1 matrix metalloproteinase (MT1-MMP) in v-*src* transformed Madin-Darby Canine Kidney Cells

Hee-Jae Cha, Akiko Okada, Kyu-Won Kim, Hiroshi Sato, Motoharu Seiki

To analyze the mechanism by which MT1-MMP becomes expressed in cancer cells, we assessed the MT1-MMP promoter region for the presence of *cis*-acting promoter elements that support transcription in transformed cells. Our tumor model consisted of Madin-Darby canine kidney (MDCK) cells transformed by v-*src* (src4 cells). MT1-MMP mRNA was only faintly detected in parental cells but was strongly expressed in the src4 cells. In parallel, src4 cells invaded into collagen gels, whereas MDCK cells did not. A plasmid was constructed that contained a fragment consisting of -3000 to -99 nt from the upstream region of the MT1-MMP gene. The ability of this fragment to promote transcription was indicated by a luciferase gene acting as a reporter. When MDCK and src4 cells were transiently transfected with this plasmid, the promoter activity was 2.6 fold higher in src4 cells than in MDCK cells, indicating the presence of src-specific enhancer element(s) in the promoter fragment. To pinpoint the location of these elements, the promoter fragment was subjected to sequential truncation and the effect of this on expression was analyzed in both cell types. The region between –399 and –356 nt was found to contain the src4-specific enhancer element(s). Tandem Sp1 binding sites were also found to be essential in promoting transcription, but this was true for both src4 and MDCK cells, indicating that the Sp1 sites are not src4-specific enhancers. An Egr-1 site that partially overlaps with the Sp1 sites was found to cooperate with the src4-specific enhancer and to also contribute weakly to the basal promoter activity. The presence of transcription factors that bind to the src4-specific enhancer site was detected by mobility-shift assays

in src4 cell nuclear extracts but only weakly in MDCK extracts.

6. Analysis of mice lacking membrane-type matrix metalloproteinase 1 (MT1-MMP)

Akiko Okada, Hiromi Hayashita, Kiyoshi Komori, Noriko Ito, Motohatu Seiki, Kenji NAKAMURA[3], Kazutaka NAKAO[3], Motoya KATSUKI[3], Hiroshi SATO[4], Syun'ichiro TANIGUCHI[5] and Yasunori Okada[6]:[3]Ctr for Experimental Medicine, Inst. of Med. Sci., Univ. of Tokyo, [4]Dept. of Mol. Oncol., Cancer Res. Inst., Univ. of Kanazawa, [5]Res. Ctr for Aging and Adaptation, Univ. of Shinsyu, [6]Dept. of Pathology, Keio Univ.

Membrane-type matrix metalloproteinase 1 (MT1-MMP), which belongs to the matrix metalloproteinase family, localizes on cell surface, and mediates pericellular proteolysis of extracellular matrix components. MT1-MMP is expressed during embryogenesis, wound healing, and progression of tissue destractive diseases, such as arthritis and cancer. Thus, it is thought to be an important molecule for tissue remodeling.

We have generated MT1-MMP-deficient mice by targeted disruption of the gene. Homozygous mutants revealed severe skeletal abnormalities those are characteristic of an absence of intramembranous bone formation, osteopenia, brachycephaly, and dwarfism. The mice also showed fibrosis of soft tissue starting in 3-4 weeks after birth, which caused increased joint rigidity. Analysis of MT1-MMP gene expression during embryonal development using a LacZ reporter gene revealed strong expression at the sites of bone formation.

Comparison between MT1-MMP deficient mice and other gene knockout mice or mutant mice showing similar phenotypes is underway.

Publications

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Department of Cancer Biology
Division of Cancer Genomics

Our aim is to elucidate molecular mechanisms of infectious diseases including DNA tumor virus infection. We are currently investigating the subject using a synthetic polynucleotide microarray system that we have independently devised and ameliorated. Transcriptome analyses with the microarray system can provide fundamental and comprehensive data on the genetic background of infectious diseases.

1. A novel microarray system with synthetic polynucleotides

Shinya Watanabe

Viruses require host factors to proliferate within cells. As the host factors may restrict viral replication, identification and characterization of the host factors and their genes should play a important role in investigation of viral pathogenesis and eventual control of viral infection. In addition, to study the host factors involved in viral life cycles as a tool may contribute to inquire about complex biological phenomena within the cell. We obtained a novel methodology to study the host factors involved in viral pathogenesis from a view point different from the conventional paradigm in biology, which utilizes DNA microarrays and leads us to survey alteration of gene expression simultaneously and comprehensively. We have independently developed methods and ameliorated the cDNA microarray system originally established in Stanford using 80mers of synthetic polynucleotides and commercially available slide glasses. When compared the Stanford cDNA microarray, our synthetic polynucleotide microarrays showed equivalent qualities on the signal-noise ratio and the detection sensitivity of the transcript levels. The synthetic polynucleotide array system have dramatically reduced the total cost of making arrays and operational labor and opened a way to utilize thousands of arrays for collaborators who are interested in transcriptome analyses in a variety of biological phenomena.

2. Global modulation of host cell gene expression during human cytomegalovirus infection reveals viral strategies for conquest and survival

Shinya Watanabe,[1,2] Michael B. Eisen, [3]Patrick O. Brown, [4]Edward S. Mocarski[2] :[1] Division of Virology, The Institute of Medical Science, The University of Tokyo, [2]Department of Microbiology & Immunology, [3]Department of Genetics, [4]Department of Biochemistry and HHMI, Stanford University

Human cytomegalovirus is a medically important pathogen encoding over 200 genes whose functions control the outcome of infection. The level to which this virus dictates the host cell response during productive infection of human fibroblasts was revealed by microarray hybridization to over 8600 human genes. 805 genes whose transcript levels changed significantly at 4, 8, 24 or 48 hours postinfection were grouped based on common expression profiles and gene function. A dramatic immediate early-type host response that peaked within a few hours after infection was followed a pronounced suppression of almost all cell surface, secreted and immune-related transcripts in a pattern that dominated through intermediate to late times. Very few genes, encoding selected cytokines, proteasome components, and metabolic enzymes, exhibited a pattern of sustained increase throughout infection. Comparison of wild type, UV-inactivated, and ie1 (UL123) mutant virus provided evidence that transcriptional control of host gene expression may occur at multiple levels and may follow regulatory mechanisms previously

only known to regulate viral gene expression. Thus, cytomegalovirus orchestrates host cell gene expression in ways that reveal the optimal environment for successful infection of the host.

