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As indicated by "Novartis(Nova=New, Artis=Technology)",
it is our never-ending hope to promote new technologies
in the fields of biological and life sciences, and related chemistry.

Foreword

To create good research environment

Recently deterioration of the moral of Japanese is questioned. In terms of “moral”, the target of criticism had been always young people, but now, it shifted to adults. Newspapers showed us examples such as an architect’s fabrication of structural calculation sheets for earthquake-resistance strength of buildings, and next, fraudulent acts carried out by new-riches called “Hills zoku” as they live in a high-rise condominium “Roppongi Hills” - the symbol of wealth and success. Also in our daily life, the bad-mannered are mostly adults. People who are using their mobile phones in a train or who throw private garbage into a trash box in a park are rather senior people. This means, adults, who normally should lead young generation to a right way, are practically to be blamed by them. Similarly, some symbolic events of moral corruption occurred in the academic research field. A fabricated report about cloning technology was uncovered in Korea, and also a certain famous Japanese university faked research data and their report. According to news reports, the Ministry of Education, Culture, Sports, Science and Technology announced that researchers who committed serious unethical acts such as falsification of report or data will not allowed to apply for research grants up to 10 years from next year. If a researcher lost his research fund even for a few years, it could be the end of his research life. Tough punishment!

In our childhood, our parents told us a saying “dishonesty does not pay”. This saying is made based on self-control. If people are self-controlled, they are all good-natured and the world is peaceful. If you assume that all the people are not good-natured, the world turns cold and cunning people gain power. Unfortunately it seems like the world we live in is rather the latter. Why did the world become so? I think the way people assess things has changed from the analog method to the digital one, namely, they do quantitatively rather than doing qualitatively. Moreover, the cycle of the assessment gets shorter. In the business world, annual sales and profits are compared with those of last term and forced to be increased unremittedly. In the academic world too, impact factors in science journals have wings and replace those in each report. Can the academic value of a report be truly quantified? As for personnel affairs in universities, they are also overwhelmed by figures like research expenditure.

In a university whose wrongful use of research fund was recently brought to the light, a delivery check center was newly set up to check if the content of purchasing slips and delivery slips match with the actually purchased materials. This way, however, seriously disturbs a smooth achievement of research in the name of “check”. Academic people now must control themselves to bring back the world for good-natured.

Akimichi Kaneko, MD, PhD
Chairman, Board of Trustees
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Part II :

Reports from the Recipients of Grants for International Meeting
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4th European-Japanese Bioorganic Conference (EJBC-4)

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Part I

Reports from the Recipients of Novartis
Research Grants (Fiscal Year 2004)

1) Biological Sciences
1-1) Molecular Biology

Molecular Design for Bioactive Small Compounds based on Phage-displayed Combinatorial Peptide Libraries

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Introduction

Combinatorial peptide libraries are widely recognized as useful sources for screening bioactive ligands that bind to receptors and enzymes. However, the selected small peptides generally possess considerable conformational flexibility, so the entropic costs of binding to target molecules are high. In addition, the flexibility does not allow one to determine the precise three-dimensional orientation of the side-chains, which impedes the structure-based design of the mimetic. A solution to these problems is to construct conformationally constrained peptide libraries. Therefore, we propose designing structured peptides as the library scaffolds and incorporating randomized peptide sequences into them.

Results

In this work, we attempt to construct a peptide library on a scaffold of a *de novo* designed helix-loop-helix (AELHALEHELAAL-(G7)-KLAALKAKLAALKA:1)¹). Since the scaffold folds by virtue of the interactions between the amino acid residues positioned inside the helix-loop-helix, the solvent-exposed, outside residues (X^{24} , X^{25} , X^{28} , X^{31} , and X^{32}) would be randomized to give a library of helix-loop-helix peptides²). A peptide library of helix-loop-helix peptides was displayed on the major coat protein of M13 filamentous phage by a modification of the pComb8 system. We obtained a phage library with the size of 1.5×10^6 , which was screened against the CRH region of G-CSF receptor.

After ten rounds of biopanning, sixteen clones were randomly selected and subjected to nucleotide sequencing. Of the sixteen, nine clones were found to possess the same sequence. The obtained peptide (**2**) was prepared as a soluble form by the solid-phase synthetic method and examined for its structural and binding properties. In surface plasmon resonance (SPR) assay, peptide **2** was found to bind to the CRH region with a K_d of 150 nM and the CD spectra suggested α -helical potency of peptide **2**. To define the correlation between the folding and the binding affinity, we examined point-mutations of the peptide to improve the α -helical potential. Since proline was considered to destroy α -helical structures, a Pro at the randomized position 31 in peptide **2** was

replaced with alanine to give peptide **4**. In addition to the Pro³¹, two histidine residues (His⁴ and His⁸) on the *N*-terminal helix were substituted with alanine to afford peptide **5**. In the CD spectra, peptide **4** exhibited an increasing α -helical content and peptide **5** showed higher α -helical content than that of the peptide **4**. As expected, peptides **4** and **5** showed K_d values of 28.0 μ M and 3.8 μ M, respectively. Thus, there was a parallel correlation between folding of the peptides into the helix-loop-helix and binding affinity against the CRH region.

In addition, the ability of the peptides to inhibit G-CSF-dependent cell proliferation was examined with NFS-60 cell lines. Cells were incubated with various concentrations of peptide in the presence of 17 pM of G-CSF; native G-CSF stimulates this cell line to proliferate with half maximal response occurring at 5 to 10 pM. The cell proliferation rate was monitored with MTT assay. Scaffold **1** was inactive at concentrations up to 100 μ M, whereas peptide **4** and **5** showed inhibitory activity in a dose-dependent manner (**4**: $ED_{50} = 79.1$ μ M, **5**: $ED_{50} = 25.0$ μ M).

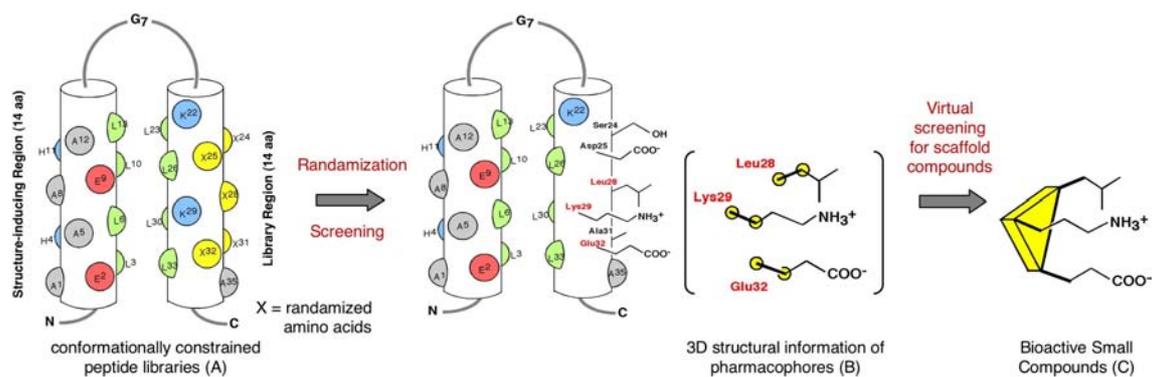
The constrained conformation of the peptides suggested a precise three-dimensional orientation of the pharmacophore (Arg, Glu, Lys, and Leu) in the interaction between the peptides and the receptor. Based on 3D information, we designed biphenyl sulfonamide derivatives, which possess the functional groups corresponding to the side chains of the active amino acids. In the SPR assay, the small compounds were found to be active against G-CSF receptor.

Discussion & Summary

In this work, we have attempted to construct a library of helix-loop-helix peptides on M13 filamentous phage. The library was screened against mouse G-CSF receptor [4], and a phage clone that bound to the target was selected. Mutations of the selected peptide to increase the α -helical content reflecting increased the stability of the two-helix template led to concomitant increases in binding affinity for the receptor, providing variants with K_d s ranging from 150 to 3.8 μ M. The receptor-binding assay with NMR showed the competitive binding of the peptide against the native G-CSF, consistent with the antagonist activity observed in MTT assay. These studies can be used to shrink the cytokine to moieties more amenable to small molecule drug design.

Figures&Tables

Molecular Design for Bioactive Small Compounds based on Phage-displayed Combinatorial Peptid Libraries



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- 1) Biological Sciences
1-1) Molecular Biology

Molecular mechanism of class switch recombination and somatic hypermutation induced by AID

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Introduction

Activation-induced cytidine deaminase (AID) is the essential molecule for two distinct events on immunoglobulin genes, class switch recombination (CSR) and somatic hypermutation (SHM). CSR is a region-specific recombination that takes place between switch (S) regions. SHM introduces point mutations at high frequency in the variable (V) region. We are asking two questions; what is the target of AID, and how AID regulates differentially CSR and SHM.

Results

1. What is the target of AID?

AID is important for the initiation of DNA cleavage in S region. However, how AID initiates DNA cleavage is still controversial. All other researchers except for us believe that AID deaminates cytosine on S region DNA directly, resulting in DNA double strand breakage in collaboration with UNG and mismatch repair enzymes. On the other hand, we think that AID is an RNA editing enzyme like APOBEC-1, which edits mRNA to make the transcript encoding the activated endonuclease, resulting in DNA cleavage. To check whether AID has cytidine deaminase activity on RNA or DNA, we first purified the recombinant AID from *E. coli* and tried cytidine deaminase assay on single-stranded DNA *in vitro*. However, purified AID from *E. coli* did not show the cytidine deaminase activity on single-stranded DNA although crude cell lysate always showed cytidine deaminase activity. Furthermore, we observed that cytidine deaminase activity on single-stranded DNA with crude cell extract even from so-called cytidine deaminase-deficient strain. We conclude that *E. coli* system is not good for deaminase assay because of contaminated activity from *E. coli* itself. Then we tried the *in vitro* transcription/translation synthesis of AID protein using wheat-germ extract and purified AID by affinity purification. *In vitro* synthesized AID showed the cytidine deaminase activity on single-stranded DNA, whereas the catalytic domain-deficient AID mutant did not. It indicates that *in vitro* synthesized AID has cytidine deaminase activity on DNA. Next, using this AID protein, we

are going to set up RNA editing assay.

2. How does AID regulate differentially CSR and SHM?

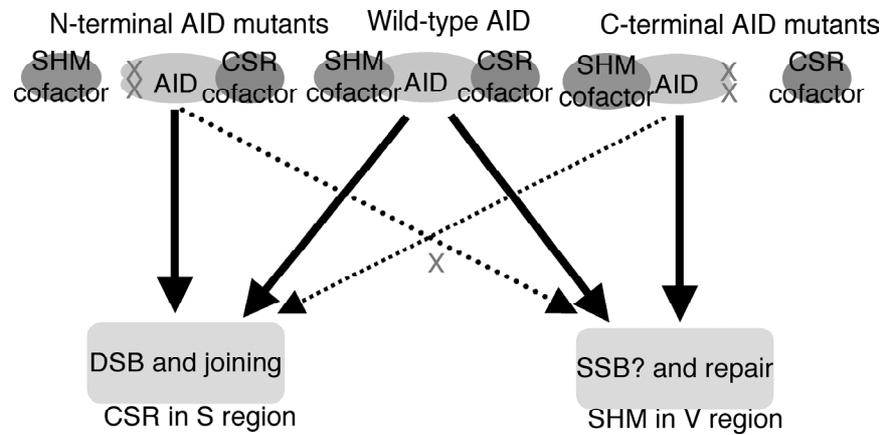
We have shown that mutants with alterations in the C-terminal region of AID retain normal SHM activity but almost completely lose CSR activity, indicating an essential function for the C-terminal region of AID in CSR. In addition, we also found that mutations in the N-terminal region of AID resulted in specific loss of SHM activity but retained CSR activity. Those mutations do not locate in the cytidine deaminase motif of AID. From these results, we hypothesize that the CSR and SHM activities of AID are regulated differentially through the interaction of CSR- or SHM-specific cofactors with C-terminus or N-terminus of AID, respectively. These mutants are useful to investigate the molecular mechanism of CSR and SHM not only *in vitro* but also *in vivo*. In physiological condition, upon antigen stimulation, B cells usually undergo both CSR and SHM. Therefore it is difficult to observe the effect of CSR and SHM separately in view of immune surveillance for whole body. For *in vivo* study, we will generate the AID knock-in mice those express the N- or C-terminal AID mutants instead of wild-type AID. Knocked-in ES cells have been established. Those ES cells will be injected into blastocyst to make chimera.

Discussion & Summary

Several groups have reported that AID purified from *E. coli* showed cytidine deaminase activity on single-stranded DNA. However, we speculate that they might observe the contaminated activity from host *E. coli*. Therefore we synthesized and purified AID using *in vitro* transcription/translation system. *In vitro* synthesized AID showed cytidine deaminase activity on single-stranded DNA, whereas deaminase-deficient mutant did not. We are going to check the RNA editing activity of AID using *in vitro* synthesized protein.

We have shown that C-terminal mutations of AID cause loss of CSR specifically, whereas N-terminal mutations of AID show SHM-specific defect. We propose that CSR and SHM activities of AID are regulated differentially through the interaction of CSR- or SHM-specific cofactors with C-terminus or N-terminus of AID, respectively. To check if these mutants show any pathological phenotype *in vivo*, we will make knocked-in mice.

Figures&Tables



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1) Biological Sciences
1-1) Molecular Biology

Mechanisms of transcriptional and posttranscriptional gene silencing by small RNA molecules

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Introduction

RNAi triggers cleavage of complementary mRNAs, which in turn results in gene silencing at the posttranscriptional level. Small dsRNA functioning in the RNAi pathway is known as siRNA. Work over the past several years has revealed that several factors such as AGO2 are necessary for RNAi and that small RNAs called miRNA also trigger posttranscriptional gene silencing. However, all the details of the RNAi/miRNA pathways are still not well understood. One of our ultimate goals is to understand how gene silencing via small RNAs (siRNA, miRNA and others) occurs at the molecular levels.

Results

Functional analyses of AGO family members in fly

We showed that in *Drosophila* both AGO1 and AGO2 possess the Slicer activity (the activity to cleave the target mRNA). Slicer activity was reconstituted with recombinant full-length AGO1. Further, reconstitution of Slicer activity with recombinant PIWI domains of AGO1 and AGO2 demonstrates that other regions in the Argonautes are not strictly necessary for siRNA-binding and cleavage activities. In circumstances with AGO2-lacking, the siRNA duplex is not unwound and consequently RNA-induced silencing complex (RISC) is not formed. We show that upon addition of siRNA duplex in S2 lysate, the passenger strand is cleaved in an AGO2-dependent manner and that nuclease-resistant modification of the passenger strand impairs RISC formation. These findings give rise to a new model in which AGO2 is directly involved in RISC formation as “Slicer” of the passenger strand of the siRNA duplex.

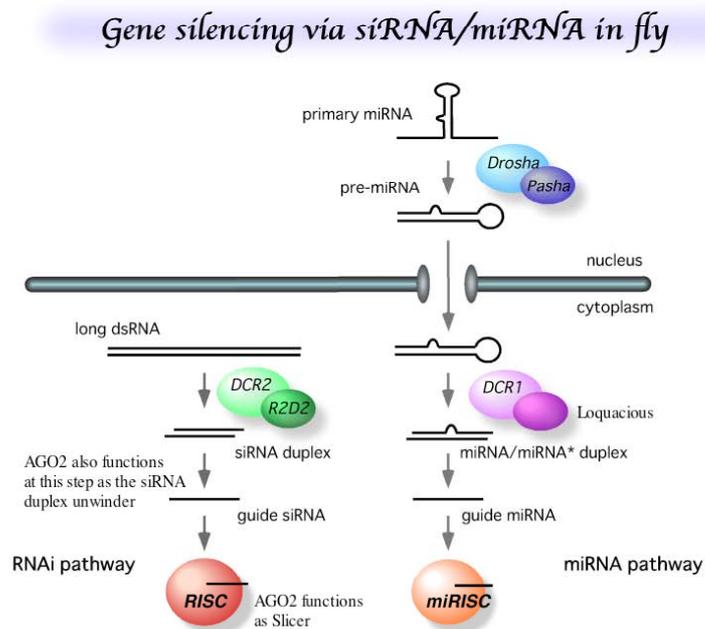
In *Drosophila*, Piwi encodes a protein of the Argonaute family, which is essential for the germ stem cell self-renewal. We show that Piwi immunopurified from ovary specifically associates with small RNAs (25 to 29 nt in length). It was found by sequencing that the Piwi-associated small RNAs are rasiRNAs (repeat-associated siRNAs), mainly derived from the retrotransposon transcripts. We are currently investigating how Piwi-associated with rasiRNA mechanistically regulates gene silencing of the target RNA.

Revealing the molecular mechanism of the miRNA processing pathway

miRNAs are a large family of 21 to 23 nt non-coding RNAs that interact with target mRNAs at specific sites to induce cleavage of the targets or inhibit the translation. miRNAs are excised in a stepwise process from pri-miRNA transcripts. The Drosha-Pasha complex cleaves pri-miRNA to release hairpin-shaped pre-miRNA. The pre-miRNAs are further processed by Dicer to mature miRNAs. We showed that *Drosophila* Dicer1 interacts with Loquacious, a dsRNA-binding protein. This data and others support a model in which Loquacious with Dicer1 mediates miRNA biogenesis and thus the expression of gene regulation by miRNAs. Lately, we found that in the miRNA biogenesis pathway, ATP hydrolysis is required. It might be that the unwinding process of miRNA/miRNA* duplex requires the reaction. We are currently investigating what factor(s) are involved particularly in the unwinding process.

Discussion & Summary

Once we could identify the miRNA unwinding factor(s), it means that we do understand throughout the miRNA biogenesis pathway starting from the pri-miRNA synthesis to mature miRNA loading onto AGO1. We are hoping that it happens soon as we expect. Regarding AGO family members in fly, we still do not know much about functional contributions of Aubergine and AGO3 to gene silencing mechanisms. Hopefully soon, though, we could identify small RNAs associated with them as we have done for Piwi; then, we will be able to get some idea of how they are mechanistically involved in the gene silencing pathways in *Drosophila*.



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1) Biological Sciences
1-1) Molecular Biology

Analysis of HSF1-mediated cell death and alive signaling pathways

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Introduction

Heat shock response is an adoptive response to proteotoxic stress, and a major transcription factor 1 (HSF1) has been believed to protect cells from cell death by inducing heat shock proteins (Hsps) that assist protein folding and prevent protein denaturation. However, it revealed recently that HSF1 also promotes cell death of male germ cells exposed to thermal stress. Recently, we found a proapoptotic *Tdag51* (T-cell death associated gene 51) gene as a direct target gene of HSF1. Therefore, here, we will reveal a novel HSF1-mediated death pathway.

Results

(1) HSF1 activates a pro-apoptotic gene

We found that a profile of time-dependent accumulation of *Tgad51* mRNA was similar to those of *Hsp70* when MEF cells were heat-shocked at 42°C. Reporter activity of *Tdag51* gene was induced in the presence of the putative HSE element, but was never induced when the HSE element was deleted or mutated. Furthermore, gel shift assay showed that an activated HSF1 bound specifically to the HSE element, but not to a mutated HSE. Moreover, ChIP analysis showed that HSF1 binds to the *Tdag51* gene. These results indicate that HSF1 directly binds to and activates *Tdag51* gene in response to heat shock.

(2) Hsps inhibit Tdag51-mediated cell death

Overexpression of GFP-*Tdag51* into HeLa cells caused detachment-induced cell death at time-dependent manner. We firstly examined effects of *Hsp40* on *Tdag51*-mediated cell death. We found that HeLa/*Hsp40* cells were more resistant to low levels of *Tdag51* than control HeLa cells. Furthermore, cells expressing high levels of *Hsp40* were more resistant to *Tdag51* than cells expressing low levels of *Hsp40*. HeLa cells overexpressing *Hsp70* and *Hsp110*, but not cells overexpressing *Hsp27*, were also resistant to *Tdag51*.

(3) Hsps bind directly to a PHL domain of Tdag51

We examined direct interaction of Hsps with *Tdag51*. We found that *Hsp110*, *Hsp70*, and *Hsp40* bind directly to *Tdag51* in vivo, whereas *Hsp27* does not. *Tdag51* is a member of the PHL domain family that has a conserved motif of ~100 amino acids, and its C-terminal domain is composed of

long proline/glutamine- repetitive and proline/histidine-repetitive tracts. We found that Hsp40 binds to the N-terminal region containing the PHL domain. Furthermore, Hsp70 and Hsp110 also bound to the same regions.

(4) A PHL domain is required for inhibiting cell death activity of C-terminal regions

We determined which regions are responsible for its ability to induce cell death. We found that cells expressing C-terminal proline/glutamine-rich and proline/histidine- rich tracts died. Interestingly, cell death induced by the C-terminal region was not inhibited by overexpression of Hsp40, Hsp70, and Hsp110. These data indicate that the PHL domain negatively regulates cell death activity of the C-terminal regions through interaction with Hsps.

(5) Tdag51 is uniquely induced by heat shock

To reveal functional relevance of HSF1-Tdag51 pathway *in vivo*, we focused on male germ cells that are susceptible to high temperatures. When the testicle was exposed to hot water at 43°C for 15 min, Tdag51 mRNA as well as Hsp70-1 mRNA was induced in wild-type mice. As we reported previously, Hsp70-1, Hsp110, and Hsp27 mRNAs were hardly detectable in unstressed testes. In contrast, Tdag51 mRNA was expressed at substantial levels in unstressed germ cells, especially pachytene spermatocytes. Surprisingly, we found marked induction of Tdag51 mRNA in wild-type germ cells including pachytene spermatocytes in response to heat shock. In HSF1-null mice, however, constitutive expression of Tdag51 mRNA was decreased, and its heat shock-induced expression was significantly impaired.

(6) Tdag51 promotes heat-induced cell death *in vivo*

We next analyzed the testis of Tdag51-null mice. Tdag51-null mice were fertile and the testis was morphologically normal. We examined apoptotic cells when the testicles were heat-shocked at 43°C for 15 min, and found less numbers of apoptotic germ cells in Tdag51-null mice than that in wild-type mice. These data demonstrated an important role of HSF1-Tdag51 pathway on germ cell death *in vivo*, and suggest that a proapoptotic Tdag51 functionally dominates antiapoptotic gene products such as Hsps in heat-shocked germ cells.

Discussion & Summary

These observations revealed a novel mechanism how HSF1 manages against proteotoxic stress. Cell fate on proteotoxic condition is determined at least by balance between Hsp and Tdag51 levels, which are differently regulated by HSF1 (Figure 1). HSF1 not only protect cell death, but also promotes it depending on cell types. Analogous to p53, HSF1 is a major factor that senses proteotoxic stress and transduces it to expression of proapoptotic and antiapoptotic genes depending on cell types (Figure 2). Thus, the HSF1-mediated pathway may be one of quality control mechanisms not only in male germ cells, but also in other somatic cells.

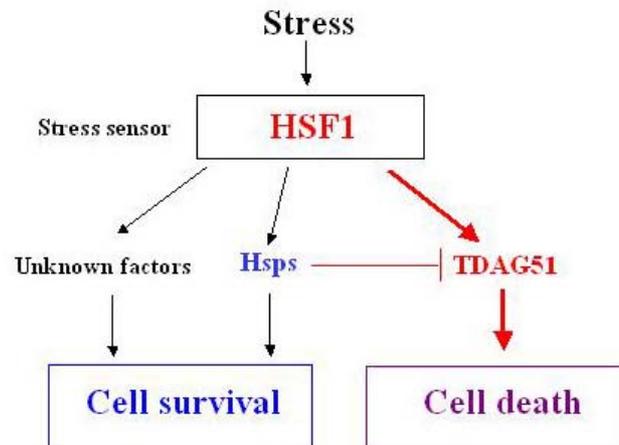


Figure 1. HSF1 determines cell survival and death

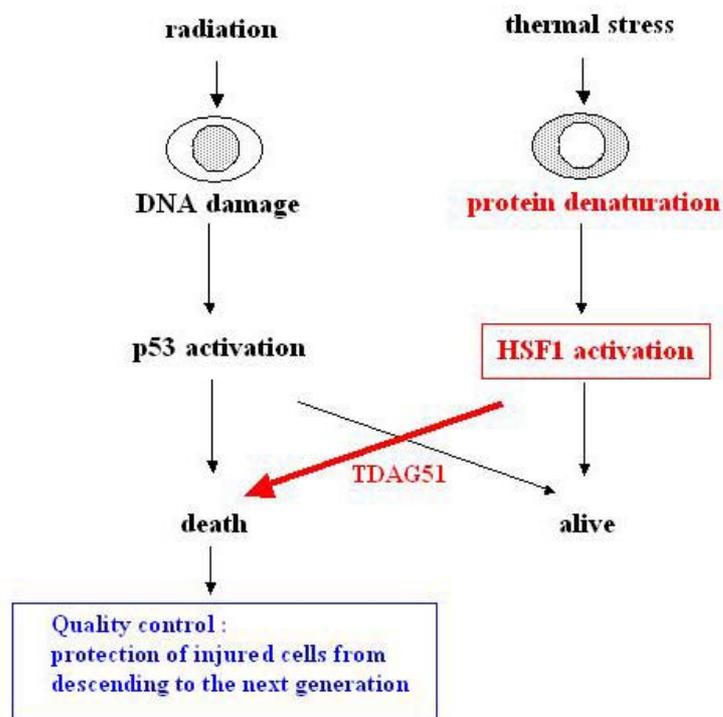


Figure 2. Physiological significance of HSF1-induced cell death

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- 1) Biological Sciences
1-1) Molecular Biology

Visualization of demyelinating neuropathic pain by lysophosphatidic acid

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Introduction

More recently, we have found the critical role for receptor-mediated LPA signaling in the initiation of neuropathic pain (Inoue et al., Nature Medicine, 10,712-718, 2004). However, we are still poorly understood LPA synthesis and the relationship between demyelination and allodynia. In this research, I tried to be find following three topics. 1) The analysis of LPA synthesis. 2) The analysis of mechanism of demyelination. 3) The visualization of allodynia and analysis of its mechanism.

Results

- 1) The analysis of LPA synthesis.

After intrathecal injection of antisense oligodeoxynucleotide (AS-ODN) for LPA synthetic enzyme, nerve injury-induced nociceptive hyperalgesia and allodynia were abolished.

- 2) The analysis of mechanism of demyelination.

We carried out the in vitro explant culture of dorsal root.. In this model, LPA induced the demyelination, and the decrease of myelinated protein mRNA and protein expression.

Furthermore, LPA-induced these events were completely abolished by Rho A inhibitor, but not other inhibitors.

- 3) The visualization of allodynia and analysis of its mechanism.

We developed the behavioral test, which we can detect specific primary afferent stimuli (each C, Adelta, Abeta)-induced responses in nerve-injured mice (please see the reference).

In nerve-injured mice, both Adelta and Abeta-stimuli-induced responses were supersensitized, whereas C-fiber stimuli-induced response was decreased.

In nerve injured mice, Adelta stimuli-induced signaling in spinal cord appeared in novel region, which may be activate by Adelta-stimuli.

Discussion & Summary

From 1) experiments, we concluded that LPA is synthesized after nerve injury. Now we also carried out the measure of LPA content in CSF following nerve injury.

From 2) experiments, we concluded that LPA induces the demyelination and the decrease of myelin protein expression directly, but not through other molecules. And these signaling are mediated through LPA1-G12/13 protein-RhoA pathway, but not Gi or Gq protein pathway, which are other downstream pathway of LPA1 receptor activation.

From 3) experiments, we concluded that both Abeta and Adelta fibers-induced signaling are mixed after nerve injury. This may be related to the development of allodynia.

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Matsumoto M, Inoue M, et al., Characterization of three different sensory fibers by use of neonatal capsaicin treatment, spinal antagonism and a novel electrical stimulation-induced paw flexion test. *Molecular Pain*, 2, 16, 2006

1) Biological Sciences
1-1) Molecular Biology

Molecular mechanism of axonal guidance of the injured central nervous system

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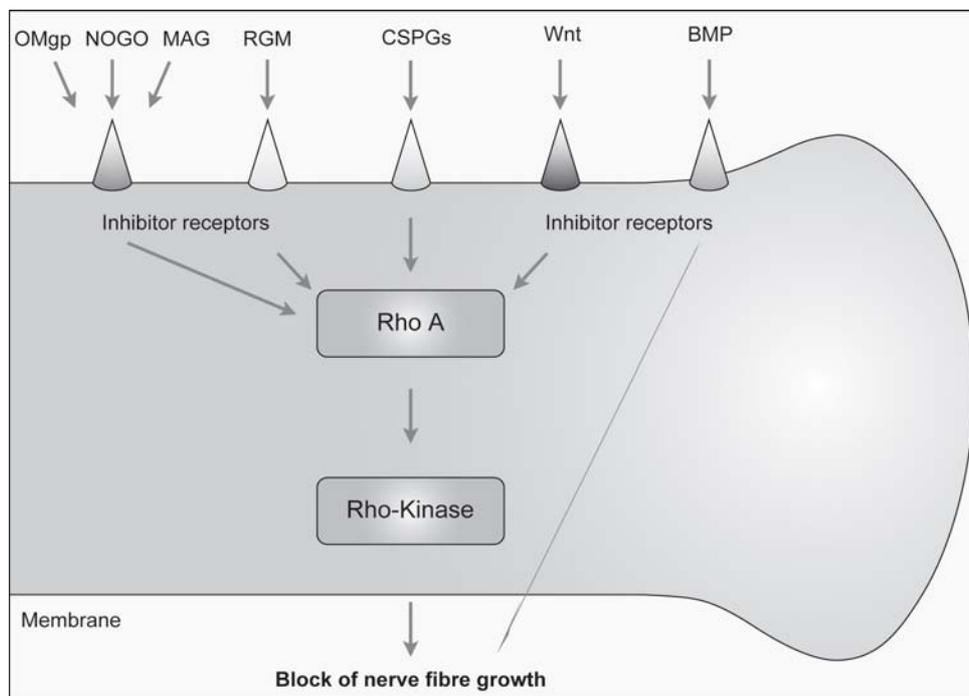
Introduction

We hypothesized that regenerating neurons can be navigated by guidance molecules that include myelin-derived inhibitors of axon growth. They may change their responsiveness to the guidance molecules, which allow regenerating neurons to make complex network. While neurons are normally repulsive to myelin-associated glycoprotein, they are attracted by it if the neurons are priorly exposed to neurotrophins. This phenomenon is designated neurotrophin priming and is a good example for intrinsic mechanisms of neuronal navigations. Through exploring the underlying molecular mechanisms of neurotrophins priming, we would be able to establish therapeutic strategies to make correct axonal and dendritic connections.

Results

First, we cultured the cerebellar neurons from p7 rat, and treated them with brain derived neurotrophic factor for 24 hours. We then screened the cDNAs using microarray techniques, and found that more than several genes were upregulated after exposure to neurotrophins in the postnatal cerebellar neurons *in vitro*. The enhanced expression of these genes was confirmed by real time RT-PCR as well as Northern blot analysis. Time course experiments show that the expression of some of these genes was upregulated gradually at 2 to 6 hours after exposure to the neurotrophin. To explore possible involvement of these genes in regeneration of the injured axons in the central nervous system, we next determined the expression of these genes *in vivo* after the spinal cord injury. Among the candidate genes, two genes were upregulated around the site of injury after spinal cord injury. They turned out to be BMP-2 and Wnt4. BMP-2 was intensively expressed in reactive astrocytes around the epicenter of the lesioned site after injury. Wnt4 was also expressed specifically in the reactive astrocytes after the injury. These interesting observations suggest that the reactive astrocytes as well as oligodendrocytes could be chemical barrier that inhibit regeneration of the injured axons in the spinal cord. To address if these proteins work as inhibitors of axon growth *in vitro*, we performed neurite outgrowth assay using the postnatal cerebellar neurons. Cultured neurons were incubated *in vitro* for 24 hours in the presence or absence of BMP-2 or Wnt4. Both

proteins significantly inhibited neurite outgrowth compared to the control. Co-incubation of Y-27632, a Rho-kinase inhibitor, completely abolished the effect of Wnt4, but not that of BMP-2. As expected, Wnt4 induced activation of RhoA in the neurons. These results demonstrate that Wnt4 inhibits neurite outgrowth by activating RhoA and Rho-kinase. Wnt family proteins comprise of several members, and we determined the expression of these proteins in the spinal cord after injury. Wnt3 as well as Wnt5a was expressed in the reactive astrocytes after injury. Thus, multiple members of Wnts work together on the neurons. We further found that neutralizing antibody to Ryk, one of the receptors for Wnts, totally abolished the effect of Wnt4, Wnt3 and Wnt5a. So, the inhibitory effect on the neurons is dependent on Wnt binding to Ryk. Interestingly, the effect of BMP-2 was not mediated by activation of RhoA or Rho-kinase. Additional experiments demonstrate that the effect of BMP-2 was dependent on the activity of LIM kinase, as the dominant negative form of LIM kinase silenced the effect of BMP-2. Indeed, BMP-2 induced activation of LIM kinase *in vitro*. Although BMP-2 also activated the Smad signal, transfection of inhibitory Smad did not modulate the effect of BMP-2 on the neurite outgrowth. In addition, BMP-2 did not activate p38MAPK, though previous reports showed that BMP-2 activated this signal in some cells. In addition, we found that the neutralizing antibody to RGM reduced the effect of BMP-2. As RGM and the LIM kinase receptors form a receptor complex that transduces the signal of BMP-2. This antibody offers an effective blocker of the BMP signal. Our findings, therefore, identified new signals involved in axon regeneration inhibition (Figure).



Discussion & Summary

So far, several proteins were identified to inhibit axon outgrowth at least *in vitro*. Among them, three molecules expressed in oligodendrocytes, myelin-associated glycoprotein, Nogo and oligodendrocyte myelin glycoprotein, have been extensively studied. All these proteins inhibit neurite outgrowth from some neurons *in vitro* by activating RhoA and Rho-kinase. However, recent studies show that inhibiting each molecule or the receptors for these molecules resulted in no or little regeneration of the injured axons or no functional recovery. These findings suggest the presence of other inhibitory molecules. We reported recently that RGM is another inhibitor of axon regeneration (Hata et al., 2006). Inhibition of RGM promoted massive axon regeneration and functional recovery. RGM inhibits axon regeneration by activating RhoA and Rho-kinase, common signals of axon regeneration. Our present study shows that two other proteins are implicated in inability of axon regeneration in the central nervous system. Importantly, the effect of BMP-2 was not dependent on RhoA or Rho-kinase, but was mediated by LIM kinase. These findings provide new molecular targets to treat patients with injuries to the central nervous system.

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1) Biological Sciences
1-1) Molecular Biology

Analysis of the new death receptor signal for the treatment of cancers and immune diseases

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Introduction

DR6 (death receptor 6) is the newest member of death receptor family (Pan et al., 1998). Disruption of DR6 gene in mice caused a reduced apoptosis and hyper-proliferation of both T and B cells (Liu et al., 2001; Schmidt et al., 2003; Zhao et al., 2001). It is suggested that DR6 has important roles for regulating activation of peripheral T and B cells. On the other hands, DR6 gene disruption also caused production of Th2 type cytokine production such as IL-4, but not Th1 type cytokine production such as IFN-gamma, (Liu et al., 2001; Zhao et al., 2001). These findings suggested that DR6 is one of critical factors for the regulation of Th balance. Interestingly, unlike other death receptors, DR6 does not interact with FADD, RIP1 or RAIDD and over-expression of dominant negative form of FADD failed to inhibit DR6-mediated apoptosis (Kasof et al., 2001; Pan et al., 1998). These data suggest that there is a unique and unknown mechanism in DR6-mediated death-signaling pathway.

Results

Identification of CLIPR-59 as a novel binding protein to the cytoplasmic region of DR6

To examine the signaling pathway mediated by DR6, we screened the molecules which bind to DR6. By the yeast two-hybrid (Y2H) screening using the cytoplasmic domain of DR6 as a bait, we identified 111 positive clones. It should be noted that among these genes, 28 independent clones encoded the same region of CLIPR-59 (cytoplasmic linker protein-170, CLIP-170 related protein 59kDa), from amino acids 387 to 547. To clarify the specificity of the interaction of CLIPR-59 with DR6 and CLIPR-59 binding region within DR6, the following Y2H and immunoprecipitation assay were carried out using mutants of DR6. We showed that the region from amino acids 370 to 428 of DR6 is necessary for its interaction with CLIPR-59.

CLIPR-59 induces apoptosis and inhibits NF- κ B activation in DR6 signal

Since DR6 is known to induce apoptosis and the regulation of NF- κ B activity, we examined the

functional roles of CLIPR-59 on DR6-mediated apoptosis and NF- κ B activation. In NF- κ B dependent reporter gene assay, we found that the expression of CLIPR-59 could inhibit the DR6-mediated NF- κ B activation but CLIPR-59 delta C, lacking DR6-binding region (Figure 1D), could not (Figure 2A). Contrary to the inhibition of NF- κ B activation, CLIPR-59 significantly augmented DR6-mediated apoptosis and this effect was not seen in CLIPR-59 delta C (Figure 2B). These data suggested that CLIPR-59 could play negative roles for NF- κ B activation and positive roles for apoptosis in DR6 signaling. Next, we examined whether the interaction of DR6 with endogenous CLIPR-59 is involved in DR6-mediated cell death, the cell viability assay was carried out using DR6WT or delta CLR. Expression of DR6WT significantly reduced the cell viability. On the other hands, absence of CLR of DR6 partially but significantly abrogated this effect (Figure 2C). In addition, in NF- κ B dependent reporter gene assay, DR6WT induced NF- κ B activation and deletion of CLR of DR6 further augmented NF- κ B activation (Figure 2D).

Discussion & Summary

CLIPR-59 was originally identified as a member of a family of proteins related to CLIP-170, which is a cytoplasmic linker protein mediating interactions of organelles with microtubules (Perez et al., 2002). CLIPR-59 consists of 547 amino acids, and several predicted protein interacting motifs, including proline and glutamic acid rich region, ankyrin like repeats, two cytoskeleton-associated protein glycine-rich (CAP-Gly) domain and serine rich region (Perez et al., 2002). It was previously shown that CAP-Gly domain of CLIPR-59 is responsible for its interaction with microtubule, and this association was inhibited by its amino-terminal region (Lallemand-Breitenbach et al., 2004; Perez et al., 2002), indicating that conformational change of CLIPR-59 is involved in the process of association with microtubules. We showed that presence of amino-terminal region of CLIPR-59 did not affect its binding to DR6. Our results suggested that the underlying mechanisms for the interaction between DR6/CLIPR-59 and CLIPR-59 and microtubules are quite distinct. It should be pointed that DR6 mutant, lacking the region from amino acids 461 to 655 was unable to induce apoptosis and mediate NF- κ B activation (Pan et al., 1998). Nevertheless, this mutant lacks death domain (DD) and still possesses CLR. Thus, it is likely that primary region for mediating apoptosis and NF- κ B activation exists in the region from DD to the carboxy-terminal end of DR6. CLR of DR6 should play a positive role for apoptosis and negative role for NF- κ B activation in DR6 signaling by interacting with CLIPR-59.

DR6 regulates the process of apoptosis and interacts with TRADD but not with FADD, RIP1 or RAIDD, suggesting that there might be a unique DR6-mediated signaling pathway, which leads to apoptosis. Here, we showed a novel mode of action for DR6-mediated signaling pathway. We identified CLIPR-59 as a novel DR6-binding molecule. CLIPR-59 is a novel signal transducer to inhibit DR6-mediated NF- κ B activation and induce apoptosis.

Fig.1

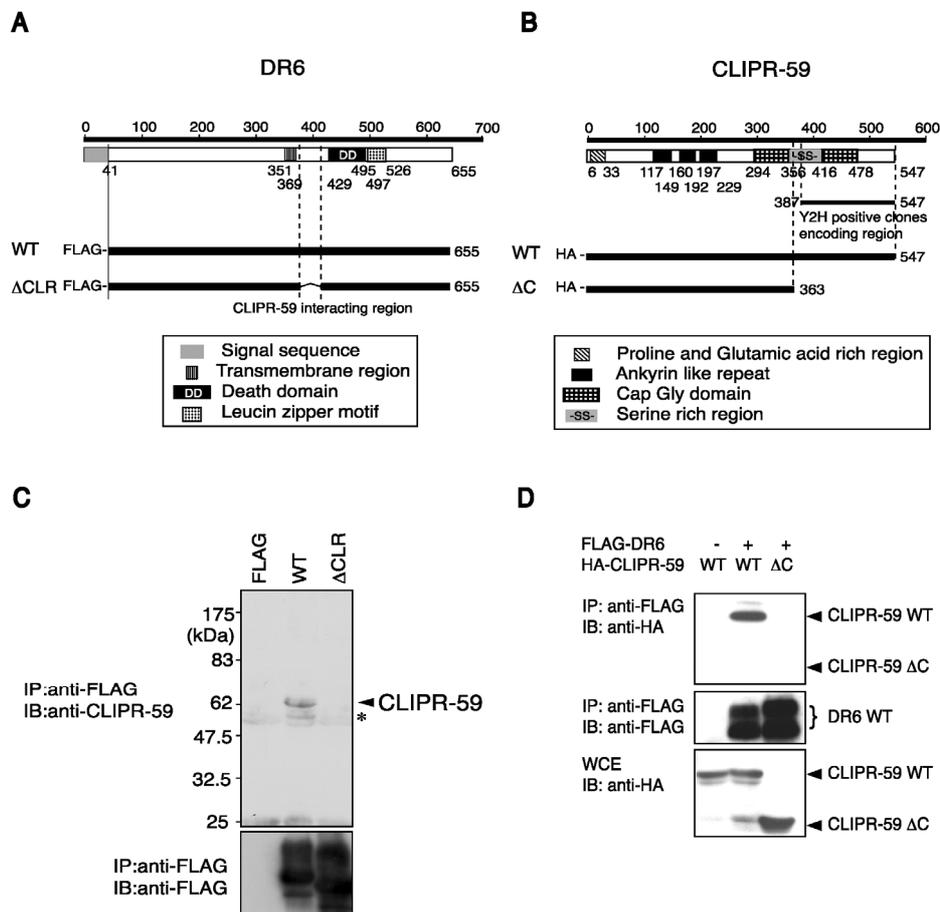


Figure 1. CLIPR-59 interacts with DR6. Amino-acid coordinates of the deletion mutants of DR6(A) and CLIPR-59(B) are indicated. (C)DR6 wild type but not DCLR mutant could interact with endogenous CLIPR-59. 293T cells were transfected with indicated plasmid. At 48hr after transfection, cell extracts were analysed by immunoblotting (IB) either directly (WCE) or after immunoprecipitation with indicated antibodies (IP). (D)CLIPR-59 wild type but not DC mutant could interact with DR6. 293T cells were transfected with the combinations of empty vector (-) and indicated plasmid, and analysed as shown in C.