3. Patents

Shinya Watanabe

In this year, we have applied the following patents that concern the microarray technology including a novel method for simple and efficient solidification of DNA onto the surface of slide glasses coated with aminosilane molecules, a system for washing a narrow slit at the pin head of an arrayer to avoid cross contamination of samples within the slit, a method to remove excess fluid outside a pin of the arrayer efficiently and reproducibly to increase a yield rate of array making in a single round of operation, and a novel sensitive method for labeling with fluorescent dyes:

i) 出願年月日：2000 年 2 月 29 日
発明の名称：試料チップ作製用分注針の洗浄方法
願書番号：特願 2000-53109 号
ii) 出願年月日：2000 年 5 月 12 日
発明の名称:ポリヌクレオチドマイクロアレイの作製方法、作製装置ならびにポリヌクレオチドマイクロアレイ
願書番号：特願 2000-139926 号
iii) 出願年月日：2000 年 5 月 19 日
発明の名称：試料チップ作製方法
願書番号：特願 2000-147606 号
iv) 出願年月日：2000 年 8 月 24 日
発明の名称：核酸標識方法および核酸標識用キット
願書番号：特願 2000-254172 号

Department of Cancer Biology
Division of Pathology

Division of Pathology has a unique research strategy to work directly on the pathogenic mechanisms and diagnosis of various human diseases through the analyses of pathological specimens. Elucidation of the primary events hidden in such specimens is the priority field of pathology. Our current targets include human lymphoid diseases and viral infections involving lymphoid tissues/cells.
(S. Mori's research group is curtailed this year again because of the burden of administrative works).

I. Malignant lymphomas

1. Maintenance of human malignant lymphomas and establishment of new lymphoma lines

Izuru Maekawa, SCID mouse team

Fresh pathology specimens are prerequisite for the molecular analysis of various diseases. Since our major interest resides in the pathogenesis of human lymphomas, we are continuing to maintain resected human lymphomas in mice with severe combined immune deficiency (SCID). We have transplanted over 260 lymphoma tissues in these 9 years and could maintain 36 cases successively. They consisted of 28 B-MLs, 5 T-MLs and 3 null-cell MLs. A T-cell lymphoma cell line expressing Epstein-Barr Virus (EBV) latent gene products is now under investigation for the secondary event in its tumorigenesis. A part of them have been submitted for the study of their pathogenesis and some of our results have been recognized widely, including the elucidation of the molecular mechanism of anaplastic large cell lymphoma, pyothorax associated lymphoma and primary effusion lymphoma.

2. Molecular pathogenesis of human MLs

a. Characterization of a novel sno-RNA gene translocating to the bcl-6 gene

Ritsuko Tanaka, Hitoshi Satoh, Toshiki Watanabe, Masatsugu Moriyama[1], Yoshikazu Nakamura[2] and Shigeo Mori:[1]Faculty of Medicine, Tottori University and [2]Division of Tumor Biology

The bcl-6 gene is known to be a promiscuous translocation gene, translocating to various different genes. Through the analysis of these chromosomal translocation partners, we have identified a novel partner gene, termed as U50HG, and found it to be a new member of sno-RNA that will work on the regulation of ribosomal protein synthesis (Genes to Cells, 2000,). This is the first report of snoRNA that is associated with human disease(s). We identified the mouse homologue of U50HG and preliminary characterization of mouse U50HG gene revealed a unique repetitive organization. Possible pathogenic roles of U50HG gene aberration are now under study.

3. Multicentic Castleman's disease; human herpes virus type 8 (HHV8) -associated diseases

Shigeo Mori, Harutaka Katano[1] andTetsuji Suda: [1]National Institute of Infectious Disease

HHV8 is a new member of human herpes virus that is known to associate with Kaposi's sarcoma, primary effusion lymphoma and Castleman's tumor. We established an in vitro cell line from a case of primary effusion lymphoma carrying HHV8. With the use of expression cloning, we isolated HHV8 transcripts and found the ORF59 to be the major antigenic proteins. We established an ELISA system for the detection of anti-ORF59 antibody by using its

recombinant protein and screened over 2500 Japanese patients, revealing the positive rate of around 1.5% (H Katano et al., J Virol, 2000). The associations of antibody-positivity with certain diseases are now under investigation. We also developed rabbit anti-ORF 59 antibody with the use of recombinant ORF59 protein and used it in the detection of virus-infected cells in pathology slides of various human diseases. We could dissect the so-called Multicentric Castleman's tumor into HHV8-positive and -negative cases.

4. Hodgkin's disease -Overexpression of CD30 drives NF-κB activation in Hodgkin-Reedsternberg cells

Horie R[1], Watanabe Ta, Morishita Y, Ito K[2], Nagai M, Aizawa S, Ishida T, Mori S and Watanabe T: [1]Kitasato University, School of Medicine and [2]Toho University School of Medicine

Hodgkin's disease (HD) is a malignant lymphoma characterized by the presence of mononucleated Hodgkin cells and multinucleated Reed-Sternberg cells (H-RS cells) in a background of reactive cells comprising lymphocytes, eosinophils, plasma cells, histiocytic cells and fibroblasts. However, the biological mechanisms of its growth, regulation, and death remained unsettled. H-RS cells are characterized by overexpression of CD30, a member of tumor necrosis factor receptor (TNFR) superfamily. Recently, constitutively activated Nuclear factor-κB (NF-κB) (p50/p65) was reported to be a unique and common characteristics of H-RS cells, which prevent these cells from undergoing apoptosis and triggering proliferation. NF-κB is a pleiotropic transcription factor that interacts with the upstream regulatory regions of numerous genes. Ligation of CD30 by its ligand or an antibody triggers signals to activates NF-κB through recruitment of TNFR associated factor (TRAF) 2 and 5. We found that, through self-association, CD30 overexpression constitutively recruits TRAF2 and 5, and drives the NF-κB activation in H-RS cells. These findings linked two major characteristics of H-RS cells, CD30 overexpression and constitutive activation of NF-κB, and provided new insights into the mechanisms by which H-RS cells grow and express immuno-regulatory cytokines that characterize clinical features of Hodgkin's disease. To find out a cytospecific gene therapy based on the mechanisms of tumor cell growth, we examined adenovirus vector as a tool to efficiently transduce an effector gene into H-RS cells. We found that adenovirus vector can infect H-RS cell lines very efficiently, which contrasts to the relative resistance to adenovirus infection generally found in lymphoid and hematopoietic cells. We demonstrated that adenovirus-mediated transduction of a mutant CD30 that lacks the cytoplasmic region or dominant negative IκBα induced apoptosis of H-RS cell-derived cell lines. These findings provide a basis for a new strategy of cytospecific gene therapy of HD. (Horie et al., submitted).

5. Development of adenovirus mediated gene therapy for lymphoid malignancies

Watanabe Ta, Horie R[1], Koga S[2], Ishida T, Yamaguchi K[3] and Watanabe T:[1]Kitasato University, School of Medicine and [2]Amakusa Chuo Sogo Hospital

Although adenovirus vectors has many advantages in efficient gene transfer, they have not been used for hematopoietic and lymphoid cells because of low transduction efficiency. As described above, we found that adenovirus vectors infect some of the neoplastic cells of Hodgkin/Reed Sternberg cells, and also HTLV-1-infected T cell lines as well as ATL cells. Furthermore, transduction of a dominant negative form of IkBα into these cells induced apoptosis. To examine the feasibility to apply adenovirus vectors to clinical situation, characterization of adenovirus infection to *ex vivo* tumor cells and screening of effector molecules are now underway.

6. Molecular analysis of 1p36 chromosome translocation found in malignant lymphoma

Hitoshi Satoh

We have arrayed 21 cosmid and five P1 phage clones along with the short arm of chromosome 1 from telomere to the centromere direction by pair-wise comparison experiment using multi-color FISH technique. The resulting order is as follows: 1pter-D1S1002 (cYS142)-D1S1053 (cYS1467)-D1Z2-D1S1085 (cYS1138)-D1S1013 (cYS1299)-D1S1032 (cYS1384)-D1S1010 (cYS1296) / D1S1047 (cYS1429)-D1S96-D1S989 (cYS1287) / D1S1131 (cYS1234)-NPPA-D1S975 (cYS1173)-D1S968 (cYS1121)-D1S1062 (cYS73)-D1S1092 (cYS1148)-D1S1130 (cYS1232) / D1S1028 (cYS1363)-D1S967 (cYS1120)-PAX7-D1S1111 (cYS1180)-D1S1073 (cYS191)-D1S1037 (cYS1406)-D1S1112 (cYS1181)-D1S1040 (cYS144)-D1S112-cen. In reference to this FISH physical ordering map, we have determined the chromosomal breakpoints within the 1p36 region in some lymphoma cell lines. Three of *in vitro* established and two of *in vivo* cell lines maintained in SCID mouse were assessed. As a result, it is revealed that all of the five cell lines had different breakpoints at cytogenetic level. However, the translocation breakpoint of HMS cell line, an *in vivo* cell line maintained in SCID mouse, was mapped between markers D1S96 and D1S989, corresponding to one of the telomeric break end detected in BALL-1, a B-cell line derived from acute lymphoblastic leukemia patient. In order to clarify there is a common causation in developing these lymphomas, screening of the gene locate on the breakpoint is now on going by probing YAC clones.

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

1. Multistep leukemogenesis of adult T-cell leukemia (ATL) -Possible involvement of PKCβII activation in the progression of HTLV-1 Tax-immortalized T-cells

Shionoiri C, Fukumoto R, Koiwa T, Matsuoka M[1], Yamaguchi K[2], Watanabe T. [1]Institute for Virus Research, Kyoto University, [2]School of Medicine, Kumamoto University

Adult T cell leukemia (ATL) develops more than 50 years of the latency period. The age distribution of ATL onset statistically fits in with the Weibull's multistep carcinogenesis model and five independent leukemogenic events are involved in ATL development. HTLV-1 infection of T cells immortalizes the infected cells by the action of viral transcriptional regulator Tax. However, HTLV-1 Tax-immortalized T cells differ significantly from *in vivo* transformed ATL cells in terms of biochemical and biological phenotypes, suggesting that more steps are involved in progression to ATL cells. Characterization of the gene expression profiles of ATL cells by the differential display analysis (DDA) resulted in identification of PKCβII overexpression as one of the characteristics that distinguishes *in vivo* transformed cells from *in vitro* immortalized ones. In addition to overexpression of the mRNA and protein, constitutive activation of PKCβII was demonstrated in cell lines derived from ATL clones but not in those immortalized by *in vitro* infection or introduction of Tax. Transduction of a constitutive active form of PKCβII by a retrovirus vector into Tax1A cells resulted in a growth advantage and resistance to apoptosis induced by IL-2 withdrawal (R Fukumoto et al., in preparation). Since GSK-3β-β-catenin/TCF pathway is reported to be a target of PKCβII in colon carcinogenesis, we examined whether GSK-3β-β-catenin/TCF pathway is also activated by PKCβII in ATL leukemogenesis. We found accumulation of β-catenin in ATL cells, ATL derived cell lines and Tax-immortalized cell lines transduced with a constitutively active PKCβII and phosphorylation GSK-3β in some of these cells. Thus, it was suggested that the GSK-3β-β-catenin/TCF pathway is involved in ATL leukemogenesis.

2. Involvement of the IL-2/IL-2R system activation by the parasite antigen in the polyclonal expansion of CD4+25+ HTLV-1-infected T-cells in dual carriers of HTLV-1 and *S. stercoralis*

Satoh M[1], Matsuoka M[2], Yamaguchi K[3], Hori E[4], Kamihira S[5], Watanabe T:[1,4]Saitama Medical School, Department of Medicine,[2]Institute for Virus Research, Kyoto University, [3]School of Medicine, Kumamoto University, [5]School of Medicine, Nagasaki University

The intermediate state of HTLV-1 infection often found in individuals dually infected with *Strongyloides stercoralis* (*S. stercoralis*) and HTLV-1, is assumed to be a preleukemic state of adult T-cell leukemia (ATL). To investigate the effects of *S. stercoralis* superinfection on the natural history of HTLV-1 infection, we characterized the peripheral blood of these individuals in Okinawa, Japan, an endemic area for both HTLV-1 and *S. stercoralis*. The results demonstrated that dually infected individuals showed a significantly higher provirus load and an increase in CD4+25+ T cell population, which showed a significant, positive correlation with each other. The increase resulted from polyclonal expansion of HTLV-1-infected cells demonstrated by inverse-long PCR analysis of the integration sites. *S. stercoralis* antigen activated the IL-2 promoter in reporter gene assays, induced production of IL-2 by PBMC *in vitro*, and supported growth of IL-2 dependent cell lines immortalized by HTLV-1 infection or transduction of Tax. Taken collectively, these results indicate that *S.stercoralis* infection induces polyclonal expansion of HTLV-1-infected cells through activation of IL-2/ IL-2R system in dually infected carriers, which is likely a precipitating factor for ATL and inflammatory diseases. (M Sato et al., submitted)

3. HTLV-1 uveitis and immune privilege of the eye

Nakazawa-Ishikawa N, Sugita S[1], Mochizuki M[1], Sagawa K[2] and Watanabe T:[1]Tokyo Medical and Dental University, [2]School of Medicine, Kurume University

The eye is a classic example of the immune privileged organ that is maintained by various proteins expressed on the cell surface or as soluble factors (M Mochizuki et al., Cornea, 2000). We have revealed suppression of cytokine gene expression by the infiltrating cells in the affected eyes of HTLV-1 uveitis patients, and found that soluble Fas ligand present in the eye (S Sugita et al., Brit J Opthalomol, 2000) is one of the suppressive factors in the aqueous humor. We showed soluble Fas ligand at the very low concentration can mediate signals that suppress IFN-g expression through Daxx-ASK-1 pathway (N Nakazawa-Ishikawa et al., submitted). The molecular mechanisms of differential signal transduction leading to apoptosis or suppression of cytokine gene expression are now under study.

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

Transcriptional repression of heterologous genetic elements such as proviruses is often observed concomitantly with their integration and packaging into chromatin in the host cell genome. Deacetylation of nucleosomal histones and methylation of cytosines, particularly in the dinucleotide 5' CpG negatively influence transcriptional activity in cis. Therefore, we have been investigating the regulatory roles of CpG methylation in latency induction and reactivation of human retroviruses, HIV and HTLV-1.

a. Reactivation of latent HIV is associated with CpG demethylation

Ishida T, Koiwa T, Nagai M, Iwakura Y[1] and Watanabe T:[1]Division of Cell Biology, Center for Experimental Medicine