Fig.2

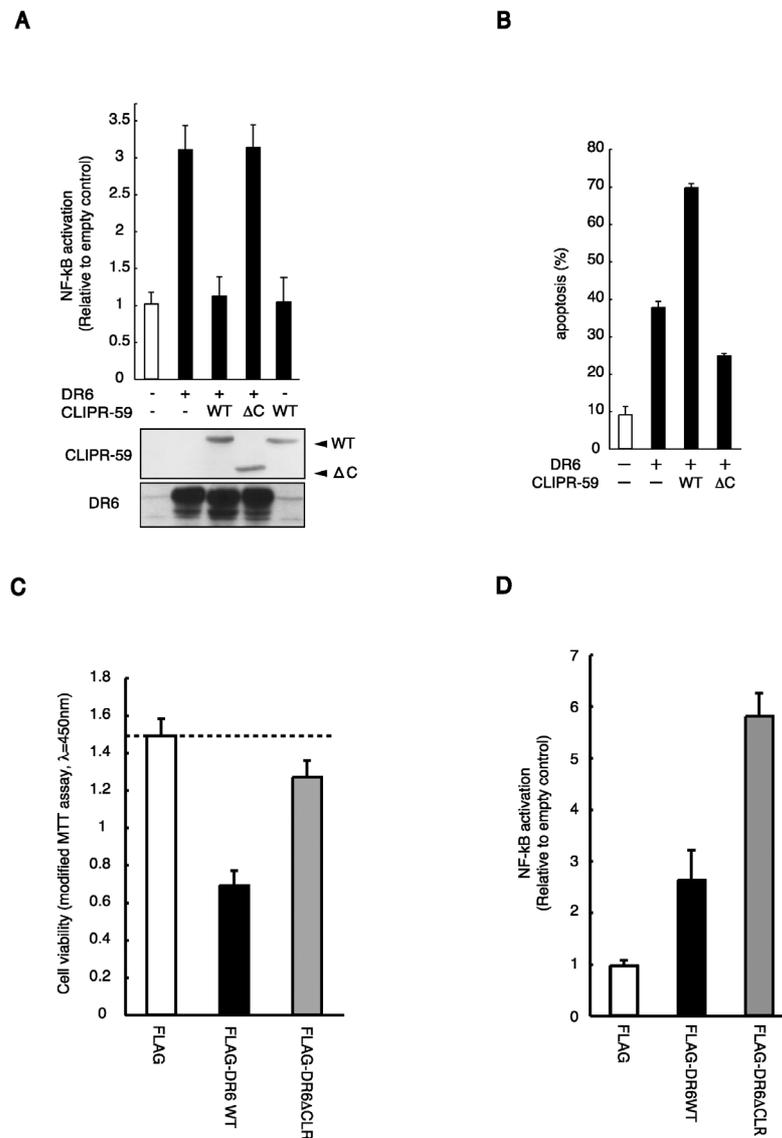


Figure 2. CLIPR-59 could inhibit NF-kB activation and induce apoptosis in DR6-mediated signaling. (A) Expression of CLIPR-59 could inhibit NF-kB activation in DR6 signaling. 293T cells were co-transfected with either empty vector (-) or indicated expression plasmid and NF-kB reporter plasmids. NF-kB activation was analysed as shown in Methods. Relative expression of the DR6 and CLIPR-59 or these mutants is shown in lower panel. (B) CLIPR-59 enhanced DR6-mediated apoptosis. Cells were transfected with indicated plasmids. At 24hr after transfection, apoptosis was determined as described in Method. Deletion of CLR on DR6 reduced cell death (C) and enhanced NF-kB activation (D). HeLa cells (5×10^4 cells) were seeded in 24well culture plate. Next day, cells were transfected with indicated plasmid, and at 48hr after transfection, cell viability assay or luciferase assay were done as described in Methods.

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- 1) Biological Sciences
- 1-1) Molecular Biology

Neuronal cell death and malfunction of intracellular-trafficking

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Introduction

ALS2, a causative gene product for a number of juvenile recessive motor neuron diseases (MNDs), acts as a guanine nucleotide exchange factor (GEF) for small GTPase Rab5 and enhances early endosome fusion through the activation of Rab5. Thus, ALS2 may play an important role in the survival and maintenance of motor neurons via regulating membrane trafficking. However, detailed functions of ALS2 are still unclear. The aim of this study was to delineate molecular function of ALS2 and its interactors, thereby clarifying the molecular mechanisms for neuronal cell dysfunction and death in the ALS2-linked MNDs.

Results

First, to examine the ALS2 localization in the cells with different developmental stages, hippocampal neurons were isolated from hippocampi dissected from post neonatal day 1 mouse, and cultured for a different period of time. In stage 3 neurons, ALS2 was distributed to cytoplasm and patchy membrane structures. ALS2 was also enriched in membrane ruffles at growthcone colocalizing with F-actin. In stage 5 neurons, ALS2 was localized to endosomes both in dendrites and in axon, which were partially labelled with Alexa-594 transferrin.

Recently, we have identified a novel ALS2 homolog, ALS2 C-terminal like (ALS2CL), which is highly homologous to the C-terminal half of ALS2. To delineate the molecular and cellular functions of ALS2CL, and the functional relationship between ALS2 and ALS2CL in the cells, we next conducted yeast two-hybrid assays, immunoprecipitation, and gel-filtration analysis. The results showed that a majority of ALS2CL was present as a homo-dimeric form, which could turn into interaction with the ALS2-oligomer, resulting in the formation of the large ALS2/ALS2CL heteromeric complex. Although formation of such heteromeric complex exhibited no significant impact on their Rab5GEF activity *in vitro*, overexpression of ALS2CL significantly increased the detergent-insoluble fraction of ALS2 with concomitant increase of the vesicular colocalization of ALS2CL with ALS2 in the cells. Further, ALS2CL dominantly suppressed the endosome enlargement induced by a constitutively active form of ALS2, and induced extensive perinuclear

tubulo-membranous phenotypes instead. Collectively, ALS2CL is implicated in the modulation of ALS2-mediated endosome function in the cells.

Finally, to further investigate the effects of ALS2 absence on cellular function, particularly receptor-mediated endocytosis in detail, we have prepared primary fibroblasts from new born *Als2*^{-/-} and *Als2*^{+/+} mice. Fibroblasts were exposed to Alexa Fluor-488 labeled epidermal growth factor (EGF) for 10 min, allowing the internalization of EGF via receptor-mediated endocytosis, and then analyzed at the 10 min, 30 min, and 60 min time points. Internalized EGF forms a punctate pattern of vesicles, representing EGF-positive endosome compartments. No gross abnormality was observed at all time points analyzed both in *Als2*-null and wild-type cells. These results imply that endocytosis of the EGF receptor *per se* does not require ALS2. However, a quantitative analysis of the fluorescence intensities of the EGF-labeled endosomes/vesicles revealed that frequency of the vesicles with stronger fluorescent signals, thus larger in size, was significantly lower at a 10 min point in the *Als2*-null mutants than in the wild-type cells. Notably, signal intensities in the wild-type cells were gradually decreased thereafter, while those in *Als2*-null mutants rather increased with highest at a 30 min point. The results imply that trafficking and fusion of EGF-positive endosomes/vesicles in fibroblasts were significantly delayed by the lack of ALS2. Thus, ALS2 might control efficiency of vesicles/endosomes trafficking and fusion in the cells.

Discussion & Summary

We here demonstrated the evidences representing a unique endosomal localization of ALS2 in matured neurons and its positive effect on endosome fusion in the cells. These findings imply that ALS2 might regulate membrane trafficking through the endosome fusion, thereby mediating the survival and maintenance of motor neurons. Further, our results suggest that ALS2CL is a novel

ALS2 binding protein and may play an important role in the ALS2-mediated membrane/endosome trafficking in the cells (Fig.1). Thus, further studies on the neuronal functions of ALS2 and ALS2CL will lead to a better understanding of the pathogenesis for the ALS2-linked as well as other MNDs.

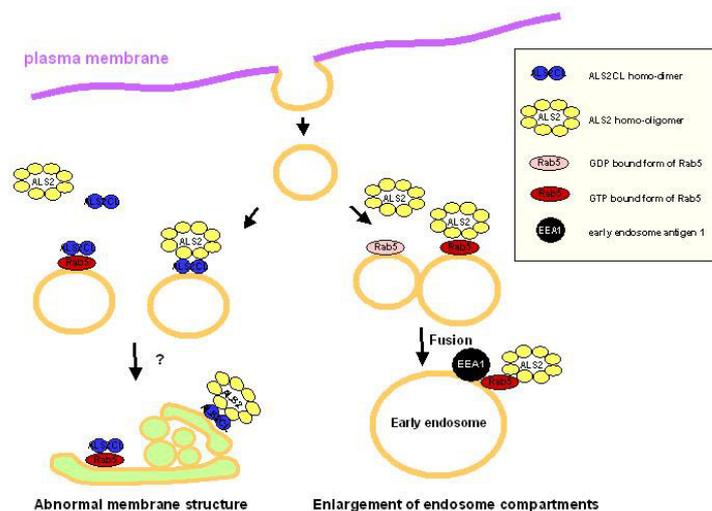


Fig. 1. Models for the molecular function of ALS2 and ALS2CL.

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- 1) Biological Sciences
1-1) Molecular Biology

Proteomic Studies of Hepatitis C Virus

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Introduction

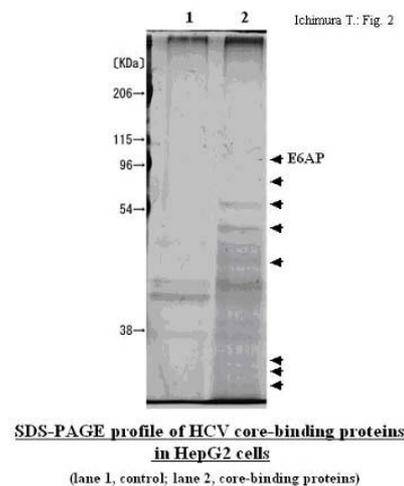
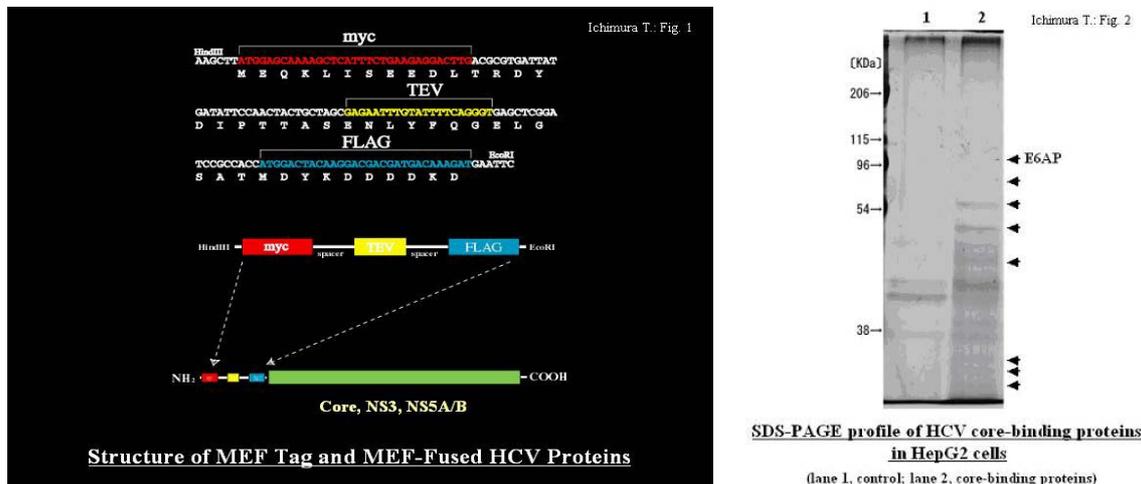
Hepatitis C virus (HCV) has recently been discovered to be the cause of majority of blood born non-A and non-B hepatitis. This virus encodes a single polypeptide of 3011 amino acids, which is then processed into 10 mature structural and regulatory proteins in infected cells. A number of host factors that could bind with these virus proteins have already been identified by use of yeast two hybrid system and GST-pull down assay. However, the molecular mechanism by which the HCV infection induces pathogenesis in host cells is still largely unknown.

Results

We recently developed a novel tandem affinity purification approach coupled with mass spectrometry-based proteomics technology to identify novel protein-protein interactions (1). The purification strategy employs an amino-terminal affinity tag (we call it MEF tag) composed of two independent epitope tags, myc and FLAG, that are fused in tandem and separated by the spacer sequence containing a TEV protease cleavage site. A series of immunoprecipitation, cleavage with TEV protease, and elution with FLAG-peptide allowed us to identify very specific protein-protein interactions (1-3). Here, we applied the MEF method to isolate novel host factors associated with virus proteins in human hepatoblastoma HepG2 cells.

We used the adenovirus expression system to express recombinant proteins, because this system permits suitable expression of exogenous proteins in most mammalian cells. The MEF cassette was fused to the N terminus of HCV structural and regulatory proteins (core, NS3, NS5A and NS5B)(Fig. 1), and the MEF-core, -NS3, -NS5 or -NS5B cDNA was inserted into the multicloning sites in an adenovirus expression vector. HepG2 cells were then transformed with recombinant adenovirus to express each MEF-fused protein. Immunoblotting using anti-myc monoclonal antibody demonstrated that all the fused proteins were expressed in HepG2 cell with relatively high amounts (data not shown). After the HepG2 cells expressing MEF-core were lysed, the MEF-core was recovered with its binding partners by the procedures of MEF method (1). Ultimately, the protein complexes bound to FLAG beads were dissociated with a synthetic FLAG peptide. SDS-PAGE

analysis revealed that several host factors including E6AP, an E3 ubiquitin ligase that had previously been identified as a core binding partner in human kidney Hek293 cells, were specifically interacted with the expressed MEF-core in HepG2 cells (Fig. 2, marked).



This procedure was also similarly useful to characterize the binding partners for NS3 and NS5B in HepG2 and Hek293 cells (data not shown), suggesting that the expression system using adenovirus and MEF cassette is suitable for the functional proteomic analysis of HCV virus proteins.

In a separate study, we further characterized the previously identified interaction between HCV core protein and E6AP. We confirmed that E6AP directly binds HCV core both *in vivo* and *in vitro*. Immunofluorescence study revealed that HCV core and E6AP are predominantly colocalized in cytoplasm. Exogenous expression of E6AP promoted proteasomal degradation of the core protein in hepatic cells and in non-hepatic cells. Knockdown of endogenous E6AP by siRNA resulted in increasing the levels of HCV core protein. Furthermore, *in vivo* and *in vitro* ubiquitylation assays revealed that HCV core protein is polyubiquitylated by E6AP. Taken together, these results suggest that E6AP functions as the E3 ubiquitin-protein ligase that mediates ubiquitylation and degradation of HCV core protein *in vivo*.

Discussion & Summary

In summary, our results identified E6AP as a novel HCV core binding partner in both Hek293 cells and HepG2 cells. We also confirmed that HCV core protein directly interacts with E6AP *in vitro*. HCV core and E6AP are found to colocalize in the cytoplasm. We further suggested that E6AP mediates ubiquitylation and degradation of HCV core protein. From these results, we propose that the abundance of HCV core protein is regulated by E6AP-mediated ubiquitin proteasome pathway. Further studies will help to understand new targets of HCV pathogenesis in hepatoblastoma cells.

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1) Biological Sciences

1-1) Molecular Biology

Analysis of physiological roles of catecholamines and vasopressin on fat and carbohydrate metabolism in insulin resistance, obesity and diabetes.

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Introduction

Catecholamines and vasopressin play critical roles in cardiovascular regulation and endocrine systems. In addition, they are involved in regulating carbohydrate and fat metabolism via insulin secretion from pancreas or glycogenolysis in liver. In order to investigate the functional roles of catecholamines and vasopressin on carbohydrate metabolism as well as in developing obesity, mutant mice lacking or overexpressing alpha 1 adrenergic receptor or vasopressin receptor are generated and assessed in vivo.

Results

We found that mutant mice lacking V1a vasopressin receptor have higher blood glucose level during feeding compared to wild type mice. By analyzing with GTT and ITT, the mutant mice showed decreased insulin sensitivity. The levels of blood glucose in the mutant mice were 165 and 103 mg/dL at feeding and fasting, respectively, while the levels in control mice were 124 and 83 mg/dL. On the other hands, fat tissue in the mutant mice was significantly heavier than that in controls, suggesting that the mutant mice could be prone to become diabetic mellitus and obesity. In addition, during feeding with high fat diet, the mutant mice showed increased weight gain and decreased insulin sensitivity.

Discussion & Summary

Our study implicated that antagonism for V1a vasopressin receptor could lead to diabetic mellitus and obesity and specific ligands for the receptor would be useful for treating these disorders.

1) Biological Sciences
1-3) Cell Biology

The molecular mechanism of activity-dependent vesicle transport regulated by synaptotagmin IV

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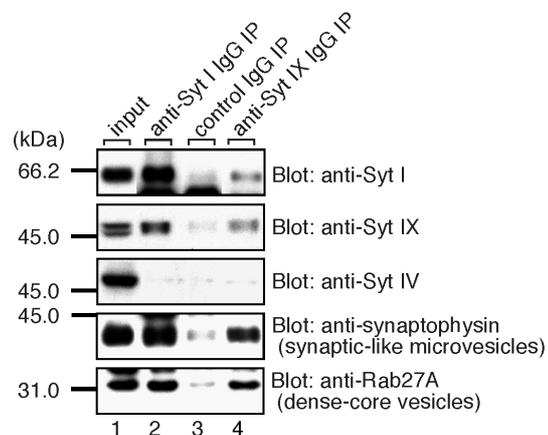
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Introduction

Synaptotagmin (Syt) is a putative membrane trafficking protein that contains an N-terminal single transmembrane domain and C-terminal tandem C2 domains. Among the Syt family members, Syt IV has been suggested to control synaptic plasticity, because its mRNA is rapidly increased by membrane depolarization (or synaptic activity) and Syt IV protein is sorted from the Golgi to the cell periphery (referred to as “activity-dependent vesicle transport”) (refs. 1-3). However, the precise role of Syt IV protein in synaptic plasticity and the cargos of Syt IV-containing vesicles still completely remains unknown. The aim of this research is to determine the specific cargo of Syt IV-containing vesicles

Results

To isolate Syt IV-containing vesicles from membrane fractions of PC12 cells or mouse brain, we produced rabbit polyclonal antibody against Syt IV, and the specificity of the antibody was confirmed by probing with COS-7 cell lysates expressing Syt I-XV as described previously (ref. 1). Specific antibody against Syt I or Syt IX was obtained from commercial sources. We coupled purified antibody against Syt I, IV, or IX with magnetic beads (Dynabeads M-280), and the beads coupled with either anti-Syt I, IV, or IX antibody was incubated for 1 hr with crude membrane fractions of PC12 cells or developing mouse brain in 0.03M sucrose, 5 mM HEPES-KOH, pH 7.2, and 5 mM EDTA (ref. 4). After washing the beads with PBS containing 2 mM EDTA, the bound fractions were analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting with anti-synaptophysin (a marker for synaptic vesicles), anti-Rab27A (a marker for dense-core



vesicles), anti-Syt I, anti-Syt IV, and anti-Syt IX antibodies. We found that Syt IV protein in PC12 cells was not co-purified with Syt I-containing vesicles, Syt IX-containing vesicles, or secretory vesicle markers (Figure 1), whereas Syt I and Syt IX proteins were co-purified with synaptophysin (i.e., synaptic-like microvesicles) and Rab27A (i.e., dense-core vesicles). We further showed by subcellular fractionation study using a 0.15 - 0.9 M sucrose gradient that Syt IV protein was absent in the Syt I-containing vesicles from mouse brain (i.e., synaptic vesicles) and instead present in the membrane fractions larger or denser than synaptic vesicles. Our result strongly indicates that Syt IV-containing vesicles derived from the Golgi are different from conventional synaptic vesicles in brain or dense-core vesicles in PC12 cells.

Discussion & Summary

In the present study, we have succeeded in isolating Syt IV-containing vesicles from developing mouse brain or PC12 cells and found that Syt IV protein is not present on conventional secretory vesicles, where Syt I (or IX) is normally present. We speculate that Syt IV is present on a novel type of vesicles that may contain molecules involved in synaptic plasticity or activity-dependent changes of synapse. We are now trying to determine the specific cargo molecule(s) of Syt IV-containing vesicles by two-dimensional electrophoresis combined with tandem Mass spectroscopy. Future study will clarify the mechanism of how Syt IV is involved in synaptic plasticity at the molecular level.

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1) Biological Sciences

1-3) Cell Biology

Relationship between carcinogenesis and the control of the ribosome and translation

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Introduction

The cytoplasmic ribosome, a huge RNA-protein complex essential for protein synthesis, has been treated as a “black box”, especially in the research on higher organisms. The structures, functions, and regulations of ribosomal proteins are largely unknown. In the present study, proteomics screening has been performed to clarify qualitative changes in the mammalian ribosome.

Results

For the purpose of this study, we selected the translational machinery in the mouse mammary gland as an analytical target, because this tissue repeats a dynamic change in protein synthesis during the periods of proliferation, differentiation, and a regressive phase, involution. The mammary tissue samples were collected from female mice on lactation day 10 (L10; time point of large production of milk proteins) and 48 hours after a forced weaning (involution day 2, I2; end point of milk protein production). The polysome fractions were isolated by biochemical methods such as sucrose density gradient ultracentrifugation. Proteins in these fractions were separated by two-dimensional gel electrophoresis, and the electrophoretic patterns were compared between L10 and I2. Most of ribosomal proteins are known to be very basic (isoelectric point > 10). The radical-free and highly reducing (RFHR) method, which we have used (instead of a conventional method based on the O’Farrell method) and is specialized for such very basic proteins, provided good separation of many protein components in the ribosomes (Fig. 1).

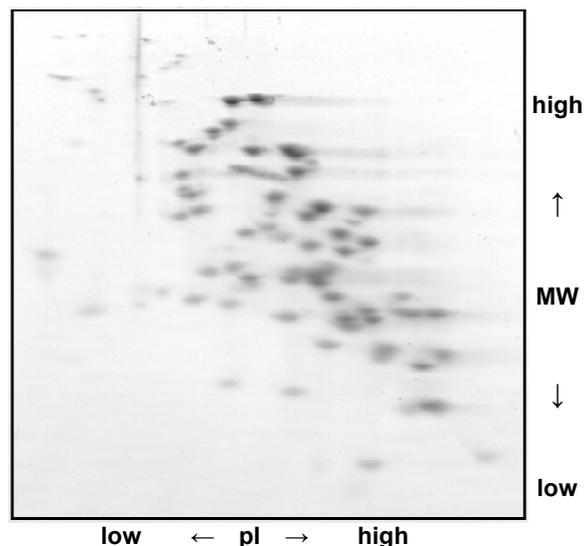


Fig.1. Two-dimensional electrophoretogram of ribosomal proteins from the polysome collected at L10.

Proteins were stained with a Coomassie brilliant blue method modified for low-molecular-weight proteins, collected from the gels, digested in gel with trypsin, and subjected to MALDI-TOF mass spectrometry (including tandem mass spectrometry). Protein spots specific to each time point were intensively analyzed. Proteins were identified using the Mascot program. Part of the results is shown in Fig. 2. By this comprehensive screening, specific ribosomal proteins have been found to be more phosphorylated at L10 than at I2, suggesting that the post-translational modifications may be linked to efficiency of protein synthesis. In addition, Rack1, receptor for activated C-kinase 1, was identified at L10 and I2. Immunoblotting using antibodies against phosphorylated ribosomal S6 and Rack1 has been a method suitable for confirmation and further analysis of these data. The findings implicate an intriguing relationship between protein phosphorylation and translational control. We have also obtained some candidates for new functional components in the ribosomes, analysis of which is in progress in our laboratory.

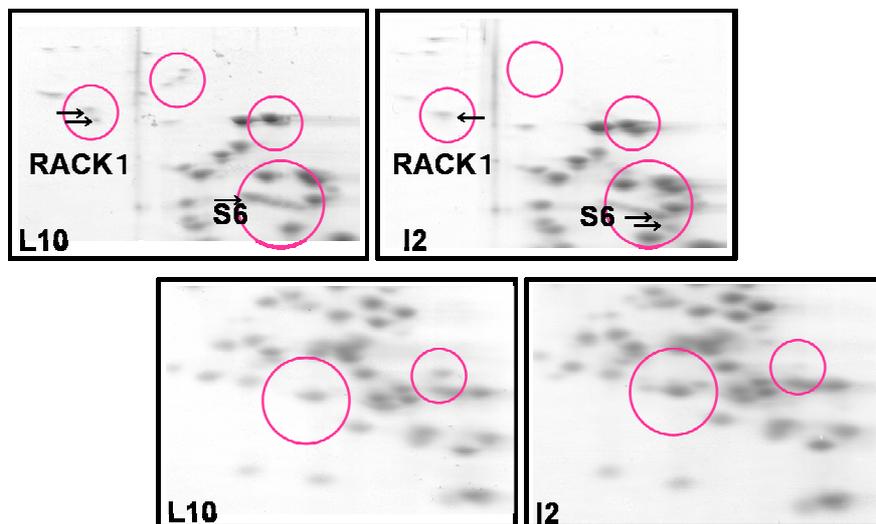


Fig. 2.

Comparison of protein bands between L10 and I2: qualitative changes in the components of the ribosome. S6, ribosomal protein S6; RACK1, receptor for activated C-kinase 1. These two proteins were identified by mass spectrometry.

Discussion & Summary

Proliferation of cancer cells accompanies the upregulation of protein synthesis. Therefore, it is easy to speculate the enhancement of functions of the ribosome and increased synthesis of the ribosome itself in these cells. Actually, fragmental data have been obtained to support this hypothesis. In other words, it is natural to consider that these mechanisms (for translation and ribosome biogenesis) are good targets for cancer therapy. The comprehensive analysis of the ribosome will be fundamental basic knowledge from this therapeutic viewpoint.

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1) Biological Sciences

1-3) Cell Biology

Studies of the regulatory mechanism in EMT mediated by the Zn signaling

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Introduction

During embryonic development, organ and tissue regeneration, and cancer progression, epithelial cell subpopulations actively downregulate cell-cell adhesion systems, and leave their “local neighborhood” to move into new microenvironments. This regulated phenotypic modulation including cell-cell dissociation and cytoskeleton remodeling, is called epithelial-mesenchymal transition (EMT). The Snail/Slug family zinc-finger transcription factors, master regulators of EMT, are responsible for the transcriptional repression of epithelial genes, and for the transcriptional activation of mesenchymal genes. The transcriptional activity of Snail is regulated by its intracellular location. We previously showed that LIV1, a breast cancer associated zinc transporter protein, is essential for the nuclear localization of Snail, however its precise mechanism is unknown. In this project, we try to reveal molecular mechanism of zinc transporter LIV1 mediated zinc signalling, which regulates subcellular location of zinc finger transcription factor Snail and induces EMT.

Results

Zinc is a structural constituent of a great number of proteins, including enzymes in cellular signaling pathways and transcriptional factors, and it is essential for their biological activity; however, in excess it is toxic to cells. Thus, the intracellular zinc concentration must be tightly controlled by the zinc importers ZIPs/SLA39s, exporters ZNTs/SLC30s, and binding proteins metallothioneins, to maintain its homeostasis. In the course of the cell cycle and differentiation, the cellular zinc level is altered, and zinc accumulates in the nucleus; however, zinc, unlike calcium, has not been thought of as a cellular signaling molecule. We previously showed that the Stat3-Liv1/Zip6 cascade is critically involved in the epithelial-mesenchymal transition (EMT) during zebrafish gastrulation, and is required for the nuclear localization of Snail1, a master regulator of the EMT. Intriguing questions are how the membrane protein Liv1/Zip6 moves Snail1 into the nucleus and whether zinc is involved in this process. Here we show that zinc transduces the signal from the zinc transporters,

ZIPs, to the zinc-finger transcriptional factor SNAIL1 during EGF-induced EMT in wound healing and cancer invasion. EGF induces the expression of ZIP10, which leads to the uptake and nuclear accumulation of zinc. This actively transported zinc is coordinated by the finger domain on SNAIL1 and localizes SNAIL1 to the nucleus to initiate the EMT in keratinocytes and cancer cells. Thus, zinc is a regulator of the EMT during wound healing and cancer progression, and may act as a second messenger to transduce extracellular stimuli to zinc metalloproteins via zinc transporters.

Discussion & Summary

Our results established that zinc is a regulator of the EMT during wound healing *in vitro* and cancer progression *in vitro* and *in vivo*, and suggest that zinc may act as a second messenger transducing extracellular stimuli to zinc metalloproteins via zinc transporters. Zinc was actively transported by ZIPs and coordinated at the zinc-finger domain of SNAIL1, precluding its nuclear export, and resulting in increased levels of SNAIL1 in the nucleus, thereby enabling the induction of EMT. These results provide a molecular explanation for the zinc transporter ZIP-dependent signaling and its relevance in the nuclear accumulation of the zinc-finger protein SNAIL that is induced by EGF stimulation, as well as a conceptual basis for understanding the intracellular zinc signaling used by other zinc metalloproteins.

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1) Biological Sciences

1-3) Cell Biology

The function of human BIG family proteins in the membrane trafficking between TGN and endosomes

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Introduction

Membrane traffic between organelles is mediated by vesicular and tubular intermediates that carry cargo proteins. Small GTPases of the ADP-ribosylation factor (ARF) family trigger budding of coated vesicles by recruiting coat protein complexes onto organelle membranes (Fig.1). ARF switches between a GDP-bound (inactive) state and a GTP-bound (active) state capable of transmitting signals to its effectors. The exchange of GDP for GTP is catalyzed by guanine nucleotide exchange factors (GEFs) (1). Among the ARF-GEFs, we have focused on the high molecular weight and brefeldin A (BFA) sensitive BIG family, BIG1 and BIG2, and investigated their function in the intracellular membrane trafficking.

Results

In order to investigate the specific and redundant function of BIG family ARF-GEFs, we set out to deplete BIG1 or BIG2, or both ARF-GEFs by utilizing an RNAi approach. First, we confirmed the specific knockdown of BIG1 or BIG2, or both by immunoblot analysis of lysate from HeLa cells subjected to RNAi. In cells subjected to RNAi for BIG1, there was a significant decrease in the level of BIG1 (70 %) but not in that of BIG2. Reciprocally, RNAi for BIG2 suppressed specifically the expression of BIG2 (90 %) but not that of BIG1. Cells subjected to RNAi for both ARF-GEFs showed depletion of both proteins (3).

Consistent with our previous study (2) showing that the expression of catalytically inactive BIG2 mutant induces the membrane tubulation of recycling endosomes, depletion of BIG2, but not that of BIG1, induces the tubulation of the recycling endosomes but not that of the Golgi complex. On the other hand, depletion of both BIG1 and BIG2, but not each of them, resulted in the disappearance of AP-1 from the Golgi complex. Consistent with this result, it has been demonstrated that AP-1 is recruited to the Golgi membrane by active ARF and its recruitment is inhibited by BFA treatment. Moreover, TGN46, which recycles between TGN and plasma membrane, partially disappeared from the TGN suggesting that depletion of BIG1 and BIG2 affects the TGN46 recycling pathway. The

depletion of BIG1 and BIG2 did not significantly affect the localization of other Golgi marker proteins, such as golgin-245 and GM130, excluding disintegration of the Golgi structure. These results indicate that BIG2 alone play a role in the integrity of recycling endosomes and BIG1 and BIG2 function redundantly in the AP-1 recruitment to the Golgi membrane (Fig.1). Moreover, BIG1 and BIG2 may function redundantly in the transport pathway between Golgi and plasma membrane via endosomes.

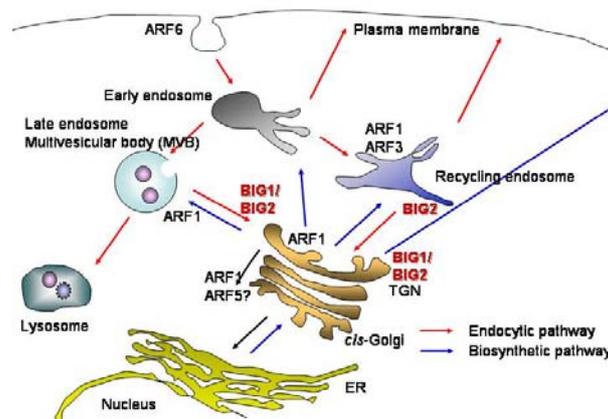


Fig.1 Intracellular membrane trafficking

We therefore, have examined whether BIG1 and BIG2 is involved in the anterograde transport pathway from the Golgi to the plasma membrane and the retrograde transport from the endosomes to the Golgi complex. To investigate the former pathway, we make use of the temperature sensitive vesicular stomatitis virus glycoprotein (VSVG). In a permissive temperature, VSVG is delivered from the ER to the plasma membrane via the Golgi complex. The VSVG delivery from the Golgi complex to the plasma membrane, however, was delayed in BIG1 and BIG2 depleting cells, but neither BIG1 nor BIG2 depleting cells (Fig.2). In contrast, the VSVG transport from the ER to the Golgi complex was not affected. Interestingly, we have frequently observed tubular VSVG carriers in BIG1 and BIG2 depleting cells suggesting that ARF dependent budding step seems to be inhibited (Fig. 2). Now we are trying to dissect this phenomena using time lapse video microscope.

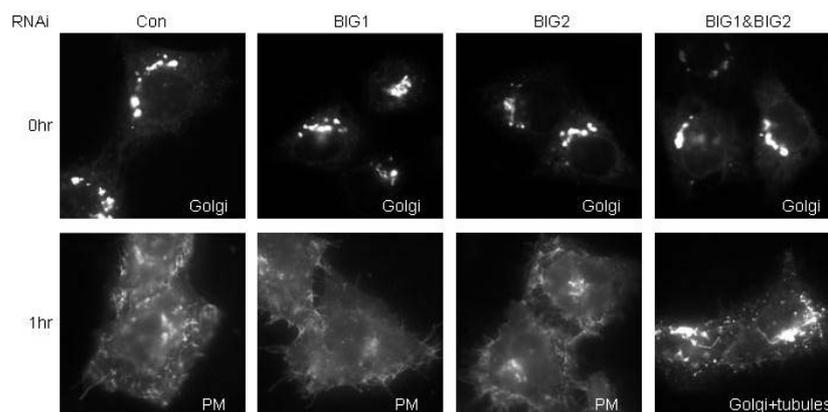


Fig.2 Depletion of both BIG1 and BIG2 inhibits the VSVG transport from the Golgi to the plasma membrane. EGFP-VSVG tsO52 was transfected in HeLa cells pretreated with con, BIG1, BIG2 or BIG1 and BIG2 siRNAs and the cells were incubated in the non-permissive temperature (42°C) for overnight. Cells were incubated in 20°C for 3hrs (0hr, VSVG is localized to the Golgi) in the presence of cycloheximide and then shifted to the permissive temperature (32°C) for 1hr (1hr, VSVG is localized to the plasma membrane (PM) except the last lower panel). Cells were fixed and performed the immunofluorescence analysis.

Next, to examine latter pathway, we made use of a chimera protein of CD4-furin which is known to recycle the Golgi and the plasma membrane via endosomes like TGN46. It has been shown that AP-1 adaptor complex is required for the retrograde transport of furin from endosomes to the TGN. The transport of furin from endosomes to the Golgi was also delayed in both BIG1 and BIG2 depleting cells but neither BIG1 nor BIG2 depleting cells.

Discussion & Summary

Here, we have extended our previous studies (2) and made three important findings concerning BIG1 and BIG2. First, BIG2 but not BIG1 is specifically required for the integrity of recycling endosomes that is consistent with our previous observations (2). Second, both BIG1 and BIG2 redundantly act on the AP-1 recruitment to the Golgi complex that is an excellent agreement with the dissociation of AP-1 from the Golgi complex upon BFA treatment. Third, BIG1 and BIG2 function redundantly in the anterograde transport of VSVG as well as the retrograde transport of furin (Fig.1). Further dissection of the function of BIG1 and BIG2 in these pathways will bring an important key to understanding how ARF regulates various transport pathways in an appropriate time and place.

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- 1) Biological Sciences
- 1-4) Developmental Biology

Studies on human hereditary disease using medaka as a model
An analysis of pc medaka mutant showing a phenotype
of polycystic kidney disease.

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Introduction

Polycystic kidney disease (PKD) is a common hereditary disease, which leads to massive kidney enlargement by the cystic dilation of renal tubules and chronic renal failure. Various murine models of PKD have been generated from naturally occurring or experimentally induced mutants and are used for the understanding of PKD. In medaka, pc (polycystic) mutant is a fish model for this kidney disorder developing bilateral massive enlargement in adulthood in an autosomal recessive trait.

Results

- 1) Developmental stage when kidney tubules start to enlarge was examined in pc mutant. In pronephros, which form during embryogenesis, enlargement of the tubule was observed just after hatching, consequently to form cysts.
- 2) Mesonephros, generated in the surrounding tissue of the pronephros, developed a number of cysts although we could not distinguish between mesonephric and pronephric.
- 3) By positional cloning, we identified the responsible gene of pc mutant. pc gene consists of at least ten exons and encodes a novel transcription factor containing five repeats of C2H2-zinc finger. In pc mutant, a large fragment of transposon-like sequence was found to be inserted in the intron 4 of this gene. This insertion was considered to alter splicing site of pc mRNA to produce C-terminal truncated pc protein.
- 4) In wild type medaka, pc gene was specifically expressed in the epithelial cells of tubules and ducts in both pronephric and mesonephric kidneys., which was shown by in situ hybridization.
- 5) Knock-down of pc gene with antisense-oligo led to cyst formation in pronephros

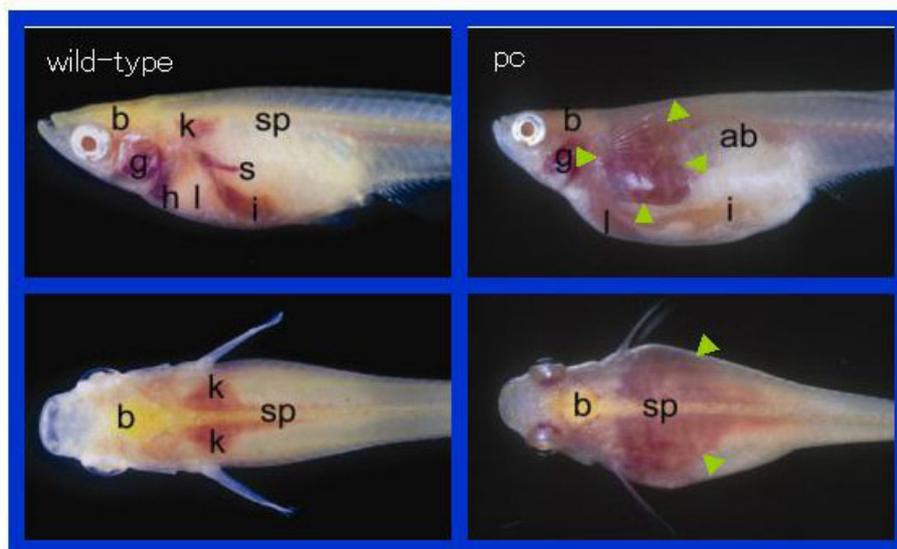
Discussion & Summary

Our histological analysis has shown that *pc* mutant medaka is the counterpart of human PKD. *pc* mutant develops renal cysts at an early stage in pronephros as well as in mesonephros. In medaka early initiation of PKD was not lethal, which might be due to the complementary function of the gill.

pc gene is a novel PKD gene, which is not known as a causal gene in mammalian models and human, provides a new insight into the mechanism underlying generation and maintenance of tube structure in kidney.

Our findings here have demonstrated that medaka is a good model animal for human genetic disease.

Figures & Tables



Appearance of transparent medaka *pc* mutant

left-side: wild-type

right-side: *pc* mutant

kidney enlargement is obvious in the living transparent medaka.

ab, air bladder; b, brain; g, gill; h, heart; k, kidney; li, liver; s, spleen; sc, spinal cord

Mochizuki et al. *Kidney International*, 68, 23-34 (2005)

- 1) Biological Sciences
1-4) Developmental Biology

The role of Notch ligand for organogenesis and regeneration of various tissues.

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Introduction

Organ-specific stem cells are maintained in organ-specific environment, which is called as niche, and produce various progenies during organogenesis and tissue repair after various injuries. These stem cells will be useful to transplant into patient who bears serious problem of an organ. However, molecular basis of these phenomena, especially molecule(s) that composes the environment, has not been determined. Recently, Notch/Notch ligand was reported to contribute for these events. In this study, I use the conditional targeting mice of Notch ligands, Dll1¹⁾ or Jagged²⁾, I've recently established, to know their physiological significance for various organogenesis and tissue repair.

Results

Jagged1- or Dll1-floxed mice were bred with Mx- (IFN-inducible) or K5-Cre (epithelial cell-specific) transgenic mice (Tgm), and administrated with IFN-inducer, poly I-C after birth for the former system, resulting in the gene deletion of the floxed allele. In these mice, the gene deletion was completely observed in bone marrow, liver (Mx-Cre) or skin (K5-Cre).

[A] The role of Notch ligand for the maintenance of the hematopoietic stem cells in vivo

At 4 weeks after the treatment of Poly I-C in Mx-Cre Tgm for the gene deletion, the cells resided in bone marrow were obtained and analyzed by flowcyometry. All hematopoietic lineages (myeloid (Gr1- or CD11b-positive), erythroid/megakaryocytic (Ter119-positive), lymphoid (CD19-positive) and their stem cells (as lineage markers-negative, Sca1-positive, c-kit-positive cells; HSC) were detected in Jagged1- or Dll1-null mice as well as that in WT mice. To know the role of Notch ligands for the maintenance of HSC precisely, GFP Tgm-derived BM cells were transferred into Jagged1- or Dll1-null mice (poly I-C treated) and traced by the expression of GFP. The development of blood cells derived from GFP Tgm was also investigated by flowcytometry. Moreover, 2nd BM chimeras were prepared and analyzed for their chimerism of blood cells. However, all blood cells developed normally in 1st and 2nd BM chimeras, and there is no difference between Notch ligand-deficient and WT mice as environment for the maintenance of HSC.

[B] The role of Notch ligand for hepatocytes

After the induction of gene deletion by the treatment of Poly I-C, lots of white spots were observed at the liver in Jagged1-null mice. In the HE staining of the liver section of Jagged1-null mice, the distinctive feature of cell death appeared at the middle area of hepatic lobules with no infiltration (Figure). This suggested that the cell death found in Jagged1-null mice was due to their intrinsic property but not infection. The dead cells lost clear-defined nucleus and seemed to fuse each other, but their boundary could be recognized. In contrast, Mx-Cre(-) or Mx-Cre(+), Dll1-floxed mice did not show this phenotype.

[C] The role of Notch ligand for the generation of epidermis.

To investigate the role of Jagged1 in adult skin, we crossed Jagged1-floxed mice with K5-Cre Tgm. The K5-Cre, Jagged1-floxed mice appeared normal at birth but went on to lose their hair and whiskers. Subsequent hair growth was sparse and the hairs were short, resembling the Notch1 deletion phenotype³⁾.

Discussion & Summary

We had established two floxed mice for Notch ligands, Dll1 and Jagged1, to know their physiological significance as an environmental factor for organogenesis or regeneration of various tissues. In this study, we investigated for the maintenance of HSC in bone marrow, of hepatocyte viability or the generation of skin and hair, by breeding with Mx- or K5-Cre Tgm.

For the maintenance of HSC, both Notch ligands were not necessary. Recently, it was reported that Jagged1 is expressed on the osteoblasts which is estimated to provide the stem cell niche, the suitable place for the maintenance of HSC in bone marrow. We could see the gene deletion of Jagged1 in CD45-negative, adherent cells including enriched osteoblast, but its efficiency was about 80% evaluated by quantitative PCR analysis. This means that a definitive conclusion could not be obtained from this experiment. To induce the gene deletion in osteoblasts efficiently, we've bred the Jagged1-floxed mice with a1Col-Cre Tgm where Cre is expressed in osteoblasts. Moreover, it is possible to establish Jagged1, Dll1, double-floxed mice, to make clear their significance for that.

For the hepatocyte viability and the generation of normal hair, it was demonstrated that Jagged1, but not Dll1, is indispensable. However, its molecular machinery remains to be determined. The relationship with the apoptosis in hepatocytes, and with regulation of the cell cycle in hair follicular cells should be investigated.

Figures & Tables

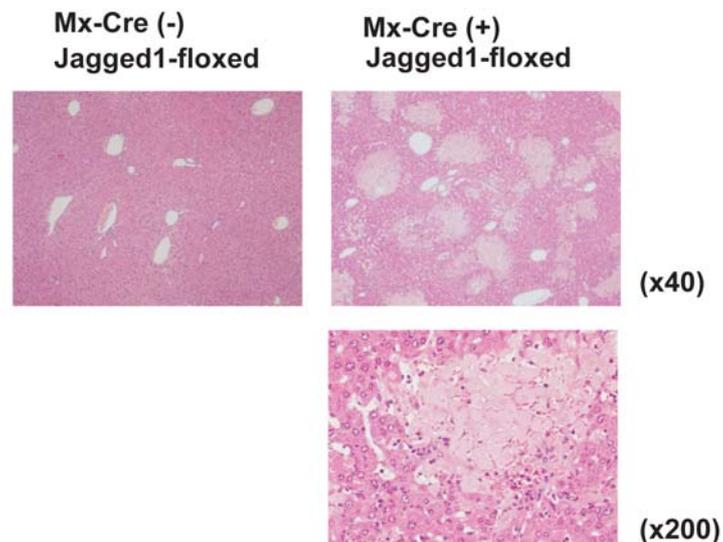


Figure. The deletion of Jagged1 gene after birth leads the cell death of hepatocytes. The Jagged1-floxed mice with or without Mx-Cre transgene were administered with poly I-C after birth to induce the gene deletion. At 4 weeks after the treatment, liver section were prepared and stained with HE.