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

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

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HTLV-1 causes leukemia and inflammatory diseases after several decades of latency period. *In vivo*, viral gene expression is detected infrequently in HTLV-1-infected T cells whether they are transformed or not. CpG methylation of HTLV-1 LTR *in vitro* by SssI-methylase profoundly suppressed the basal activity and response to activating stimuli of LTR. In HTLV-1-infected cell lines, the levels of viral gene expression were inversely correlated with those of CpG methylation of the LTR studied by the bisulfite genomic sequencing method. In latently infected T cell lines, LTR was heavily methylated and viral gene expression was reactivated by 5-AzaC treatment after two days. CpG methylation levels were also high in PBMCs from asymptomatic carriers. The proviruses integrated in the leukemic cells of adult T-cell leukemia (ATL) were grouped into two depending on the levels of CpG methylation: one group with high density methylation and the other with almost complete demethylation. Structural analysis of the integrated proviruses revealed that the former has a complete form provirus whereas the latter has 5' defective one. These results suggest important roles of CpG methylation in the latency of HTLV-1 and possible requirement to inactivate HTLV-1 for malignant transformation of the virus-infected cells *in vivo*. (T Koiwa et al., in preparation)

III. Miscellaneous

1. Molecular study of tumor metastasis-associated genes

Endoh H, Kanno T

The mts1 gene (S100A4) was a mouse gene highly expressed in tumor with high metastatic potential. The molecular and cellular function of this gene is under investigation to know its role in metastasis. We have identified a gene, MetAP2, that interact with S100A4 by two-hybrid method. MetAP2 is an inhibitor of translation initiation by inhibiting phosphorylation of α sub-unit of eIF2 and also a target of anti-angiogenesis drug TNP-470. To study its expression in various tissues, we prepared polyclonal antibody against MetAP2 using a GST-fusion protein and a peptide. Immunohistochemical screening revealed high expression in germinal center B cells. Subsequent screening of lymph node biopsy samples obtained from patients with lymphoma showed frequent expression in lymphoma cells of B cell origin and Hodgkin's lymphoma. Biological function of MetAP2 in lymphocytes of B cell lineage is now under study. (T Kanno et al., in preparation).

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Our interest is to elucidate the regulation of gene expression in relation to development and tumor formation. We are currently focusing on the function of a tumor suppressor gene, WT1 which is involved in Wilms' tumor, leukemia and sex determination.

WT1 gene was originally identified as a tumor suppressor gene for Wilms' tumor, a pediatric kidney tumor. Several lines of evidence show that WT1 is required for development of kidney and reproductive organs. Surprisingly, recent progress demonstrated that WT1 is overexpressed in leukemic cells and thought to function as an oncogene in leukemogenesis. However, molecular mechanism of WT1 remains to be elucidated. We are trying to answer the questions, why WT1 exerts such two distinct functions.

1. Analysis of "oncogenic" function of WT1 in leukemia

Kentaro Semba and Yoshimasa Sakamoto

In contrast to the previous reports which showed that overexpression of WT1 inhibited growth of a variety of cultured cells, WT1 is required for growth of leukemic cells. To analyze the "oncogenic" function of WT1 in those cells, we isolated cDNAs encoding WT1-associated proteins from human erythroleukemia cell line, K562. 1.6 x 10^7 clones were screened and 467 clones have been shown to bind to WT1 specifically. 263 of 467 clones are human ubiquitin conjugating enzyme 9 as previously reported. In addition, four small nuclear ribonucleoprotein particles genes have been identified. Furthermore, eight clones were likely to encode transcription factors. Among them, we focused on GATA family proteins. GATA family plays a critical role in differentiation of hematopoietic cells. We showed i) WT1 binds to GATA-1 and GATA-2 *in vitro* and in cultured cells, ii) WT1 and GATA-1 synergistically activates transcription from a promoter which contains GATA binding sequence, iii) DNA pull down assay showed that WT1 was recruited on GATA-binding sequence in a GATA-dependent manner, iv) WT1 competed with FOG-1, a cofactor of GATA-1 in luciferase reporter assay. In this assay, FOG-1 repressed GATA-mediated transcription, whereas, WT1 activated GATA-mediated transcription in a dose-dependent manner even in the presence of FOG-1, v) WT1 formed ternary complex with GATA-1 and FOG-1 in a GATA-dependent manner.

Our current hypothesis is that WT1 may perturb the differentiation process of hematopoietic cells by inhibition of FOG-1 and subsequently constitutive activation of GATA-mediated transcription. We are currently testing this hypothesis.

2. Analysis of WT1 function in sex determination

Kentaro Semba and Yumiko Matsuzawa-Watanabe

In humans, as in other mammals, sex determination is controlled by a dominant switch termed TDF for Testis Determining Factor. The SRY gene is thought to be the TDF, which encodes a transcription factor with one HMG box as a DNA binding domain. Mutations in the SRY gene have been identified in 15% cases of XY sex reversal in humans. Introduction of mouse SRY gene (Sry) into XX female mice induced testis differentiation and subsequent male development. However, little is known about mechanism of transcriptional regulation by SRY. WT1 mutations have frequently been observed in Denys-Drash syndrome (DDS) patients with urogenital malformation. During analysis of WT1-associated

proteins, we found that WT1 bound to several types of transcription factors. One of them was Sox30, which encodes a novel transcription factor with one HMG box. Further analysis showed that WT1 bound to its HMG box. This observation prompted us to analyze interaction between WT1 and SRY. To date, we showed i) WT1 binds to SRY *in vitro* and in cultured cells, ii) this binding is mediated by the zinc finger domain of WT1 and the HMG box of SRY, iii) WT1 and SRY synergistically activates transcription from a promoter which contains SRY binding sequence, while WT1 mutants found in DDS did not show this activity, iv) the SRY gene itself is a candidate for target genes which are regulated by WT1 and SRY synergy, v) WT1 is recruited on SRY-binding sequence in a SRY-dependent manner, while recruitment of DDS mutants is significantly reduced, vi) one SRY mutant (L163ter) found in a Japanese family with complete gonadal dysgenesis had reduced affinity with WT1.

We still need to examine the WT1-SRY interaction in biological systems. Recently we were able to isolate SRY mutants which lost the binding affinity with WT1 by yeast two hybrid screening. Thus we hope we may be able to examine the biological significance of WT1-SRY interaction by introduction of such SRY mutants into female mice.

3. Characterization of a novel Sox family gene, Sox30

Kentaro Semba, Gensuke Takayama, Emiko Osaki, Yukio Nishina[1], Johji Inazawa[2], Jun-ichi Hata[3], Iwao Fukui[4] and Haruo Sugano[4]:[1]Department of Biology, Graduate School of Integrated Science and Faculty of Science, Yokohama City University, [2]Department of Molecular Cytogenetics, Division of Genetics, Medical Research Institute, Tokyo Medical and Dental University, [3]Keio Univ., School of Medicine and [4]Japanese Foundation for Cancer Research

During searching for WT1-associated proteins, we cloned Sox30 cDNA which encodes a novel transcription factor with a SRY-like HMG box (Sox). Sequence analysis of Sox30 identified a novel subclass H of Sox family transcription factors. Sox30 protein showed sequence-specific binding to ACAAT motif and activated transcription from synthetic promoter containing this binding motif. Immunohistochemical and northern blot analyses revealed that Sox30 was highly expressed in germ cells. Interestingly, RT-PCR analysis showed that aberrant Sox30 transcripts were observed in two human germ cell tumor cell lines and 17 out of 18 tumor specimens. Sequence analysis of those transcripts and corresponding genomic clones revealed that aberrant splicing occurred in tumor cells but not in normal testis, which resulted in missing the DNA binding domain of Sox30. Furthermore aberrant splicing was observed not only in primary lesion but also in metastatic lymphnodes. Those mutants did not show any activation of transcription. It still remains to be tested whether reduced expression of Sox30 is involved in development of germ cell tumors, nevertheless, our finding may be served as molecular marker of male germ cell tumors.