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1) Biological Sciences
1-4) Developmental Biology

Regulation of mesenchymal stem cell differentiation by phosphoinositide metabolism

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Introduction

Phosphoinositide metabolism is an important intracellular signaling system that is involved in a variety of cell functions, including secretion of hormones, transduction of neurotransmitter, growth factor signaling, and regulation of the cytoskeleton. Phospholipase C (PLC) is a key enzyme in this system that acts by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate two-second messengers, diacylglycerol and inositol 1,4,5-trisphosphate. Among the PLC family isozymes, PLC δ is evolutionally conserved and is thought to be the primary form in mammals.

We have reported that PLC δ 1 is required for skin stem cell lineage commitment and PLC δ 4 is involved in acrosome reaction in sperm by generating PLC δ 1- and PLC δ 4 gene-deficient mice. We also found that PLC δ 4 gene-deficient mice got fat, suggesting that PLC delta-type may be involved in differentiation of mesenchymal stem cells to adipocytes, osteoblasts and myocytes.

Results

PLC δ 4 gene-deficient mice showed obesity

First of all, we compared the weight of PLC δ 4 gene-deficient mice to that of wild-type mice (WT). Both male and female PLC δ 4 gene-deficient mice were about 20% heavier than WT at any age. We also observed the accumulation of many lipid-drops in liver and skeletal muscle of PLC δ 4 gene-deficient mice by histochemical staining of Oil-red-O.

Enhanced adipogenesis of primary MEF derived from PLC delta-type gene-deficient mice

Primary MEFs have multi-potencies to differentiate into adipocyte or cardiocyte. Then we examined the difference of adipogenesis in primary MEFs derived from PLC δ 1 gene-deficient, PLC δ 3 gene-deficient, and PLC δ 4 gene-deficient mice. Insulin-induced adipogenesis of MEFs from PLC δ 4KO mice was quite enhanced in compared to MEFs from WT mice by oil-red O-staining. On the other hand, basal level of adipogenesis was increased in MEFs from PLC δ 3 gene-deficient mice.

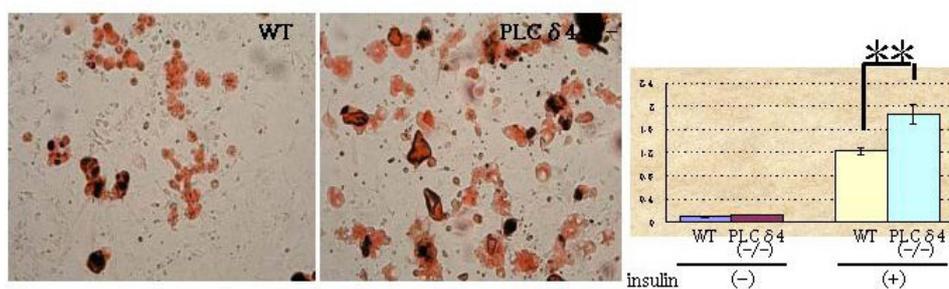
Abnormal differentiation of mesenchymal stem cells from PLC δ 3 gene-deficient mice into adipocytes and osteoblasts

We observed the remarkable decrease in osteogenesis, instead, the marked increase in adipogenesis of mesenchymal stem cells from PLC δ 3 gene-deficient mice. In addition, we noted a correlation between the expression of PLC δ 3 and the inductive osteogenesis by measuring alkaline phosphatase activity of C310T1/2 cells, which is a mesenchymal stem cell-line and established from bone marrow. Furthermore, when we carried out PLC δ 3-RNAi in C310T1/2 cells, osteogenesis of C310T1/2 cells was partially inhibited. These results suggest that PLC δ 3 is involved in lineage commitment of mesenchymal stem cells between adipocytes and osteoblasts.

Discussion & Summary

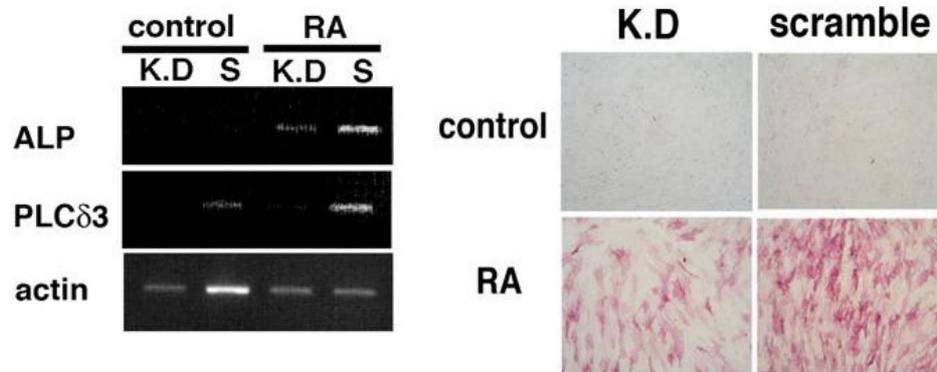
This time we have indicated that PLC δ 4 gene-deficient mice got fat and adipogenesis of MEFs derived from PLC δ 4 gene-deficient mice was enhanced. These results suggest that PLC δ 4 may have important role in adipogenesis. We also showed that mesenchymal stem cells from PLC δ 3 gene-deficient mice differentiated abnormally into adipocytes and osteoblasts, indicating that PLC δ 3 may be involved in lineage commitment of mesenchymal stem cells. It may be necessary to understand the mechanism of how these PLC delta-type proteins are involved in adipogenesis or osteogenesis.

Figures & Tables



Insulin-induced adipogenesis of mouse embryo fibroblast (MEF)s from PLC δ 4KO mice was enhanced.

The primary MEFs were prepared from E14.5 embryo of wild-type (WT) mice or PLC δ 4 KO (-/-) mice. The MEFs were then treated by insulin to differentiate into adipocytes and adipocytes were stained by Oil-Red-O.



PLC δ 3-RNAi inhibits differentiation of C3H10T1/2 cells into osteoblasts.

The expression of PLC δ 3 was knock down by PLC δ 3-RNAi (K.D.) in C3H10T1/2 cells. The cells were then treated by retinoic acid (RA) to differentiate into osteoblasts. Left panel: The expression of PLC δ 3 and alkaline phosphatase (ALP) were examined. Right panel: Osteogenesis was measured histochemically by the activity of alkaline phosphatase.

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1) Biological Sciences
1-4) Developmental Biology

Molecular genetic analysis of the signaling pathway of the DIF, an anti-tumor substance involved in the development of a model organism, *Dictyostelium discoideum*.

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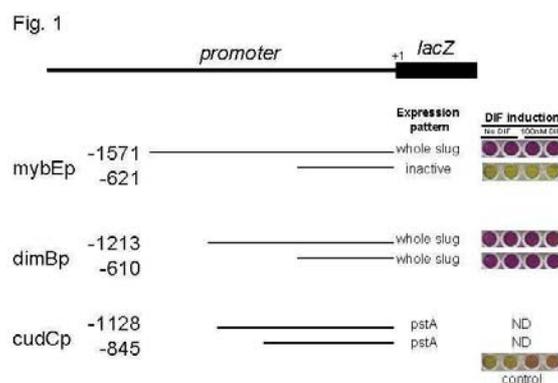
Introduction

DIF (Differentiation-Inducing Factor) made by *Dictyostelium discoideum* cells acts as an anti-tumor and differentiation-inducing substance on mammalian cells. In order to understand the DIF action on mammalian cells it is necessary to reveal the intrinsic DIF signaling pathway in *Dictyostelium*. I have characterized two transcription factors, MybE (a SHAQKY family Myb) and DimB (a bZIP family), as the endpoint of the pathway. In this report I have cloned gene regulatory regions of these two factors and analyzed their expression pattern to define the place of action within multicellular stage.

Results

Analysis of the expression patterns of the promoters

Promoter fragments to each transcription factor gene were generated by PCR and cloned into pDdGal, a beta-galactosidase (*lacZ*) reporter with G418 selection. Figure 1 shows the structure of the reporter constructs. To dissect the promoter region roughly, a half-length of the longer ones (ca -600 fragment of *mybEp* and *dimBp*, respectively) was also tested for its expression. The -621 promoter fragment of *mybE* was inactive, showing that the cis regulatory region locates between -1571 to -621. For *dimBp*, the -610 fragment is sufficient for its expression. When multicellular structure (*slug*) was stained for beta-galactosidase activity, both *mybEp* and *dimBp* showed ubiquitous expression, with a slight gradient of expression from anterior tip towards posterior (Fig. 2, A and C). The promoter activity of the transformants was so high and, together with the stability of



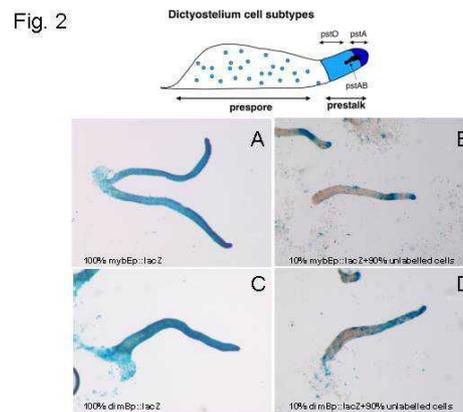
beta-galactosidase, the strong uniform staining observed might be due to saturation of the staining. To overcome this the transformant cells were diluted 10 times with unlabelled wild-type cells to reduce the overall staining intensity. The resultant slug with mybEp construct showed a distinct pattern of expression; high expression at the tip and the front 1/3 of the posterior prespore region, and no expression within pstO region (Fig. 2B). To my knowledge, this complex pattern has never been shown by any other promoter construct previously described. In Arabidosis, CAPRICE(CPC), a Myb-like protein, is transported from atrichoblasts (where it is expressed) to trichoblasts (where it functions). It is very interesting if MybE would be transported from the front of the prespore region (where it is expressed) to pstO region (where it functions).

Staining-positive cells for dimBp are distributing throughout the front half of a slug when they were diluted with unlabeled cells and there was not much difference to undiluted staining in terms of patterning (Fig. 2D). Taken together, these results suggest that the promoter structure and expression pattern is totally different between mybE and dimB.

I also analyzed cudCp, whose expression is confined to pstA cells. Although the -1128 and -845 fragments of cudCp exhibit strong pstA expression, neither of them are DIF-inducible (not shown). Since recent studies suggest that differentiation of pstA population is not directly related to DIF signaling, cudCp was not analyzed further.

DIF inducibility of the promoters

The mybEp and dimBp constructs were examined on microtiter assay for DIF inducibility and raw data was presented with duplicate wells for each experimental point (Fig. 1, right). Both promoters were constitutively activated in a DIF-independent manner, suggesting that DIF regulations of MybE and DimB are at post-transcriptional level. This notion is likely to be directly applied for DimB, where nuclear translocation is induced by DIF treatment within 3 minutes (ref. 2). However, it seems that MybE does not respond to DIF in terms of nuclear translocation, because either anti-MybE antibody or GFP-MybE are shown to be constitutively nuclear localized with or without DIF (ref.1). This would lead to a prediction that DIF acts on MybE indirectly; there should be an interacting protein that regulates MybE activity within pstO cells.



Discussion & Summary

Evidences suggest that DIF induces pstO cell differentiation, hence ecmO-lacZ expression within pstO region. MybE was identified as a binding factor for ecmO promoter fragment, and when MybE is inactivated by gene knockout, the ecmO-lacZ is not expressed (ref. 1). The simple explanation for this activation is that MybE localizes in pstO region. However, this study suggests that the promoter for mybE is not active in pstO region but mainly active in the tip and in the anterior part of prespore region. This implies that expression pattern of MybE and the place of action do not coincide, and suggests another mode of complexity for its regulation. The gradient spatial pattern of expression observed in dimBp is consistent with the proposed role of DimB, that is to establish a gradient of ecmA gene expression in the slug tip (ref. 2). This suggests that transcriptional regulation of dimB is partly involved in maintaining the identity of the prestalk cells within a slug. Because of lack of good antibodies, the precise localizations of these factors are still not clear. It is necessary to combine the promoter results and antibody localization to define the place of action for these two factors. Also, identification of binding partners would greatly help to reveal the signaling pathway.

This study showed spatial expression patterns of mybE and dimB, transcription factors that regulate DIF responses in Dictyostelium.

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1) Biological Sciences

1-5) Physiology

Analysis of primary afferent-evoked synaptic plasticity in spinal dorsal horn by simultaneous imaging of nitric oxide and presynaptic neuronal excitation.

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Introduction

Synaptic long-term potentiation (LTP) in the spinal dorsal horn is believed as a mechanism for induction of hyperalgesia, an increased response to noxious stimuli, following peripheral inflammation or nerve injury. Recently, we have shown that LTP at primary afferent synapses on lamina I neurons projecting to the periaqueductal gray matter (PAG) may be induced by the action of nitric oxide (NO) released from glial cells on presynaptic terminals (Ikeda et al., *J. Neurosci.*, in press). The purpose of this study, therefore, is to construct optical imaging system to visualize the distribution of NO and the pre- and postsynaptic neuronal excitation in spinal dorsal horn simultaneously, and to reveal the action of NO on presynaptic terminals in detail so as to clarify the underlying mechanism of hyperalgesia.

Results

As in our previous reports (Ikeda & Murase, 2004), high-intensity single-pulse test stimulation to the dorsal root, which activates A- and C-afferent fibers, evoked an increase in light absorption, an indication of neuronal excitation, in the spinal dorsal horn. The neuronal excitation was strongest in lamina I-III of dorsal horn, which contains nociceptive neurons (Fig. 1).

After LFS, the neuronal excitation was gradually potentiated to as much as $141 \pm 5\%$ of control at 2 hours after LFS (Fig.1, $n = 5$). The LTP of neuronal excitation was strongest in the superficial dorsal horn, which includes many projection neurons that convey nociceptive signals to the brain.

Although LFS-induced LTP of optically recorded neuronal excitation in the spinal dorsal horn is strongly inhibited by NOS inhibitors As in our previous reports (Ikeda & Murase, 2004), it is not clear if NO is produced as a result of this stimulation. Thus, we measured the spatiotemporal distribution of NO during LFS in the spinal dorsal horn with the fluorescent NO indicator, DAR-4M. The NO signal gradually increased during LFS (Fig. 2A,B,C, $2.4 \pm 0.3\%$, $n = 5$) especially in lamina I and II. The same region as that LTP was induced.

Depending on the slice, the degree of LTP varied from 130 % to 160 %. In five slices showing different degrees of LTP, there is close linear correlation between degree of LTP in the neuronal excitation and the intensity of NO signal (Fig. 2D)

We also examined the effect of NOS inhibitors on both neuronal excitation and NO production. 100 μ M L-NAME significantly inhibited both the LTP ($105 \pm 2\%$, $p < 0.01$ compared with control, $n = 5$) and NO signals induced by LFS ($0.5 \pm 0.3\%$, $p < 0.01$ compared with control, $n = 5$). L-NMMA also strongly inhibited both LTP (10 μ M, $141 \pm 6\%$, $n = 5$; 50 μ M, $117 \pm 5\%$, $n = 5$; 100 μ M, $99 \pm 3\%$, $n = 4$) and NO signals (10 μ M, $2.6 \pm 0.6\%$, $n = 5$; 50 μ M, $1.1 \pm 0.4\%$, $n = 5$; 100 μ M, $0.5 \pm 0.3\%$, $n = 4$) induced by LFS in a dose-related manner.

It is well known that NO synthase activity is controlled by NMDA receptors (Kawamata & Omote, 1999; Takata et al., 2005). Therefore, we examined contribution of NMDA receptor to LTP and NO signals induced by LFS. A NMDA receptor antagonist D-AP5 (50 μ M) did not inhibited neither LTP ($166 \pm 8\%$, $n = 5$) nor NO signals ($2.8 \pm 0.5\%$, $n = 5$).

Since it is reported that carbon monoxide (CO) also acts as a retrograde messenger during LTP (Zhuo et al. 1999), we tested the inhibitor of heme oxygenase, the synthetic enzyme for CO. Both LTP and NO signal induced by LFS were significantly inhibited in the presence of a heme oxygenase inhibitor zinc protoporphyrin IX 10 μ M (LTP: $100 \pm 1\%$, $n = 5$; NO signal: $0.9 \pm 0.3\%$, $n = 5$).

Discussion & Summary

In behavioral studies, hyperalgesia induced by intrathecal injection of NMDA is blocked by administration of the NOS inhibitor, L-NAME (Kitto et al., 1992). The enhancement of formalin-induced paw licking behavior in rats treated with L-glutamate or substance P is reversed by pretreatment with L-NAME (Coderre & Yashpal, 1994). Topical application of L-NAME onto spinal cord reduces both the first and second peaks of the response of dorsal horn neurons to formalin injection (Haley et al., 1992). The sensitization of spinothalamic neurons induced by intradermal injection of capsaicin is prevented by pretreatment of the dorsal horn with the NOS inhibitor L-NAME or 7-NINA (Lin et al., 1999). The allodynic response, and increased response of dorsal horn neurons to noxious and innocuous stimuli induced by intradermal capsaicin injection are reversed by the administration of the NOS inhibitors 7-NINA or AMT (Wu, 2001). The NO-dependent LTP in the spinal dorsal horn reported in this study indicates a possible cellular mechanism for the induction of these NO-dependent abnormal pains.

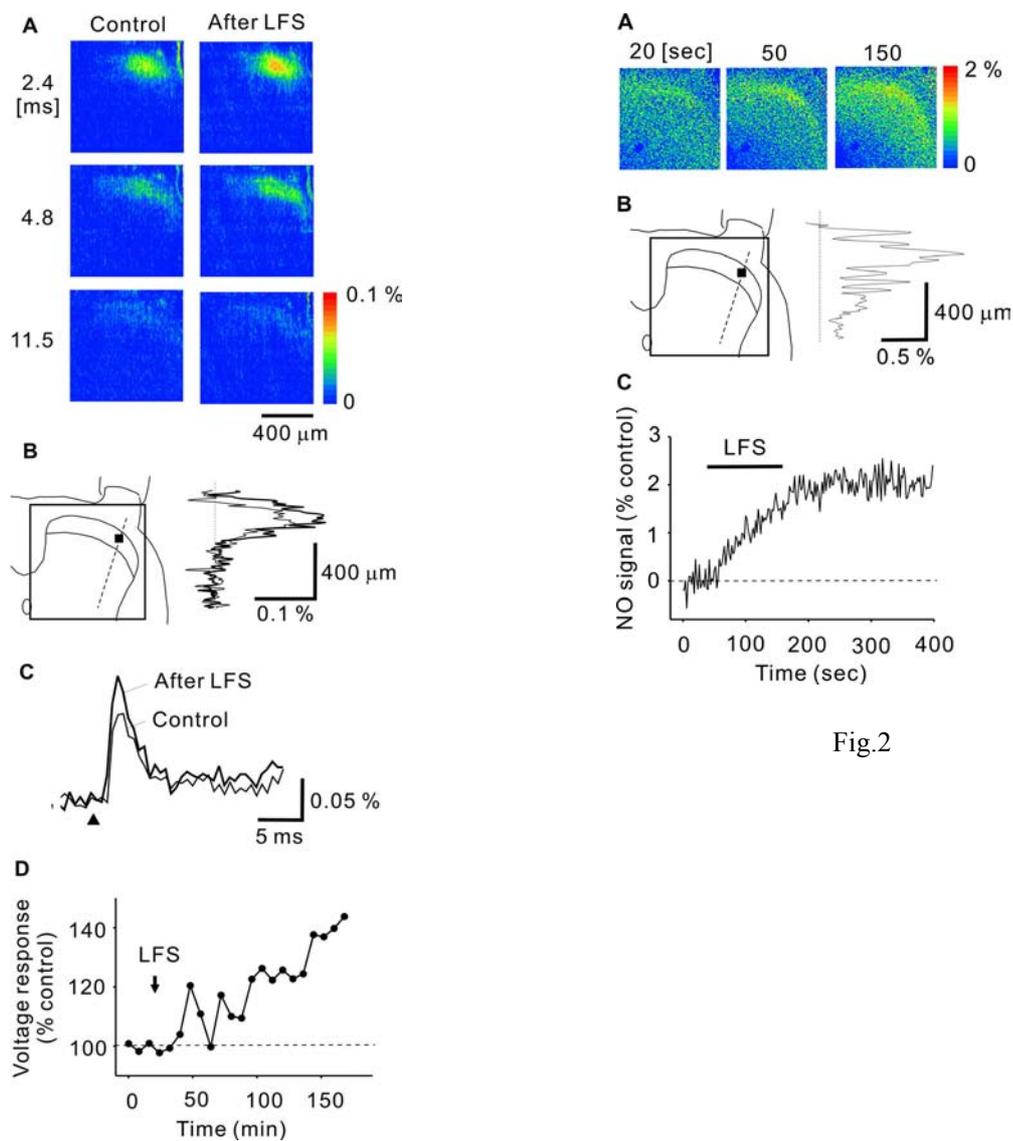


Fig.2

Fig.1

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1) Biological Sciences

1-5) Physiology

Roles of presynaptic calcium store in synaptic plasticity

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Introduction

Calcium ions play important roles in neurotransmitter release and its use-dependent modulation. However, the roles of intracellular calcium stores in regulating calcium dynamics within presynaptic terminals were not examined well. In this study, I aimed to elucidate the roles of presynaptic calcium stores in synaptic plasticity at the hippocampal mossy fiber synapses by using simultaneous recordings of presynaptic calcium dynamics and EPSPs in the slice preparations.

Results

To examine the presence of presynaptic calcium stores at the hippocampal mossy fiber synapses, we used simultaneous recordings of fluorescent measurement of presynaptic calcium dynamics and electrophysiological recording of EPSPs in mouse hippocampal slices. We first examined the effect of caffeine, which causes calcium release through ryanodine receptor channels. Application of caffeine caused robust enhancement of mossy fiber synaptic transmission. This synaptic enhancement was accompanied by reduction of paired-pulse facilitation of field EPSPs (excitatory postsynaptic potentials) or monosynaptic NMDA receptor-mediated EPSCs (excitatory postsynaptic currents) measured in the presence of AMPA receptor blocker CNQX. Caffeine also caused an increase in frequency, but not in amplitude, of miniature EPSCs recorded from CA3 neurons. Frequency of kainate receptor-mediated miniature EPSCs recorded in the presence of AMPA receptor-selective antagonist GYKI 53655, which reflect spontaneous transmitter release exclusively from mossy fiber terminals, were also increased by application of caffeine. Optically monitored presynaptic calcium level was also increased by application of caffeine. These results suggest presynaptic origin of the caffeine-induced synaptic enhancement, i.e. caffeine may cause calcium release from the presynaptic calcium stores via ryanodine receptors and thereby increasing transmitter release from the presynaptic terminals. To elucidate the physiological roles of presynaptic ryanodine receptors, the effect of ryanodine receptor blocker TMB-8 was also examined. EPSCs evoked at low frequency (e.g. 0.1 Hz) stimulation of mossy fibers were not significantly affected by application of TMB-8. However, presynaptic calcium transients evoked by train of high frequency

stimulation (100 Hz for 1 s) were significantly suppressed by TMB-8. Notably, initial phase of calcium transients was hardly affected by TMB-8, but prominent inhibition was occurred at the late phase during high frequency stimulation. These results suggested the notion that presynaptic ryanodine receptors were activated in a use-dependent manner, and caused amplification of presynaptic calcium transients by calcium-induced calcium release (CICR) mechanism. Since long-term potentiation (LTP) and long-term depression (LTD) at the mossy fiber-CA3 synapse are known to be a unique form of synaptic plasticity independent from postsynaptic NMDA-receptors but dependent on presynaptic calcium accumulation within the mossy fiber terminals, it was suggested that activity dependent amplification of presynaptic calcium transients facilitate induction of the presynaptic LTP and LTD at this synapse. In support of this notion, prolonged application of caffeine reliably induced long-term depression lasting longer than at least 60 min after washout of the drug. This caffeine-induced LTD was accompanied by enhancement of paired-pulse facilitation, suggesting that presynaptic LTD could be induced by calcium release within the presynaptic terminals. We also examined different conditions to activate presynaptic ryanodine receptors at the mossy fiber-CA3 synapse. Synaptic strength at this synapse exhibits robust frequency facilitation (FF), another form of presynaptic short-term synaptic plasticity elicited by repeated stimulation of afferent fibers at relatively low frequency (e.g. 1 - 5 Hz). Application of CPA, an inhibitor of calcium uptake into intracellular stores, reversibly suppressed the magnitude of FF tested at 1Hz stimulation. This suggested that amplification of presynaptic calcium raise by ryanodine receptors could be activated at rather low frequency stimulation of mossy fibers.

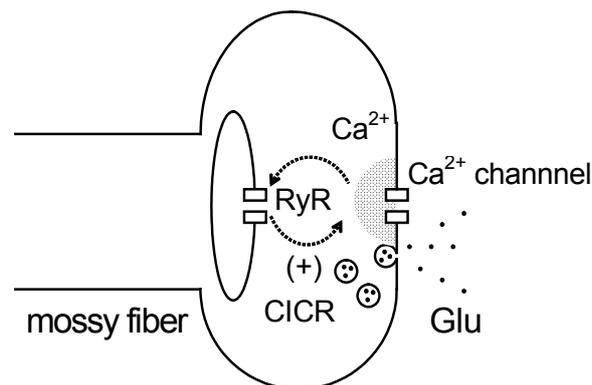


Figure 1

Ryanodine receptors reside within presynaptic terminals of hippocampal mossy fiber synapses. They are activated by calcium accumulation following repetitive stimuli, and cause amplification of presynaptic calcium raise by calcium-induced calcium release (CICR) mechanism.

Discussion & Summary

It has been demonstrated that presynaptic calcium stores are specifically localized in certain synapses in the central nervous system. In this study, I examined the roles of presynaptic calcium stores at hippocampal mossy fiber synapses using fluorescence measurement of presynaptic calcium and electrophysiological recordings. All the results are consistent with the notion that ryanodine receptors within the presynaptic terminals are involved in the presynaptic forms of short- and long-term synaptic plasticity at the hippocampal mossy fiber terminals. Since these forms of presynaptic plasticity have been shown to depend on accumulation of calcium in the presynaptic terminals, presynaptic ryanodine receptors may serve to amplify robust presynaptic plasticity at the mossy fiber-CA3 synapse.

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1) Biological Sciences

1-6) Pharmacology

Photodynamic therapy for cancer using photoreactive nano-micelles with the favorable property of accumulation in a tumor

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Introduction

Photodynamic therapy (PDT) is a minimally-invasive local therapy for solid cancers. In order to enhance efficacy of PDT, the property of photosensitizer requires achievement of high singlet oxygen quantum efficiency, accomplishment of their tumor-selective delivery, and minimization of side effects such as skin photosensitivity.

We have synthesized a novel potent photosensitizing agent composed of dendrimer-based photosensitizers and polymeric micelle incorporating them. Dendrimer-based photosensitizers achieve high quantum efficiency by preventing photosensitizers from aggregating. Polymeric micelle can accomplish tumor-selective delivery by EPR (enhanced permeability and retention) effect and prolonged blood circulation.

In this study, we therefore evaluated the therapeutic effect of PDT using polymeric micelle incorporating dendrimer-based phthalocyanine.

Results

We designed the novel photosensitive nano-carrier using Zn(II)-phthalocyanine as a photosensitizer since it can be excited by longer wavelength light (~ 680 nm). In the region ranging from visible to near-infrared, a light can penetrate deeper in tissue dependent on its wavelength. In addition, ~680-nm light is less absorbed by hemoglobin than 630-nm light, which corresponds to the second-maximum absorption of Photofrin, the most popular photosensitizer in clinical practice. As a result, the light at ~680 nm can penetrate tissues approximately 2 times deeper than that at 630 nm can.

The synthesis of the ionic dendrimer phthalocyanine (Pc/d) has been reported previously (1). The synthesized Pc/d is composed of the second-generation aryl ether dendrimer with a Zn(II)-phthalocyanine center and 32 negatively charged (COO⁻) groups on its periphery (Fig. 1A). Poly(ethylene glycol)-poly(L-lysine) block copolymer (PEG-b-PLL) (Fig. 1B) was synthesized by the polymerization of the N-carboxy anhydride of N ϵ -Z-L-lysine initiated by CH₃O-PEG-NH₂ in

dimethylformamide (DMF), followed by deprotection of the Z group according to a previously reported method (2). The polymeric micelle encapsulating Pc/d (Pc/m) was prepared with a stoichiometric ratio of negatively charged Pc/d and positively charged PEG-b-PLL (Fig. 1D). The synthesized Pc/m showed a size of ~ 50 nm. Zn(II)-phthalocyanine tetrasulfonic acid (Pc) (Fig. 1C) was used as a comparative photosensitizer.

The photo-cytotoxicities of Pc, Pc/d, and Pc/m were quantitatively evaluated by MTT assay 24 h after photoirradiation. 50% inhibitory concentration (IC_{50}) in human lung cancer cells (A549) by PDT using Pc/m was 7 - 44 folds lower than PDT using Pc or Pc/d.

Morphological changes of A549 cells after PDT using Pc, Pc/d or Pc/m were also observed in time lapse manner by a differential interference contrast (DIC) microscope. As a result, PDT with Pc/m induced a cellular expansion with blebs on cellular membrane, whereas PDT with Pc or Pc/d caused cellular shrinkage. Remarkable morphological changes of cells in PDT using Pc/m were considered to be associated with the immediate cell death.

Anti-tumor effect of PDT using Pc, Pc/d or Pc/m was evaluated in tumor xenograft mice model. One of the tested photosensitizing agents was administered intravenously as the administration amounts were proportional to molecular weights. 24 h after the administration, tumor sites were irradiated. This animal study protocol was approved by the Ethics Committee for Laboratory Animals of the National Defense Medical College. Mice with PDT using Pc/m showed significantly slow proliferation of tumor compared with those using Pc or Pc/d, or control (untreated) mice. The ratio of tumor volume in mice with PDT using polymeric micelle was about half as large as that in PDT-untreated mice on 30 days after PDT. Note that single PDT after the single injection of Pc/m was enough to significantly reduce the proliferation rate of solid tumors. The dosage of agent and effective photoirradiation should be further studied to achieve more effective PDT in *in vivo* study. In addition, tumor-selective delivery may be accomplished by polymeric micelle formation with PEG shell, so that side effects such as skin photosensitivity can be minimized.

Discussion & Summary

We have demonstrated that PDT using polymeric micelle encapsulating dendrimer-based phthalocyanines induced a unique and high cytotoxic effect in *in vitro* study. This treatment modality also exerted anti-tumor effect in tumor-bearing animal model. By incorporating Pc/d into polymeric micelle, more effective cytotoxicity, immediate cell death and characteristic morphological change can be induced in PDT. We expect that PDT using polymeric micelle encapsulating dendrimer-based photosensitizers can be more effective and safe treatment, and research in this direction is now going in our laboratory.

Figures & Tables

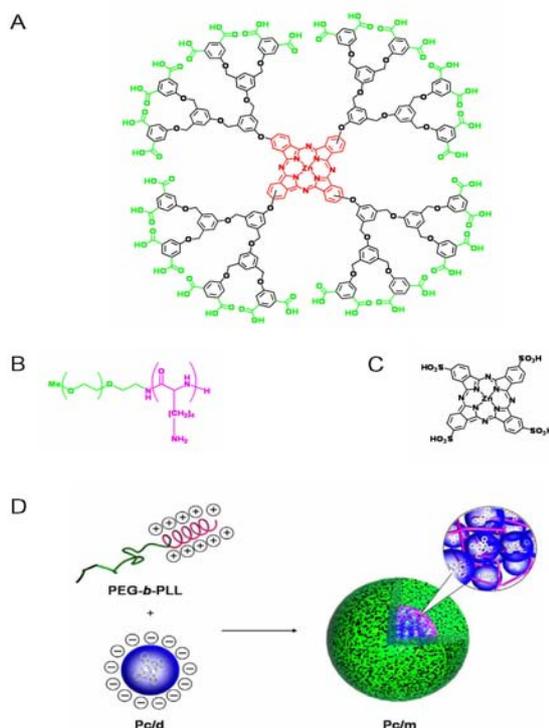


Fig. 1

Fig. 1 Chemical structure of ionic dendrimer Zn(II)-phthalocyanine (A), poly(ethylene glycol)-poly(L-lysine) (PEG-b-PLL) block copolymer (B) and Zn(II)-phthalocyanine tetrasulfonic acid (C). (D) Formation of Pc/m via electrostatic assembly of Pc/d and PEG-b-PLL block copolymers.

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1) Biological Sciences

1-6) Pharmacology

“Studies on the molecular mechanisms for physiological regulation of urinary bladder smooth muscle and etiology of overactive bladder
Analysis from the viewpoint of large-conductance,
Ca²⁺-sensitive K⁺ (MaxiK) channel role”

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Introduction

Overactive bladder (OAB) is a urinary bladder dysfunction usually accompanied with frequent urination and urinary incontinence. Although OAB itself is not life-threatening, its incidence is increasing in developed countries decreasing patients' QOL. Against OAB, new therapeutic drugs other than anticholinergic drugs are expected to emerge. One of the key molecules targeted at the understanding of OAB and its drug therapy, is the large conductance, voltage-dependent and Ca²⁺-sensitive K⁺ (MaxiK) channel. In the present study, roles of MaxiK channel in the regulation of urinary bladder smooth muscle electrical and mechanical activities and the effects of its openers were examined.

Results

1) Effects of Ca²⁺ channel modulators on the generation of action potential (AP)

In the isolated guinea-pig urinary bladder smooth muscle (UBSM) preparation, spontaneous AP was generated when membrane potential changes of UBSM cells were recorded with microelectrode. AP was myogenic in nature since its generation was not inhibited by receptor antagonists for neuronal transmitters in the UBSM wall. On the other hand, the amplitude and frequency of AP were significantly decreased by L-type Ca²⁺ channel inhibitors (nisoldipine and diltiazem), while BayK 8644, its channel activator, dramatically increased them, indicating that the upstroke of the AP recorded is provided by an activation of L-type Ca²⁺ channel.

2) Effects of K⁺ channel modulators on AP

UBSM cell AP was composed of depolarizing phase followed by repolarizing phase without a clearly defined plateau phase. Since the repolarizing phase of AP is assumed to be attributed to the activation of K⁺ channels, effects of several K⁺ channel inhibitors on the AP were examined: they

were iberiotoxin (IbTx) vs. MaxiK channel; apamin vs. small conductance Ca^{2+} -activated K^+ channel; 4-aminopyridine (4-AP) vs. voltage-gated K^+ channel; E-4031 vs. HERG channel. Among these K^+ channel inhibitors, IbTx (50 nM) was found to affect most dramatically the generation of AP: both the amplitude and frequency of AP were significantly increased by IbTx (Fig. 1A). IbTx was also the most powerful enhancer of the spontaneous mechanical activity of the guinea-pig UBSM (Fig. 1B). These electrical and mechanical studies with IbTx indicate that (1) IbTx enhances spontaneous mechanical activity of UBSM in part by enhancing AP frequency and (2) MaxiK channel functions as a primary negative feedback element to limit extracellular Ca^{2+} influx through affecting AP generation in the UBSM contraction.

To confirm the primary role for MaxiK channel in the UBSM electrical and mechanical activities, effects of openers of this channel were examined: they are NS-1619 and niflumic acid (MaxiK channel α -subunit activator), and estradiol and tamoxifen (MaxiK channel β -subunit activator). As expected, both types of MaxiK channel activators abolished the AP generation and significantly reduced the UBSM spontaneous contraction (Fig. 2B, C). NS-1619 also suppressed the spontaneous contractions generated in the balloon-inserted urinary bladder of the anesthetized guinea-pig. These findings strongly support the central role for MaxiK channel in the UBSM functional regulation and imply a usefulness of the opener of this K^+ channel in suppressing excessive UBSM excitability and contractility.

3) Effects of MaxiK channel openers on the channel activity of the isolated UBSM cells

In the enzymatically isolated guinea-pig UBSM cells, MaxiK channels with a slope conductance of $\approx 200\text{pS}$ were recorded. The channel activity was enhanced by BayK 8644 and a Ca^{2+} -pump Ca^{2+} -ATPase inhibitor, thapsigargin. In this UBSM tissue, both α - and β -subunits of MaxiK channels were abundantly detected at the mRNA level. These findings indicate that MaxiK is expressed in guinea-pig UBSM cells and functions as a negative feedback element to suppress cell excitability after the intracellular Ca^{2+} concentrations are increased. MaxiK channel openers, both α -subunit activators (NS-1619, niflumic acid) and β -subunit activators (estradiol and tamoxifen), significantly increased the channel open probability (P_o) (Fig. 2A). These results substantiate a crucial role for this channel as indicated by the electrical and mechanical studies mentioned above.

4) Possible roles for MaxiK channel in β_3 -adrenoceptor (β_3 -AR)-mediated inhibitory effect on the contractile responses of UBSM

Isoprenaline (ISO) and noradrenaline (NA) suppressed acetylcholine-induced contractions of UBSM in the presence of propranolol. These inhibitory effects were judged to be mediated through β_3 -AR since they were suppressed by bupranolol that is able to block β_3 -AR. The β_3 -AR-mediated relaxations by ISO and NA were significantly reduced in the presence of IbTx, which suggests a

possible functional coupling between MaxiK channel and β_3 -AR.

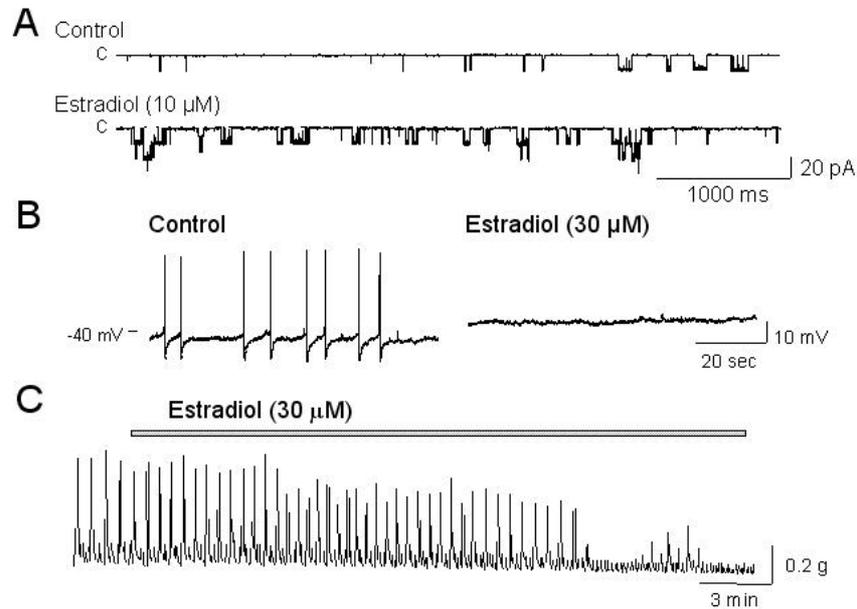


Fig.2. Effects of estradiol on the MaxiK channel activity (A), action potential (B) and muscle contraction (C).

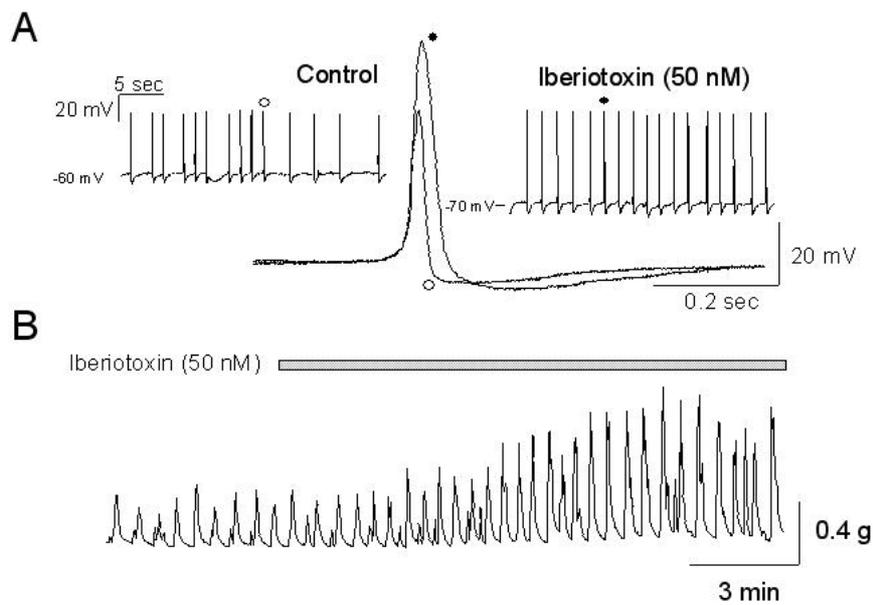


Fig.1. Potentiating effects of iberiotoxin on the action potential (A) and muscle contraction (B) recorded in guinea-pig UBSM.

Discussion & Summary

In the present study, we clearly indicate a key role for MaxiK channel which functions as a negative-feedback element to restrain over excitability and contractility of UBSM. This inhibitory effect of MaxiK channel is attained in part through suppressing the generation of AP. Therefore, functional disturbance of this type of K^+ channel would lead to causing OAB. On the other hand, the activation of MaxiK channel limits the generation of AP and contraction of UBSM. Accordingly, MaxiK channel openers would be useful drugs for the treatment of OAB. Another important finding of this study is that MaxiK channel might be functionally coupled with β_3 -AR. This is of interest since β_3 -AR is also expressed in UBSM cells and its agonist is a potent candidate for OAB treatment. For the better understanding of the UBSM functions and OAB, establishment of molecular mechanisms between MaxiK channel and β_3 -AR coupling will be a critical issue that should be settled in future.

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- 1) Biological Sciences
- 1-6) Pharmacology

Molecular dissection of the role of CLICK-III/CaMKI γ in fear- and eating-related behavior

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Introduction

CLICK-III/CaMKI γ is a novel CaMKI isoform isolated and cloned by the Bito laboratory. This CaM kinase distinguishes itself from all other CaM kinases through three features: 1) the expression of this kinase is CNS- and neuron-specific; 2) it is physically inserted into lipid bilayers through its lipidifiable C-terminal domain; 3) this kinase is abundant in the CeA of the amygdala and the VMH of the hypothalamus, indicating a possible function in regulation of fear- and eating-related behavior.

Results

In our 3-year proposal plan (to be ended on March 2008), we sought to identify the biological significance of CLICK-III/CaMKI γ through histological, biochemical and behavioral analyses of CLICK-III/CaMKI γ -KO mice. As a first step towards this goal, heterozygous mice for a CLICK-III/CaMKI γ -KO line were successfully generated in a BL/6 ES cell background.

Analysis of expression pattern of CLICK-III/CaMKI γ in embryonic and adult CNS revealed that this kinase is relatively ubiquitously expressed in the central nervous system, and in particular in the cortical plate. This was unexpected as its expression pattern was largely confined to the CeA and the VMH in the adult tissues. In a preliminary analysis of the substrates of this kinase, we have identified a membrane-bound signaling molecule whose role is known in neuritogenesis.

In the next two years, the availability of the knockout mice will be capitalized to further help us investigate the role of CLICK-III/ CaMKI γ in CeA- and VMH-based regulation of fear and eating-related behavior.

Discussion & Summary

CLICK-III/CaMKI γ is a novel CaMKI isoform that has no similarity with other CaM kinases in terms of cellular and subcellular distribution. Thus, a knockout analysis and identification of its physiological substrates will be critical to dissect its role. Availability of the knockout mice will now facilitate intensive screening of membrane-bound substrates while also allowing behavioral analyses.

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1) Biological Sciences

1-7) Plant Biology

Molecular mechanism of stem cell maintenance in the plant root meristem

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Introduction

In contrast to animal development where basic body plans are completed in embryogenesis, higher plants continue to form new organs after germination. This post-embryonic organ formation relies on the maintenance of undifferentiated cell populations (stem cell pools) in both shoot and root meristems (Ref. 1). How plants control stem cell numbers has not been clearly elucidated, though a few genes have been implicated in the shoot stem cells maintenance. In this study the role of WUSCHEL-LIKE HOMEODOMAIN 5 (WOX5) in the root stem cell maintenance was investigated by using mutants and transgenic plants. A new approach using the novel activation tagging method was also applied.