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Department of Cancer Biology
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Our major projects are to clarify (1) signal transduction systems in cell growth, reorganization of cytoskeleton and morphogenesis (2) roles of inositolphospholipids as biomodulators. Currently we are focusing on the clarification of mechanisms in cortical actin reorganization and cell movement, and the roles of inositolphospholipid-mediated signallings in nuclear events.

1. IRSp53 is an essential intermediate between Rac and WAVE in the regulation of membrane ruffling

Hiroaki Miki, Hideki Yamaguchi, Shiro Suetsugu, and Tadaomi Takenawa

Neural Wiskott-Aldrich syndrome protein (N-WASP) functions in several intracellular events including filopodium formation, vesicle transport and movement of Shigella frexneri and vaccinia virus, by stimulating rapid actin polymerization through the Arp2/3 complex. N-WASP is regulated by the direct binding of Cdc42 which exposes the domain in N-WASP that activates the Arp2/3 complex. A WASP-related protein, WAVE/Scar, functions in Rac-induced membrane ruffling; however, Rac does not bind directly to WAVE, raising the question of how WAVE is regulated by Rac. We demonstrate that IRSp53, a substrate for insulin receptor with unknown function, is the 'missing link' between Rac and WAVE. Activated Rac binds to the amino terminus of IRSp53, and carboxy-terminal Src-homology-3 domain of IRSp53 binds to WAVE to form a trimolecular complex. From studies of ectopic expression, we found that IRSp53 is essential for Rac to induce membrane ruffling, probably because it recruits WAVE, which stimulates actin polymerization mediated by the Arp2/3 complex.

2. A novel N-WASP binding protein, WISH induced Arp2/3 complex activation independent of Cdc42

Maiko Fukuoka, Shiro Suetsugu, Hiroaki Miki, Kiyoko Fukami, Takeshi Endo[1] and Tadaomi Takenawa [1]Department of Biology, Faculty of Science

We found a novel adaptor protein, with a SH3 domain, SH3 binding proline-rich sequences and a leucine zipper-like motif and named it WASP Interacting SH3 protein(WISH). WISH is predominantly expressed in neural tissues and testis. It bound Ash/Grb2 through its proline-rich regions and N-WASP through its SH3 domain. WISH strongly enhanced N-WASP-induced Arp2/3 complex activation independent of Cdc42 *in vitro*, resulting in a rapid actin polymerization. Furthermore, co-expression of WISH and N-WASP induced marked formation of microspikes in Cos 7 cells even in the absence of stimuli. H208D mutant N-WASP which can not bind to Cdc42 still induced microspike formation when WISH was co-expressed. We also examined the contribution of WISH to a rapid actin polymerization induced by brain extract *in vitro*. Arp2/3 complex was essential for brain extracts-induced rapid actin polymerization. The addition of WISH to the extracts increased actin polymerization as Cdc42 did. But, WISH unexpectedly could activate actin polymerization even in the N-WASP-depleted extracts. It is suggested that WISH activates Arp2/3 complex by N-WASP-dependent and –independent pathways without Cdc42, resulting in a rapid actin polymerization required for microspike formation.

3. Two tandem verprolin homology domains are necessary for a strong activation of Arp2/3 complex-induced actin polymerization and induction of microspike formation by N-WASP

Hideki Yamaguchi, Hiroaki Miki, Shiro Suetsugu, Le Ma[2], Mark Kirschner[2] and Tadaomi Takenawa: [2]Department of Cell Biology, Harvard Medical School

All WASP family proteins share a common C terminus that consists of the verprolin homology domain (V), cofili homology domain (C), and acidic region (A), through which they activate Arp2/3 complex-induced actin polymerization. In this study, we characterized the Arp2/3 complex-mediated actin polymerization activity of VCA fragments of all of the WASP family proteins: WASP, N-WASP, WAVE1, WAVE2, and WAVE3. All of the V fragments stimulated the nucleating activity of Arp2/3 complex. Among them, N-WASP VCA, which possesses two tandem V motifs, had a more potent activity than other VCA proteins. The chimeric protein experiments revealed that the V motif was more important to the activation potency than the CA region; two V motifs were required for full activity of N-WASP. COS7 cells overexpressing N-WASP form microspikes in response to epidermal growth factor. However, when a chimeric protein in which the VCA region of N-WASP is replaced with WAVE1 VCA was overexpressed, microspike formation was suppressed. Interestingly, when the N-WASP VCA region was replaced with WAVE1 VCA, having two V motifs, this chimeric protein could induce microspike formation. These results indicate that strong activation of Arp2/3 complex by N-WASP is mainly caused by its two tandem V motifs, which are essential for actin microspike formation.

4. Distinct roles of profilin in cell morphological changes: microspikes, membrane ruffles, stress fibers, and cytokinesis

Shiro Suetsugu, Hiroaki Miki and Tadaomi Takenawa

We report the functional importance of profilin in various actin-mediated morphological changes using H119E mutant profilin I, which is deficient only in actin binding. In the case of actin-protrusive structures from the plasma membrane, H119E-profilin was shown to suppress the formation of Cdc42-induced actin microspikes and Rac-induced membrane ruffles. Conversely, Rho-induced stress fiber formation seemed to occur independently of H119E-profilin introduction. Furthermore, H119E-profilin blocked cleavage furrow ingression and subsequent adhesion to the substratum during cell division, a process in which actin plays indispensable roles.

5. Autophosphorylation of type I phosphatidylinositol phosphate kinase regulates its lipid kinase activity

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Phosphatidylinositol phosphate kinases (PIPKs) have important roles in the production of various phosphoinositides. For type I PIP5Ks (PIP5KI), a broad substrate specificity is known. They phosphorylate phosphatidylinositol 4-phosphate most effectively but also phosphorylate phosphatidylinositol (PI), phosphatidylinositol 3-phosphate, and phosphatidylinositol (3,4)-bisphosphate (PI(3, 4)P(2)), resulting in the production of phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P(2)), phosphatidylinositol 3-phosphate, phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P(2)), phosphatidylinositol (3,5)-bisphosphate (PI(3,5)P(2)), and phosphatidylinositol (3,4,5)-trisphosphate. We show that PIP5KIs have also protein kinase activities. When each isozyme of PIP5KI (PIP5KIalpha, -beta and -gamma) was subjected to *in vitro* kinase assay, autophosphorylation occurred. The lipid kinase-negative mutant of PIP5KIalpha (K138A) lost the protein kinase activity, suggesting the same catalytic mechanism for the lipid and th protein kinase activities. PIP5KIbeta expressed in Escherichia coli also retains this protein kinase activity, thus confirming that no co-immunoprecipitated protein kinase is involved. In addition, the autophosphorylation of PIP5KI markedly enhanced by the addition of PI. No other phosphoinositides such as phosphatidylinositol phosphat e phosphatidylinositol bisphosphate, or phosphatidylinositol trisphosphate have such an effect. We also found that the PI-dependent autophosphorylation strongly suppresses the lipid kinase activity of PIP5KI. The lipid kinase activity of PIP5KI was decreased to one-tenth upon PI-dependent autophosphorylation. All these results indicate that the lipid kinase activity of PIP5KI that acts predominantly for PI(4,5)P(2) synthesis is regulated by PI-dependent autophosphorylation *in vivo*.