Results

1. Functional analysis of WOX5 gene

In the *Arabidopsis* root meristem, stem cells surround a small number of cells termed quiescent center (QC). It has been proposed that QC signals the surrounding stem cells and maintains their undifferentiated status (Fig.1A). A homeobox transcription factor, WOX5, is expressed specifically in the QC. Here the role of WOX5 in the root stem cell maintenance was studied by two approaches. First, loss-of-function *wox5* phenotype was analyzed by using T-DNA insertion mutants. *wox5* mutants displayed abnormal cell patterns in the root cap. Marker analysis indicated differentiation of the stem cells that would give rise to the root cap cell lineage (Fig. 1B). In contrast, stem cells proximal to the QC were not affected. Because the latter stem cells account for the root growth, *wox5* mutants showed comparable root growth rate to that of wild-type. Second, transgenic plants that express WOX5 throughout the root in an inducible manner were generated. Upon induction, these plants accumulated supernumerary layers of undifferentiated cells in the root cap region (Fig. 1B). When WOX5 expression was turned off, these cells rapidly differentiated into root the cap cells (data not shown). These observations indicate that WOX5 acts to maintain undifferentiated status of the root cap stem cells non-cell-autonomously. This strongly suggests that WOX5 is a part

of the postulated QC-derived intercellular signal that maintains stem cell activity in the Arabidopsis root meristem.

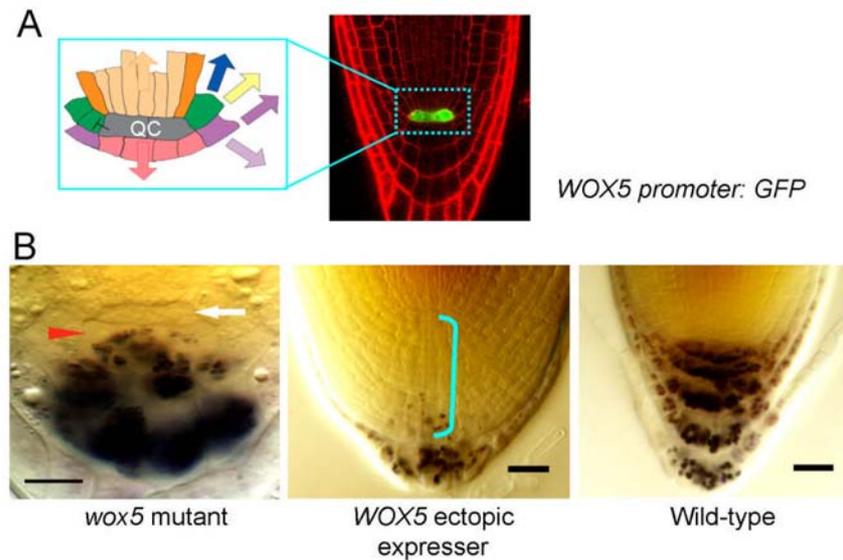


Fig.1. WOX5 maintains undifferentiated status of the root cap stem cells. **A,** WOX5 promoter:GFP reporter line showing its specific expression in the QC. **B,** In *wox5* mutants cells at the root cap stem cell position accumulate starch grains which indicates their differentiation to root cap cells (red triangle). Ectopic expression of *WOX5* resulted in supernumerary layers of undifferentiated cells (blue bracket).

2. Identification of novel genes involved in root stem cell maintenance by the GAL4/UAS activation-tagging system.

The number of Arabidopsis root meristem mutants is considerably fewer than the expected number of genes involved. This is most likely due to the gene redundancy. I am establishing a new activation-tagging system in which random genes in the genome are forced to express ectopically by the use of artificial transcription activator GAL4:VP16 (GV) and its target sequence UAS. This method allows isolation of gain-of-function mutants of redundant genes for which loss-of-function would not cause a visible phenotype (Fig. 2). Here this method was applied to screen for the mutants defective in the cell pattern formation in the root meristem (Ref. 2).

After screening about 17,000 independent lines, eight UAS-tagged root patterning (*urp*) mutants, *urp1D* through *urp8D* were isolated. Four of the eight mutants were defective in the root meristem maintenance, with their root growth terminating early after germination, whereas other four mutants showed severe patterning defects likely arose from disorganized cell divisions in the root meristem. Genes causal to the mutant phenotype were identified by determining the UAS insertion sites, followed by co-segregation and recapitulation experiments. In all cases UAS was found within the 5'

upstream regions of annotated genes, except for one, in which novel ORF was found to cause the mutant phenotype. All seven annotated genes encoded putative transcription factors belonging to the AP2, NAC, Zinc finger, MYB, DOF and RWP-RK families. Expression studies indicated that all these genes are expressed specifically in certain cell types in the root meristem, and two of them have been already implicated in root patterning by other research groups through an elaborated marker-based screening or homology-based functional studies.

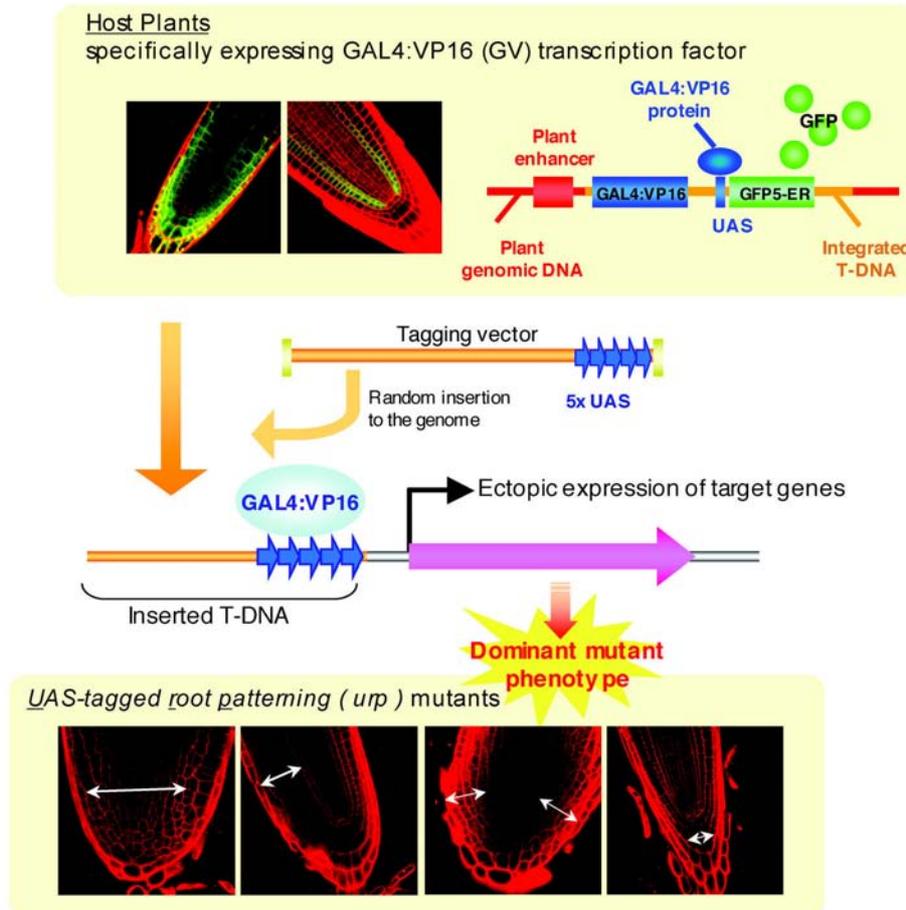


Fig.2. Isolation of novel root patterning mutants by the GAL4/UAS activation-tagging system. Arrows in the bottom panels indicate regions with abnormal cell division patterns.

Discussion & Summary

Based on classical laser-ablation studies, it has been postulated that QC cells in the root meristem function as a “stem cell niche” that emits unknown cellular signals to the surrounding stem cells and maintains their undifferentiated status. Results obtained in this study strongly suggest that a homeobox transcription factor *WOX5* plays a pivotal role in the QC-derived niche signal for the

root cap stem cells. The current data, however, does not provide evidence that WOX5 acts for proximal stem cells. Because root growth depends on the activity of proximal stem cells, in the future it is essential to identify genes responsible for the proximal stem cell maintenance.

The second part of this study proved that the GAL4/UAS activation tagging system is effective in identifying novel genes that have escaped from conventional mutant screenings. In the future this system will be applied to identify genes that can rescue known root meristem mutants.

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- 1) Biological Sciences
- 1-7) Plant Biology

Molecular and genetic studies on the function of polyamines in plant development

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Introduction

Stem elongation is a trait of particular significance in determining gross morphology of higher plants. Loss-of-function mutants of the *Arabidopsis thaliana* *ACAULIS5* (*ACL5*) gene, which encodes spermine synthase, result in severe defects in the stem elongation (1). To elucidate how *ACL5* regulates the stem elongation, we isolated four dominant or semi-dominant suppressor of *acaulis* (*sac*) mutants that suppress the *acl5* dwarf phenotype. The *sac51-d* mutation disrupts a short upstream open reading frame (uORF) of the *SAC51* gene which encodes a basic helix-loop-helix (bHLH) transcription factor. We suggest that *ACL5* plays a role in the translational control of uORF-containing genes.

Results

Mutations that suppressed the dwarf phenotype of the *acl5* mutant were identified by screening for tall individuals among M_2 plants descended from EMS-mutagenized homozygous *acl5-1* seeds. A total of four putative suppressors were isolated and designated as *sac* (suppressor of *acaulis*) 51 through 54 (Fig. 1). Microscopic observation of stem longitudinal sections revealed that the recovery of the plant height is attributable to that of the cell length in all *sac acl5* mutants. The *Arabidopsis* genome has two genes encoding spermine synthase, *ACL5* and *SPMS*. *acl5-1 spms-1* double mutant plants have no detectable levels of endogenous spermine but are morphologically indistinguishable from *acl5-1* plants (2). To examine the effect of *sac* alleles on the stem elongation under the complete depletion of spermine, we generated *sac acl5 spms* triple mutants. All *sac* mutants suppressed the *acl5* phenotype in the absence of *SPMS* in a similar degree to those in the presence of *SPMS*, indicating that the recovery of the stem growth involves no spermine.

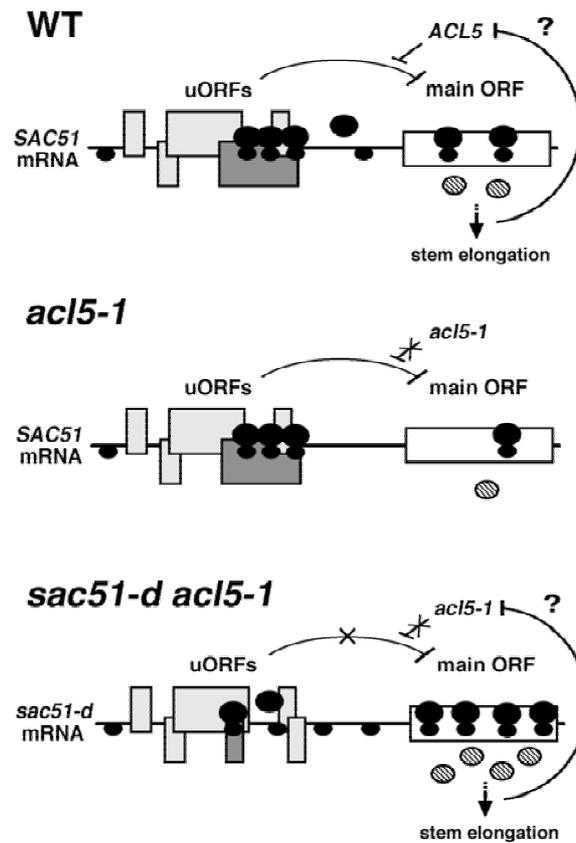
We chose *sac51-d acl5-1*, which showed the recovery of the shoot growth comparable to the wild type, for further study. Fine mapping experiments placed the *SAC51* locus on a 60-kb region of chromosome V. We found a single C-to-T mutation in the *At5g64340* gene. To confirm that *At5g64340* is the *SAC51* gene, a genomic fragment including 990 bp upstream of the transcription

Discussion & Summary

The *sac51-d* dominant allele was found to have a point mutation in one of these uORFs, which introduces a premature stop codon. Our results suggest that the *sac51-d* allele results in the deregulation of translational repression of the SAC51 bHLH protein and its overproduction is responsible for suppression of the *acl5* phenotype in *sac51-d acl5-1*. This is consistent with the dominant trait of *sac51-d*.

We propose a model whereby ACL5 directly or indirectly acts as a negative regulator of the translational repression of SAC51 and probably its homologues with their uORFs (Fig. 2). In this model, we hypothesize that the premature termination of a 53-amino-acid polypeptide encoded by the fourth uORF of SAC51 facilitates the release of the ribosome from the uORF and the translation reinitiation at the main ORF in *sac51-d*. Restoration in *sac51-d acl5-1* of the *acl5-1* mutant transcript level, which is increased in *acl5-1*, suggests that negative feedback control of the ACL5 expression does not require spermine itself. In conclusion, our findings shed first light on the uORF-mediated translational control in plant development.

Figure 2. A model of uORF-mediated translational control of SAC51 expression via ACL5 function



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Biological Sciences

1-7) Plant Biology

Molecular mechanism of phototropin kinase regulation by blue light

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Introduction

Phototropin [1] is a blue light receptor in plants for phototropism, chloroplast relocation, light-induced stomata opening and so on. Phototropin molecule has two photoreceptive domains and a kinase domain. The kinase activity is regulated by blue light received by the photoreceptive domains, however, the details of the regulation mechanism has been obscure. We tried to elucidate the role of the photoreceptive domains in this photoregulation using a newly developed phosphorylation assay system.

Results

Phototropin molecule has two photoreceptive domains named LOV in their N-terminal region and a Ser-Thr kinase domain at the C-terminus (Fig. 1a). We prepared 3 constructs of *Arabidopsis* phototropin 2 (phot2), 1) S/T kinase domain (KD), 2) KD + LOV2, 3) KD + LOV2 + LOV1 by an *E-coli* expression system. Until this study, only autophosphorylation has been reported and no other substrate than phot itself has been detected for phot kinase [2]. We surveyed systematically common substrates for Ser/Thr kinase if it can be a substrate for phot kinase, and found casein is the best substrate. Using casein as a substrate, we found the followings [3].

- 1) KD constitutively phosphorylates casein independently on light conditions.
- 2) In KD + LOV2 polypeptide, LOV2 prohibit the kinase activity of KD in the dark, that is canceled upon light illumination indicating light activation of kinases. This light effect was not observed on introduction of an amino acid mutation that prohibited the photoreaction of LOV2.
- 3) In KD + LOV2 + LOV1 polypeptide, LOV1 attenuate the light activation of the kinase by LOV2.
- 4) When isolated LOV2 is mixed with KD, the LOV still bind to KD and shows the inhibition of the kinase activity. Light illumination induced desorption of LOV2 and the kinase activation that were not observed with isolated LOV1.

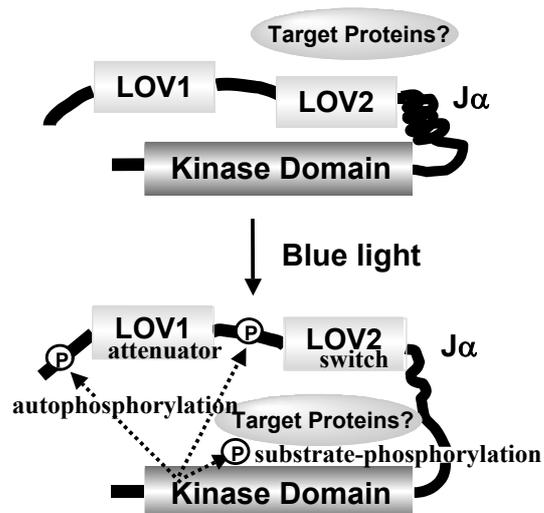
These are summarized schematically in Fig. 2.

To go into the details of the molecular mechanism underlying the light regulation of phot kinase, photoreaction and conformational changes leading to the regulation were studied by low-temperature

FTIR of hydrated films of *Adiantum* phytochrome 3 that comprises a N-terminal chromophoric domain of plant phytochrome and a C-terminal full-length phototropin (Fig. 1b). We found that LOV2 showed a secondary structural changes, however, it was partially frozen in LOV1 suggesting a different conformational changes between them [3]. Furthermore, we found a mutation in a β -strand E that forms so-call β -scaffold abolished the structural changes in the LOV2. The mutation is suggested to cancel the interaction between the β -scaffold and one α -helix residues downstream of LOV2 domain to KD. Importance of α -helical unfolding upon light perception is suggested including our present results. These are also illustrated schematically in Fig. 2.



Fig. 2



Discussion & Summary

We have proved for the first time that phot kinase can phosphorylate the other substrate that phot itself. This suggests presence of a possible substrate(s) *in vivo* that is involved signal transduction of phot. The present LOV2 + KD polypeptide gives a useful *in vitro* assay system to detects the “real substrate(s)” involved in the light-signal transduction. Investigation along this line is under progress.

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- 1) Biological Sciences
- 1-7) Plant Biology

Plant-plant communication mediated by volatile compounds.

～Elucidation of its molecular mechanisms～

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Introduction

It has been reported that plants emit a special blend of volatile compounds when herbivorous insects attack them. The surrounding plants can perceive the volatiles. Upon perception of the volatiles, the plants evoke the defense systems in order to manage the future disaster. The instances of this ‘plant-plant communication’ are accumulating, however, it has not been known what compounds mediate the communication and how plants can perceive them. In this study, I would like to identify the compounds and also to dissect the molecular machinery to perceive the volatiles with a model plant, Arabidopsis.

Results

When intact and healthy Arabidopsis plants were exposed to volatile C6-aldehydes, (E)-2-hexenal and (Z)-3-hexenal, a subset of defense genes was induced, and plants’ resistance against a necrotrophic pathogen (*Botrytis cinerea*) was induced. We attempted to dissect the components involved in the induced resistance. The penetration of *B. cinerea* hyphae into Arabidopsis epidermis and the growth of hyphae after penetration were suppressed on the C6-aldehyde-treated leaves when compared with those on the control leaves. We found that after the volatile-treatment, the vascular bundles and cell walls of the leaves were intensely lignified. Higher lignification could be observed with (E)-2-hexenal treatment compared to (Z)-3-hexenal treatment, which coincided with the degree of overall resistance. Increase of high antifungal activities could be found with the treated leaves. C6-aldehydes also induced accumulation of PDF1.2 (plant defensin1.2) and PR-3 (chitinaseB) transcripts, and camalexin. Taken together, these data suggest that C6-aldehydes made Arabidopsis resistant to the pathogen due to induced lignification, which formed a physical barrier to the penetration of the hyphae, and to the accumulation of antifungal substances including PDF1.2, PR-3 and camalexin, which inhibited the growth of the hyphae in the plant cells.

In order to elucidate the signaling pathway mediating the defense responses induced by C6-aldehydes in *Arabidopsis*, we compared the responses of *Arabidopsis* mutants deficient in the signaling pathways; i.e., *etr1-1* (ethylene resistant), *jar1-1* (jasmonate resistant), *npr1-1* (salicylic acid insensitive), or *pad2-1* (phytoalexin-deficient) with those of wild type (WT) plants. Induction of some, but not all of the defense genes in response to C6-aldehydes was significantly repressed in *jar1-1*, *etr1-1*, and *pad2-1*, but not at all in *npr1-1*. C6-aldehyde-treatment enhanced accumulation of camalexin with WT and *npr1-1*, but only partially with *etr1-1* and *jar1-1*. *pad2-1* showed little accumulation of camalexin. *npr1-1* accumulated the antifungal substances as WT did, however, *etr1-1*, *jar1-1* and *pad2-1* exhibited only partial accumulation. The treatment enhanced resistance of *etr1-1*, *jar1-1* and *npr1-1* against *B. cinerea*, but failed to enhance the resistance of *pad2-1*. Taken together, it was suggested that ETR1-, JAR1-, and PAD2-dependent signaling pathways were simultaneously activated by C6-aldehyde-treatment. Among these, PAD2-dependent signaling appeared to be most important. In contrast, involvement of NPR1-dependent signaling was minimal.

Other than C6-aldehydes, monoterpenes are also emitted from plants after biotic attack. Previously we found that volatile allo-ocimene enhanced resistance of *Arabidopsis thaliana* against *Botrytis cinerea*. Because the structure of allo-ocimene is totally different from C6-aldehydes, it has been assumed that responses caused by allo-ocimene are different from those caused by C6-aldehydes. The penetration of *B. cinerea* hyphae into *Arabidopsis* epidermis and the growth of hyphae after penetration were suppressed on allo-ocimene-treated leaves. allo-Ocimene also induced lignification on cell walls and veins of the leaves. The treatment induced accumulation of antifungal substances including *Arabidopsis* phytoalexin, camalexin. allo-Ocimene enhanced resistance against *B. cinerea* in *etr1-1*, *jar1-1* or *npr1-1*. Thus, it was suggested that a signaling pathway independent on ETR1, JAR1 and NPR1 was operative to induce the resistance. The series of responses observed after allo-ocimene-treatment was mostly similar to that observed after C6-aldehyde-treatment. The effect of C6-aldehyde-treatment has been largely accounted to the chemical reactivities of the compounds, however, from this result it was suggested that resistance responses of *Arabidopsis* could be induced by the volatiles mostly independent on their reactivities and that a common signaling pathway unaffected by the reactivities of compound was activated by the volatiles.

Discussion & Summary

Volatile compounds formed and emitted by plants while they were attacked by invaders can be perceived by neighboring plants, and they could start defense responses even without real invaders. In this study, we used *Arabidopsis*-*Botrytis* system to dissect molecular mechanism of volatile perception. It has been shown that C6-aldehydes could cause pleiotropic effects on the defense system including lignification of plant tissues and accumulation of antifungal substances. Jasmonate and ethylene signalings were at least partly involved in this response, and a signaling mediated by

glutathione must be also important. Interestingly, there was no apparent structure-dependency for the volatiles to affect defense systems in plants. Thus, there might be a volatile perceiving system independent of specific receptors. It is still controversial whether this kind of plant-plant communication, or eavesdropping, has ecological and evolutionary relevance; thus, further study to reveal whole the system must be carried out. The novel knowledge waiting for us must be useful to develop a novel tactics for the sustainable agriculture.