6. Phosphatidylinositol 4-phosphate 5-kinase type I is regulated through phosphorylation response by extracellular stimuli

Sun-Joo Park, Toshiki Itoh and Tadaomi Takenawa

Phosphatidylinositol 4-phosphate 5-kinase (PIPK) catalyzes a final step in the synthesis of phosphatidylinositol 4,5-bisphosphate (PIP2), a lipid signaling molecular. Strict regulation of PIPK activity is thought to be essential in intact cells. Here we show that type I enzymes of PIPK (PIPKI) are phosphorylated by cyclic AMP-dependent protein kinase (PKA), and phosphorylation of PIPKI suppresses its activity. Serine 214 was found to be a major phospho-

rylation site of PIPK type Ialpha (PIPKIalpha) that is catalyzed by PKA. In contrast, lysophosphatidic acid (LPA)-induced protein kinase C (PKC) activation increased PIPKIalpha activity. Activation of PIPKIalpha was induced by dephosphorylation, which was catalyzed by an okadaic acid (OA)-sensitive phosphatase, protein phosphatase 1 (PP1). *In vitro* dephosphorylation of PIPKIalpha with PP1 increased PIPK activity, indicating that PP1 plays a role in LPA-induced dephosphorylation of PIPKIalpha. These results strongly suggest that activity of PIPKIalpha in NIH 3T3 cells is regulated by the reversible balance between PKA-dependent phosphorylation and PP1-dependent dephosphorylation.

7. Identification and characterization of a novel inositol polyphosphate 5-phosphatase

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We have identified a cDNA encoding a novel inositol polyphosphate 5-phosphatase. It contains two highly conserved catalytic motifs for 5-phosphatase, has a molecular mass of 51 kDa, and is ubiquitously expressed and especially abundant in skeletal muscle, heart, and kidney. We designated this 5-phosphatase as SKIP (Skeletal muscle and Kidney enriched Inositol Phosphatase). SKIP is a simple 5-phosphatase with no other motifs. Baculovirus-expressed recombinant SKIP protein exhibited 5-phosphatase activities toward inositol 1,4,5-trisphosphate, inositol 1,3,4,5-tetrakisphosphate, phosphatidylinositol (PtdIns) 4,5-bisphosphate, and PtdIns 3,4, 5-trisphosphate but has 6-fold more substrate specificity for PtdIns 4,5-bisphosphate (K(m) = 180 microM) than for inositol 1,4, 5-trisphosphate (K(m) = 1.15 mM). The ectopic expression of SKIP protein in COS-7 cells and immunostaining of neuroblastoma N1E-115 cells revealed that SKIP is expressed in cytosol and that loss of actin stress fibers occurs where the SKIP protein is concentrated. These results imply that SKIP plays a negative role in regulating the actin cytoskeleton through hydrolyzing PtdIns 4,5-bisphosphate.

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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 establishment of new methods for glycobiology.

1. Carbohydrate recognition mechanism involved in sperm-egg interaction

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Mammalian eggs are surrounded by an extracellular matrix called the zona pellucida (ZP) which consists of a few glycoproteins. The ZP plays important roles in sperm-egg binding, induction of sperm acrosome reaction, and block to polyspermy. It has so far been suggested that the mechanism recognizing glycans on the ZP is working in the process of sperm-egg interaction, it is still unveiled. Therefore, recognition of defined carbohydrate structures by boar sperm was studied on the basis of oligosaccharide structures of porcine zona pellucida glycoproteins so far elucidated. Boar sperm abundantly adhered to Sepharose beads coupled with a glycoprotein containing sialyl oligosaccharide chains or asialo-oligosaccharide chains, but not at all to Sepharose beads coupled with a glycoprotein containing agalacto-oligosaccharide chains. The sperm also adhered to avidin-Sepharose beads coupled with Le^x oligosaccharide probe. These adhesive activities were divalent cation-independent. Inhibition study of sperm adhesion to the glycoprotein-beads by soluble ligands demonstrated that boar sperm express at least two kinds of carbohydrate recognition molecules, one recognizing both sialyl and non-sialyl *N*-acetyllactosamines but not the Le^x structure and the other recognizing the Le^x structure but not N-acetyllactosamines. Similar results were also obtained by inhibition of sperm binding to the zona pellucida on fixed porcine oocytes by several monovalent and multivalent oligosaccharides. Thus, it is sugested that multiple carbohydrate recognition mechanisms are involved in the sperm-egg interaction.

Various kinds of neoglycoconjugates have been synthesized as probes to detect or characterize the carbohydrate recognition molecules. Most of them carry simple monosaccharides or small synthetic oligosaccharides. However, physiological molecular interactions between carbohydrates and their counter proteins occur in a complex manner. The fine structures of oligosaccharides themselves such as glycosyl linkages and antennary structures, and densities of the oligosaccharides in the carrier proteins are quite important for the interaction with carbohydrate binding proteins. Therefore, it is desirable to use native oligosaccharides particularly for the understanding of the detailed carbohydrate recognition mechanism. As native oligosaccharides are usually available in a very limited quantity, further development of efficient synthetic methods for oligosaccharide probes is expected. Therefore, we developed a micro-scale method for the synthesis of dextran-based multivalent probes containing N-linked oligosaccharides which is efficient even in a small scale. The method contains two procedures, conversion of oligosaccharides into hydrazide derivatives with a dihydrazide reagent and subsequent conjugation of the oligosaccharide hydrazides to periodate-oxidized dextran. The conjugates were

further labeled with fluorescent reagent or biotinylation reagent containing hydrazino group by the use of the unreacted aldehyde groups of the oxidized dextran, yielding the probes with similar densities of fluorophores or biotin groups. We could demonstrate a successful application of the probes to the solid-phase lectin binding assay and the binding inhibition assay.

The probes were also applied to the analysis of carbohydrate recognition molecules of porcine sperm. The results are summarized as follows. Two kinds of molecules, one recognizing the sialo-/asialo-*N*-acetyllactosamine structures and the other recognizing Le^x structure in a divalent cation-independent manner, were detected on the head of boar sperm prepared from cauda epididymis by fluorescence-labeled or biotinylated dextran-based multivalent oligosaccharide probes. The *N*-acetyllactosamine recognition molecule(s) is weakly detected on uncapacitated sperm and becomes strongly detectable on capacitated sperm. On the other hand, the Le^x recognition molecule is detected at a moderate level before capacitation and at a high level after capacitation. Both molecules disappear from the sperm head after induction of acrosome reaction and also by mild detergent treatment. Thus, the two kinds of carbohydrate molecules are expressed on the plasma membrane of boar sperm depending on their physiological state. Inhibition study of the oligosaccharide-dextran probe binding to isolated sperm plasma membrane by various glycoproteins, oligosaccharides and sulfated polysaccharides also supported the occurrence of the two distinct kinds of molecules. We are currently trying to isolate the recognition molecules.

2. Altered protein glycosylation in β1, 4-galactosyltransferase (GalT-I) knockout mice and its biological effects

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The outer chains of complex-type N-glycans consist of two backbone structures called type 1 chain (Galβ1→3GlcNAc) and type 2 chain (Galβ1→4GlcNAc). These structures are also found in O-glycans and glycolipids. Modifications of the two chains by several transferases leads to the synthesis of a variety of terminal carbohydrate structures; for example, Le^a, Le^b or sialyl Le^a antigenic structures on type 1 chains and Le^x, Le^y, sialyl Le^x and polylactosamine structures on type 2 chains, respectively. These modified structures have been suggested to have physiologically and pathologically important roles in cell-cell interactions. For example, sialyl and/or sulfated Le^x serve as ligands for selectins, and mediate leucocyte-endothelial cell interactions. These ligands as well as sialyl Le^a plays important roles in interaction of tumor cells with endothelial cells during the process of metastasis.

In collaboration with Drs. Asano and Iwakura who generated mice deficient in β1, 4-galactosyltransferase (β4Gal-T), now called β4Gal-T1, which is involved in the synthesis of type 2 chain, we comparatively analyzed several glycoproteins from wild-type and β4Gal-T1-knockout mice. Unexpectedly, we found that both hepatic and plasma glycoproteins from the knockout mice expressed considerable amounts of sialylated, galactosylated N-glycans. A striking contrast is that galactose residues are mostly β1, 4-linked to GlcNAc in the wild-type samples but β1, 3-linked in the knockout samples. Thus, the backbone structure of N-glycans shifted from type 2 chain (Galβ1→4GlcNAc) to type 1 chain (Galβ1→3GlcNAc) in the knochout mice. Detailed structural analysis of plasma glycoprotein samples further revealed that the Siaα2→6Gal is predominantly expressed in the wild-type sample, but the Siaα2→3Gal is in the knockout sample. In addition, oversialylation of type 1 chains were remarkably found in the knockout sample. Thus, the altered backbone structures and sialyl linkages in the knockout samples suggests that galactosylation is primarily compensated by β3Gal-Ts in liver of the β4Gal-$T1^{-/-}$ mice, and then different sialyl linkages are formed according to the acceptor specificities of sialyltransferases. These results and the previous finding that galactosylation of glycans included in erythrocyte plasma membrane glycoproteins is partially blocked indicate that effects of deficiency of β4 Gal-T1 on glycosylation varies tissue to tissue. Some of the glycans that changed tissue-dependently might be involved in the abnormal phenotypes of the knockout mice, and contribution of the glycans to pathological changes is under investigation.

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Our major research interest is to elucidate the molecular mechanisms involved in signal transduction for malignant transformation and for tumor angiogenesis. During several years we have been mainly focusing on the functions of tyrosine kinases (VEGF receptors Flt-1/KDR, EGF receptor and Bcr-Abl) and adaptor proteins, Shc and Vav. This year we found a new type of nuclear localization signal in HIF transcription factor essential for VEGF gene expression. In addition, we worked on VEGF-resistant apoptosis of vascular endothelial cells, and extensively characterized the biological role of VEGF receptors and the association molecule for Bcr-Abl.

1. Regulation of VEGF gene expression: A variant of nuclear localization signal of bipartite-type is required for the nuclear translocation of Hypoxia Inducible Factors (1α, 2α and 3α)

Jin Cai Luo, Kenji Wakiya, and Masabumi Shibuya

An endothelial cell-specific growth factor VEGF (Vascular Endothelial Growth Factor) and its receptors have recently been shown to be deeply involved in physiological as well as most of the pathological angiogenesis. Hypoxia inducible factors (HIF1, 2 and 3), consisting of α and β subunits, play an essential role in various responses to hypoxia such as upregulation of VEGF. Nuclear entry of α subunits is a necessary step for the formation of DNA-binding complex with β subunit, which is constitutively localized in the nucleus.

We found that the nuclear accumulation of HIF2α induced by hypoxia is mediated through a variant of bipartite-type nuclear localization signal (NLS) in the C-terminus of the protein, which has an unusual length of spacer sequence between two adjacent basic domains. We further showed that when the ubiquitin-proteasome system was deficient or inhibited, HIF2α accumulated in the nucleus even under normoxia, also mediated through the bipartite NLS. These findings indicate that the protein stability is critical for the nuclear localization of HIF2α and hypoxia is not a necessary factor for the process. Importantly, the NLS of HIF2α is also conserved in the other HIF family members, HIF1α and HIF3α. Mutational analyses proved that the NLS mediating the nuclear localization of HIF1α is indeed bipartite-, but not monopartite-type as thought before. Our results suggest that the newly identified NLS is crucial for the functional regulation of HIF family.

2. VEGF-resistant apoptosis in primary vascular endothelial cells: The overexpression of PKCδ is involved in VEGF-resistant apoptosis in cultured primary sinusoidal endothelial cells

Tomoko Takahashi and Masabumi Shibuya

We have previously reported that primary sinusoidal endothelial cells (SE cells) from the rat liver are highly dependent on VEGF for cell proliferation in *in vitro* culture. However, even in the presence of VEGF, essentially all the SE cells could not survive longer than 7 days, leading to growth factor-resistant cell death. The death had characteristics typical of apopotosis, such as DNA fragmentation, staining with TUNEL reagent and nuclear condensation. We found that the cell death was blocked by the treatment of TPA in a dose-dependent manner and was preceded by a remarkable increase in PKCδ at a protein level. Furthermore, PKCδ-specific inhibitor, Rottrelin, significantly suppressed this VEGF-resistant apoptosis of cultured SE cells, whereas

conventional PKC-specific inhibitor, Gö6976 could not. TPA was found to downregulate the overexpression of PKCδ. Thus, we suggest that the VEGF-resistant apoptosis is a new type of endothelial cell death and that PKCδ is an essential mediator for this process.

3. KDR signaling and site-specific antibody: A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-dependent activation of PLC-γ and DNA synthesis in vascular endothelial cells

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KDR/Flk-1 tyrosine kinase, one of the two VEGF receptors induces mitogenesis and differentiation of vascular endothelial cells. To understand the mechanisms underlying VEGF-induced growth signaling pathway, we constructed a series of mutants of human KDR and examined their biological properties. An *in vitro* kinase assay and subsequent tryptic peptide mapping revealed that Y1175 as well as Y1214 are the two major VEGF-dependent autophosphorylation sites. Using an antibody highly specific to the phosphoY1175 region, we demonstrated that Y1175 is rapidly phosphorylated *in vivo* in primary endothelial cells. When the mutated KDRs were introduced into the endothelial cell lines by adenoviral vectors, only the Y1175F KDR, tyrosine-1175 to phenylalanine mutant, lost the ability of PLC-γ tyrosine-phosphorylation, and significantly reduced MAP kinase phosphorylation as well as DNA synthesis in response to VEGF. Furthermore, primary endothelial cells microinjected with the anti-phosphoY1175 antibody clearly decreased DNA synthesis compared with the control cells. These findings strongly suggest that autophosphorylation of Y1175 on KDR is crucial for the endothelial cell proliferation, and that this region is a new target for anti-angiogenic reagents.

4. Dual biological roles of Flt-1: Involvement of Flt-1 tyrosine kinase (VEGF Receptor-1) in pathological angiogenesis

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VEGF and its two receptors, Flt-1 (VEGFR-1) and KDR/Flk-1 (VEGFR-2) have been demonstrated to be an essential regulatory system for blood vessel formation in mammals. KDR is a major positive signal transducer for angiogenesis through its strong tyrosine kinase activity. Flt-1 has a unique biochemical activity, 10-fold- higher affinity to VEGF, whereas much weaker tyrosine kinase activity compared to KDR. Recently we and others have shown that Flt-1 has a negative regulatory function for physiological angiogenesis in the embryo, possibly with its strong VEGF-trapping activity. However, it is still open to question whether the tyrosine kinase of Flt-1 has any positive role in angiogenesis at adult stages. In this study we examined whether Flt-1 could be a positive signal transducer under certain pathological conditions such as angiogenesis with tumors overexpressing a Flt-1-specific VEGF-related ligand. Our results clearly showed that murine Lewis lung carcinoma cells overexpressing PlGF-2, an Flt-1 specific ligand, grew in wild type mice much faster than in Flt-1 tyrosine kinase (TK) domain-deficient mice. Blood vessel formation in tumor tissue was higher in wild type mice than in Flt-1 TK-deficient mice. On the other hand, the same carcinoma cells overexpressing VEGF showed no clear difference in the tumor growth rate between these two genotypes of mice. These results indicate that Flt-1 is a positive regulator using its tyrosine kinase under pathological conditions when the Flt-1-specific ligand is abnormally highly expressed. Thus, Flt-1 has a dual function in angiogenesis, acting in a positive or negative manner in different biological conditions.

5. The tubulogenic activity associated with an activated form of Flt-1 kinase is dependent on focal adhesion kinase

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Focal adhesion kinase (FAK) is known to be located at the intersection between extracellular matrix and growth factor signaling pathways to regulate cell motility. We have previously shown that an activated form (BCR-FLTm1) of Flt-1 kinase, a receptor for vascular endothelial growth factor, had a tubulogenic activity not only in endothelial cells but also in fibroblastic cells. Here we show that tubulogenesis by BCR-FLTm1 depends on FAK and that FAK is not only activated by vascular endothelial growth factor (VEGF) in NIH3T3 cells that overexpress Flt-1 but also binds to VEGF-activated Flt-1.

6. Other projects for the characterization of VEGF receptors in vertebrates

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Kazuhiro Masubuchi, Satsuki Kobayashi, Takeshi Hara, Takaahiro Honda, Masato Murakami, Naoyuki Yabana, and Masabumi Shibuya

In addition to those projects described earlier, we have been working on (1) the angiogenic activities of Flt-1(VEGFR-1) or KDR(VEGFR-2)-specific ligand, PlGF or VEGF-E in transgenic mice, (2) domain structure analysis of VEGF-E by chimeric molecules between VEGF-E/PlGF, (3) the role of Flt-1 in embrygenesis and placental development in mammalian and avian species (4) Flt-1 as a new cell surface marker of monocyte/macrophage lineage (5) molecular mechanism of vascular degeneration. The results of these projects will be reported in details in the next time.

7. Characterization of chronic myelogenous leukemia P210BCR-ABL protein

a. The human leukemogenic oncoprotein P210BCR-ABL alters TFIIH functions

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We have previously reported that expression of P210BCR-ABL counteracted against the complementary effect of XPB on DNA repair in ultraviolet-treated 27-1 cells. Cells behaved in a similar fashion when treated with cisplatin but not with hydrogen peroxide. Wortmannin, an inhibitor of PI3 kinase which plays a major role in BCR-ABL signaling did not affect its antirepair effect. Cisplatin treatment induced enhanced recruitment of p44 with XPB that was introduced into 27-1 cells for complementation. This was not observed when P210BCR-ABL was doubly expressed or when the activity of doubly-expressed temperature-sensitive P210BCR-ABL was suppressed at nonpermissive temperature. However, purified TFIIH from P210BCR-ABL expressor and nonexpressor showed almost no difference in molar ratios of each component. Interestingly, the *in vitro* activity of TFIIH was slightly decreased in repair assay with cisplatin-treated DNA but was increased in transcription assay. We conclude that mature TFIIH formation is impaired *in vivo* and that *in vitro* function of purified TFIIH by itself is also altered by P210BCR-ABL in this system.

b. The Dbl-homology domain of BCR is not a simple spacer in P210BCR-ABL of Philadelphia chromosome

Yoshitora Kin, Guang Li, Masabumi Shibuya, and Yoshiro Maru

The Dbl-homology domain of BCR in P210BCR-ABL(P210/WT) has been thought to have a negative effect on the activation of BCR-ABL because P185BCR-ABL in which this region is physically deleted has stronger biochemical as well as biological activities. In order to study the role of the Dbl-homology domain of BCR in the background of P210/WT, the region was replaced with homologous sequences derived from Dbl (P210/Dbl) or DCD24(P210/Dbl), or with irrelevant sequences from LacZ (P210/LacZ) or luciferase (P210/Luci). Surprisingly the abilities to transform Rat1 cells or mouse bone marrow cells and to induce growth factor independence in interleukin 3 (IL-3)-dependent mouse Ba/F3 cells were retained only in the P210/Dbl. However, even P210/Dbl could not achieve the wild-type level of surviving potential against genotoxins in Rat1 cells as well as in Ba/F3 cells. Activation status of Akt correlated with the biological difference in Rat1 but not in Ba/F3 cell system. The DH domain was neither tyrosine-phosphorylated *in vitro* nor could we find any difference in peptide mapping between *in vitro* phosphorylated P210/WT and P210/Dbl or P210/CDC24. Although mechanism of how the DH domain makes a functional contribution to biological activity of P210BCR-ABL remain uncovered, we make a paradoxical proposal that the DH domain makes positive contributions to P210BCR-ABL.

c. Growth suppression of Ph1 clone by the siege of Epstein-Bar virus-transformed cells of non-Ph1 karyotype

Yoshitora Kin, Masabumi Shibuya, and Yoshiro Maru

We report a human cell line KM established from a patient of blastic crisis of chronic myelogeneous leukemia that showed spontaneous reduction of leukemic cells. Cells had been infected with Epstein-Bar virus during treatment and needed aggregation for survival in culture. Ph1 clone was lost in the passage of culture. When BCR-ABL-expressing leukemic cells were purposely cocultured with KM, they were embedded in the KM cell aggregates which did not allow their growth. Increased expression of cell cycle inhibitor p27 was correlated with the suppression.

d. Protein kinase C delta mediates transformation by the BCR-ABL oncogene of Philadelphia chromosome

Yoshitora Kin, Masabumi Shibuya, and Yoshiro Maru

The BCR-ABL oncoprotein transmits transformation signals mainly through pathways involving Ras, Myc and PI3 kinase. Here we report that protein kinase C (PKC) delta also plays a role in transformation.

Transformation of Rat1 cells by BCR-ABL was blocked by delta isoform-specific inhibitor rottlerin, but not by Go6976 that inhibits only conventional isoforms. The kinase activity of delta isoform was found to be roughly 2 fold higher in BCR-ABL-expressing Rat1 cells than that in mock. Although overexpression of wild type PKC delta did not enhance BCR-ABL-mediated transformation of Rat1 cells, that of dominant-negative delta isoform blocked it by approximately 40%.

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