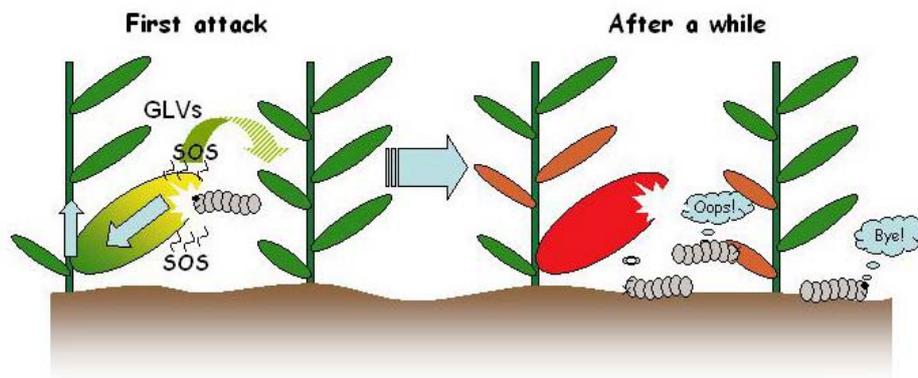


Figure 1. GLVs might function as intra- and interplant volatile signal. When leaves are damaged by herbivores, they start to form GLVs. A portion of GLVs diffuses into the leaves and induces defense responses (indicated in reddish color). The others are vaporized and reach the other leaves of the same plants or the other plants, where they induce the response. This is just one of hypotheses and needs further study.

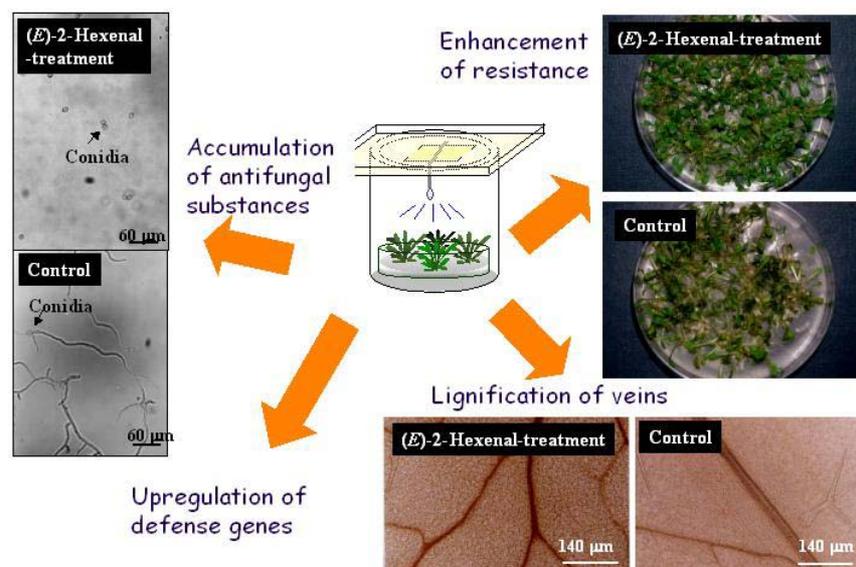


Figure 2. GLVs can cause pleiotropic effect on the plants. GLVs can upregulate defense genes, prompt plants to accumulate antifungal substances, lignify veins, and as a result, enhance resistance against pathogenic fungi.

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1) Biological Sciences

1-7) Plant Biology

Non-Cell-autonomous flowering signal of FT is conferred by its protein trafficking.

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Introduction

FLOWERING LOCUS T (FT) is one of the flowering pathway integrators of Arabidopsis. FT integrates long-day responsive pathway of flowering and promotes flowering strongly. FT is expressed in the vascular tissue of leaf apical region and its expression is not detectable in the shoot apical meristem (SAM). FT function is, however, required in the SAM because flowering is promoted when FT is expressed in the SAM specific manner. These results suggest that FT functions non-cell-autonomously, that is FT is expressed in the leaves and functions in the SAM.

Results

In order to visualize FT protein, we made FT-GFP fusion gene and introduced into *ft* mutants. By using constitutive promoter, 35S, FT-GFP fusion gene rescued *ft* mutant phenotype dependent on the FT-GFP conjunction sequences. This suggests protein function of FT is impaired by the addition of some peptide sequences. Then, we transformed *ft* mutants by FT and functional FT-GFP gene by using FT promoter. We found that FT gene complemented *ft*, but FT-GFP did not. This suggesting that FT-GFP lost its protein mobility. We examined protein localization of FT and FT-GFP, but we failed to detect due to the low level of expression driven from FT promoter.

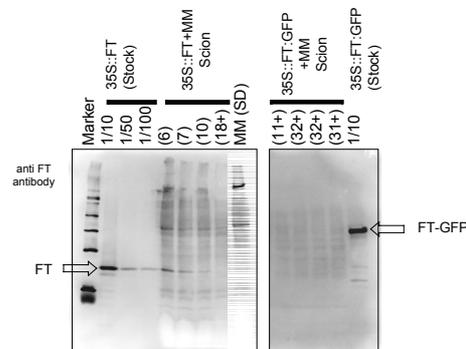
We moved our assay system to *N. tabacum* (tobacco), because tobacco is one of the best plants for grafting experiments. First, we tested whether Arabidopsis FT can promote flowering of tobacco or not, because FT functions as a "florigen" which works in the wide range of flowering plants. As expected, FT as well as functional FT-GFP promoted flowering in the tobacco driven by 35S promoter. These transgenic tobacco, 35S::FT and 35S::FT-GFP were used as rootstock and grafted wild type tobacco var. Maryland Mammoth (MM) as scions. MM is a cultivar that flowers in the short day conditions and never flowers when it is grown in the long day conditions, though native tobacco is a day-neutral plant. While scions grafted on 35S::FT flowered after producing eight to twelve leaves, scions on 35S::FT-GFP never flowered. These results suggest that FT produced graft transmissible flowering signal and FT-GFP did not. This is consistent with the

Arabidopsis experiments. Together, these results suggest that FT or flowering signal produced by FT can move beyond the grafting surface but FT-GFP cannot.

Recently, Huang et al., (2005) showed that FT RNA moves from Arabidopsis leaves to the SAM and which is the molecular basis of systemic flowering signal transduction. Our previous data, however, show that FT as well as its homolog TFL1 proteins moves cell to cell in the SAM. This protein trafficking is not diffusion but targeted movement (manuscript in preparation). These results suggest protein trafficking of FT contributes flowering signal transduction. Or at least, FT trafficking helps to deliver FT protein, which is translated from RNA after unloaded from vascular system, to the SAM because vascular bundle is not developed in the SAM.

We next tried to detect FT RNA and protein in the grafted scion. In the scions, grafted on the 35S::FT, we could detect FT protein about 10 ng per 1 g-fresh weight, but protein is under detectable amount in the scions grafted on 35S::FT-GFP. FT RNA was not detected in both grafted scions, even by using Taqman real-time quantitative RT-PCR, which is the most sensitive detection method at present. It is pretty hard to prove that RNA is not transferred from rootstock to scion because it remains a possibility that underdetectable small amount of RNA still exists. However, our results strongly suggest that protein trafficking through the grafting surface plays primary role for flowering signal transmission produced by FT.

FT protein was detected in the scion but FT-GFP was not.



Discussion & Summary

While graft transmissible FT protein is detected in the scion, not transmissible FT-GFP protein is not detected. These results suggest the possibility that FT protein trafficking is the molecular basis for the systemic flowering signal transduction produced by FT. Our results do not contradict the previous report showing that FT RNA movement transfer the flowering signal. There is, however, no molecular mechanism of FT RNA movement is shown. What sequence is essential for FT RNA transmission or how FT RNA selectively transferred? Farther analyses are required to determine whether protein trafficking or RNA movement is the molecular basis or essential for the flowering signal transduction produced by FT.

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- 2) Medical Sciences
- 2-1) Immune System

Role of negative regulators of cytokine signaling in atopic disorders

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Introduction

Atopic diseases, such as asthma, allergic rhinitis, and atopic eczema, have increased dramatically in prevalence, and patients with severe/refractory asthma are at great risk of death. Allergic airway inflammation is an important feature of asthma, and its inflammatory process is mediated via various cytokines. T helper 2 (Th2)-type cytokines, interleukin (IL)-4, IL-5, and IL-13, play a critical role in the development of asthma. These cytokines transmit signals through the JAK/STAT and the Ras/ERK signaling pathways. The suppressor of cytokine signaling (SOCS) family proteins and Sprouty-related EVH1-domain-containing protein (SPRED) family proteins have been shown to regulate the STAT pathway and the ERK pathway, respectively. However, the precise roles of IL-13, SOCS, and SPRED in the pathophysiology of allergic asthma have not been clarified.

Results

Local administration of recombinant IL-13 to non-immunized mice induced the asthma phenotype, and glucocorticoids were not sufficient to suppress IL-13-induced airway hyperresponsiveness or goblet cell hyperplasia. Chloride channels have been implicated in the regulation of mucus production in epithelial cells, and we studied the effects of chloride channel inhibitor, niflumic acid, in IL-13-induced asthma. Niflumic acid inhibited not only IL-13-induced goblet cell hyperplasia but also airway hyperresponsiveness and eosinophilic infiltration. Niflumic acid suppressed JAK2/STAT6 activation and eotaxin expression in epithelial cells.

High levels of SOCS3 expression in T cells resulted in Th2 skewing, and relate to allergic diseases, and this was because SOCS3 binds to IL-12R β 2 and inhibits IL-12 mediated STAT4 activation, thereby blocking Th1 development. T cell-specific SOCS3 conditional knockout mice showed reduced responses to OVA-induced asthma, while little difference in the Th1 responses. STAT3 and SOCS3 reciprocally modulate Th3 differentiation characterized by the production of TGF- β 1 and IL-10.

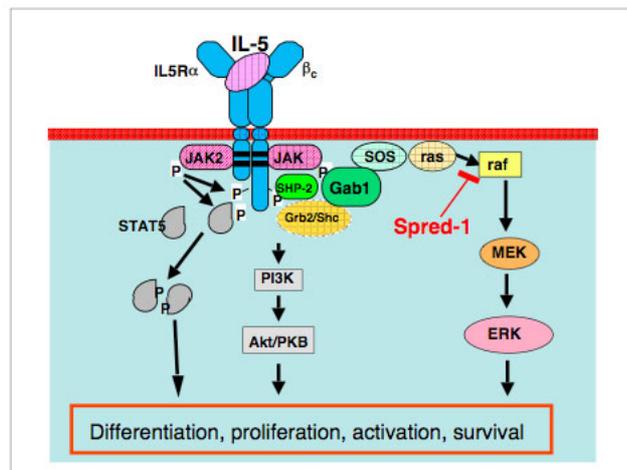
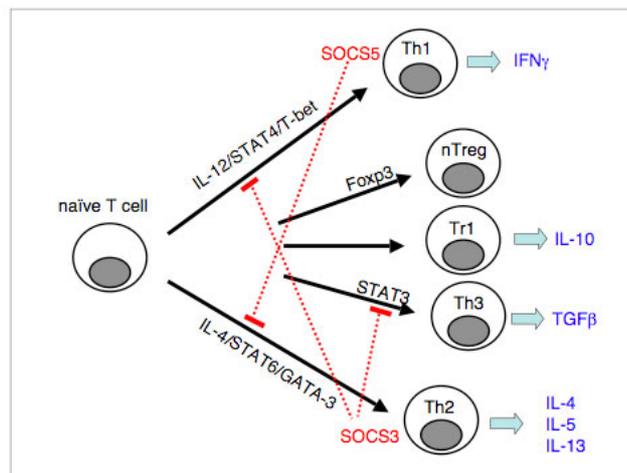
Using SPRED1-deficient mice, we demonstrated that SPRED1 negatively regulates

allergen-induced asthma without affecting helper T cell differentiation. SPRED1 suppresses IL-5-dependent cell proliferation and ERK activation.

Discussion & Summary

These findings suggest that a) chloride channel inhibitor can control IL-13-mediated airway features at least by suppressing JAK/STAT6 activation, b) the high expression of SOCS3 in T cells led to skewing to Th2 differentiation. SOCS3 also regulates Th2/Th3 balance, and the low levels of SOCS3 in T cells enhanced Th3 differentiation. c) SPRED1 negatively controls eosinophil numbers and functions by modulating IL-5 signaling in allergic asthma.

We consider the possibility that SOCS and SPRED proteins might be novel therapeutic targets for the treatment of allergic diseases.



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- 2) Medical Sciences
- 2-1) Immune System

Identification of novel immune adjuvants from parasites or fungi

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Introduction

Toll-like receptors (TLR) 7 and 9 recognize ssRNA and CpG DNA, respectively. Their signaling can induce production of not only inflammatory cytokines but also type I interferons (IFNs). It remains unclear whether TLR7 and TLR9 are involved in recognizing helminthes or how TLR7/9 induces type I IFNs.

Results

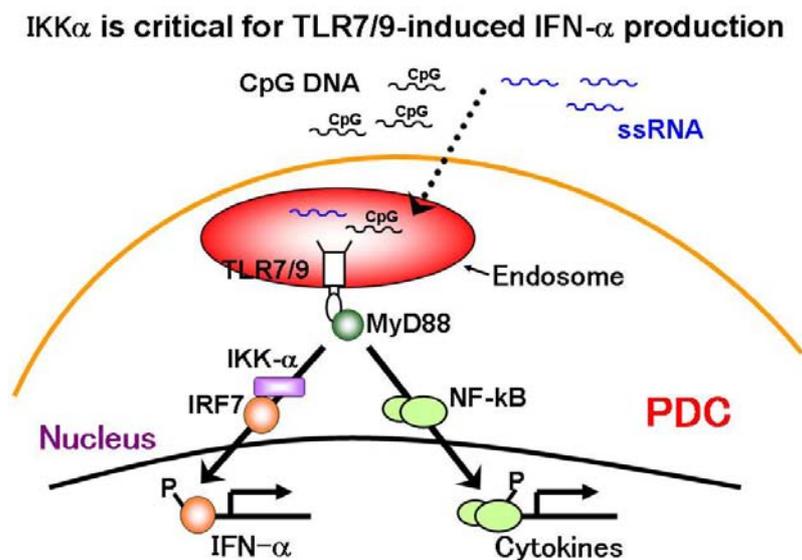
(1) IL-12 inducing activity from murine DCs was detected in nematode extracts. This activity was abolished in mutant DCs lacking a cytoplasmic TLR adapter, MyD88, indicating possible involvement of certain TLRs. Among several TLR-deficient DCs, TLR7 or TLR9 deficient DCs were found to deteriorate the responses to nematode extracts.

(2) A DC subset, plasmacytoid DC (PDC), express TLR7 and TLR9 exclusively among TLRs. TLR7 and TLR9 are expressed also on non-PDC (conventional DC, cDC) and both PDC and cDC could produce inflammatory cytokines in response to TLR7/9 agonists. However, only PDC can produce IFN- α in response to TLR7/9 agonists. All of TLR7/9 functions require a cytoplasmic adapter, MyD88, but at the downstream of MyD88, signaling cascades are bifurcated into NF- κ B and IRF-7 activation pathways, which can lead to induction of inflammatory cytokines and type I IFNs, respectively. IRF-7 should be phosphorylated to be activated, but the responsible kinases were not clear. We focused on I κ B kinase- α , because it is a member of IKK family and the other three IKK family members were known for their critical roles in TLR signaling. We have found that DCs from the mutant mice lacking IKK α , failed to produce IFN- α in response to TLR7/9 agonists. TLR7/9 signaling could activate the mutant DCs to produce inflammatory cytokines and the mutant DCs could normally respond to other TLR agonists. Meanwhile, virus-induced type I IFN production in embryonic fibroblasts, which depends on cytosolic virus recognition system, was normal in the absence of IKK α . These results indicate TLR7/9-induced type I IFN production specifically requires IKK α (Fig.). IKK α could enhance the ability of IRF-7 to activate the IFN- α promoter and dominant negative form of IKK α could inhibit MyD88-mediated activation of IRF-7. IKK α could associate

with IRF-7 in DCs and in vitro kinase assays revealed that IKK α can phosphorylate IRF-7. Notably, electrophoretic mobility shift assay showed TLR-induced IRF-7 activation was impaired in IKK α -deficient PDC. Taken together, these results demonstrate that IKK α is critically involved in TLR7/9-induced IRF-7 activation, and IFN- α production (Fig and ref. Hoshino et al.).

Discussion & Summary

Our results indicate that TLR7 and TLR9 may be involved in immune responses against helminth. It should be investigated in the future how TLR7 or TLR9-deficient mice respond to helminth infection. We could also identify IKK α as a critical molecule for TLR7/9-induced type I IFN production. TLR7/9-induced type I IFN induction is supposed to be critical not only for antiviral immunity but also for pathogenesis of certain autoimmune disorders such as SLE. Therefore, IKK α should be a potential target for manipulating host defense and autoimmunity.



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- 2) Medical Sciences
- 2-1) Immune System

Molecular mechanism of the immune regulation maintained by CD8⁺CD122⁺ immuno-regulatory T cells.

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Introduction

We identified CD8⁺CD122⁺ regulatory T cells that are important in regulating other T cells. In the absence of these regulatory cells, other T cells become dangerously activated T cells that are harmful for self and kill the individual host. Understanding the molecular action of the regulatory cells is a necessary step for the application of these cells to diseases. The aim of this study is to determine the molecular mechanism responsible for the suppressive activity of CD8⁺CD122⁺ regulatory T cells.

Results

We have already established the *in vitro* culture system to evaluate the activity of CD8⁺CD122⁺ regulatory T cells. We proceeded to identify the molecules involved in the regulation by adding specific neutralizing antibodies into the culture system.

1. Molecular mechanism in the recognition of target cells to be regulated.

First, CD8⁺CD122⁺ regulatory T cells need to recognize the target cells that should be regulated. We proved that CD8⁺CD122⁺ regulatory T cells recognize already-activated T cells stimulated by specific antigen or non-specific reagent such as anti-CD3 antibody. In the recognition phase, interaction between MHC class I on the regulated cells and T cell receptor (TCR) on the regulatory cells is essential. Furthermore, interaction between CD28 and CD80/CD86 molecules is critically involved for the regulatory cells to become active regulatory cells. We also performed experiments using T cells from MHC class I-deficient mice and those from CD28 knockout mice, and confirmed that these molecules are involved in the process of recognition or development into active regulatory T cells.

2. Molecular mechanism in the effect-transmitting phase.

Culture supernatant of CD8⁺CD122⁺ cells under the stimulation by plate-bound anti-CD3 antibody effectively suppressed the proliferation and IFN- γ production of CD8⁺CD122⁻ cells. This result means that CD8⁺CD122⁺ cells produce medium-soluble factor(s) that transmit the suppressive

effect. Experiments using specific antibodies that neutralize the function of various cytokines revealed that IL-10 was the most important and essential factor that transmitted the effect of CD8⁺CD122⁺ cells. Experimental results using the CD8⁺CD122⁺ cells derived from IL-10 knockout mice further confirmed that IL-10 was the responsible factor *in vitro*. However, CD8⁺CD122⁺ cells from IL-10 knockout mice showed reduced but significant effect to regulate CD8⁺CD122⁻ cells, indicating that the function of IL-10 is partially compensated by other factor(s) *in vivo*.

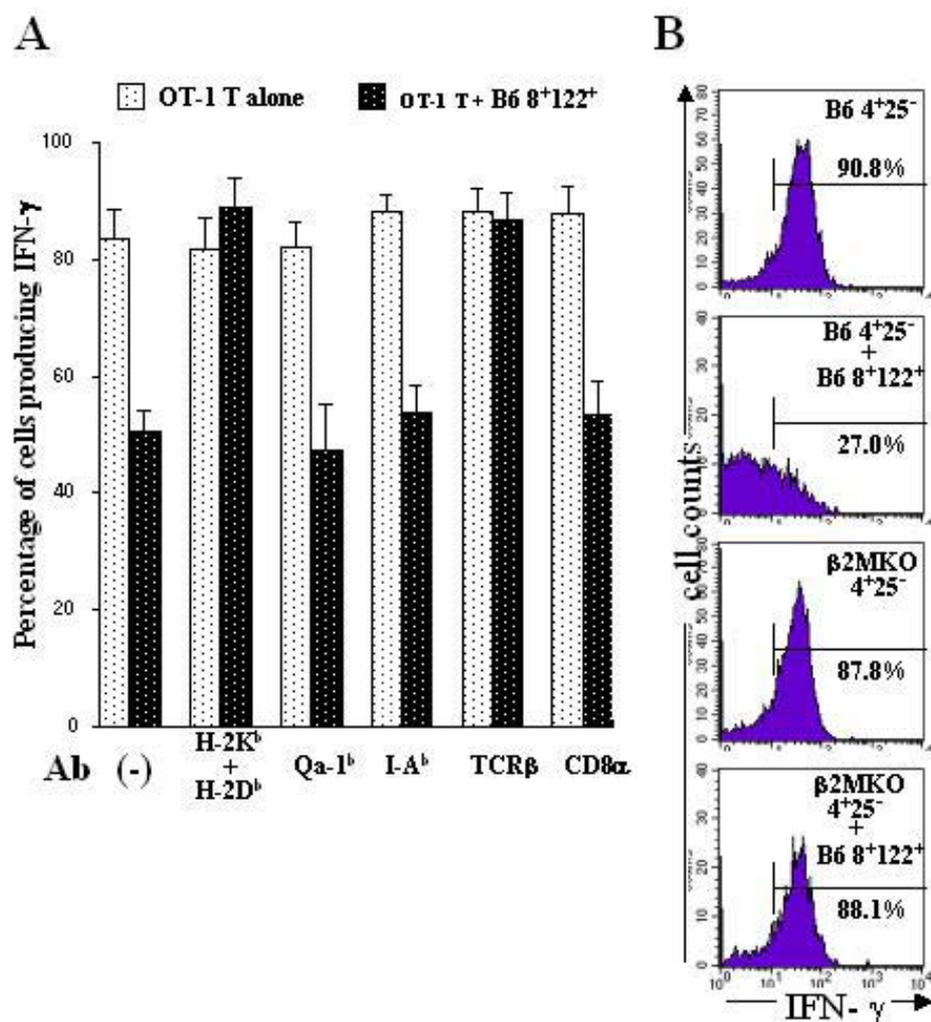
Discussion & Summary

In this study, we investigated on the molecular mechanism of CD8⁺CD122⁺ regulatory T cells. These regulatory cells first recognize the target cells depending on the cell-cell interaction with a MHC class I-restricted manner. It was highly suggested that TCR on the regulatory cells recognized the antigen on MHC class I molecule on the target cells. Once the CD8⁺CD122⁺ regulatory cells recognize target cells, they become active regulatory cells. In the step of recognition and activation of the regulatory cells, interaction between CD28 on the regulatory cells and CD80/CD86 on the target cells is also required. Then, the active regulatory T cells produce soluble factors to suppress proliferation and IFN- γ production of the target cells. The most important and essential soluble factor produced by CD8⁺CD122⁺ regulatory cells is IL-10. However, the function of IL-10 may be compensated by other factors in an *in vivo* condition.

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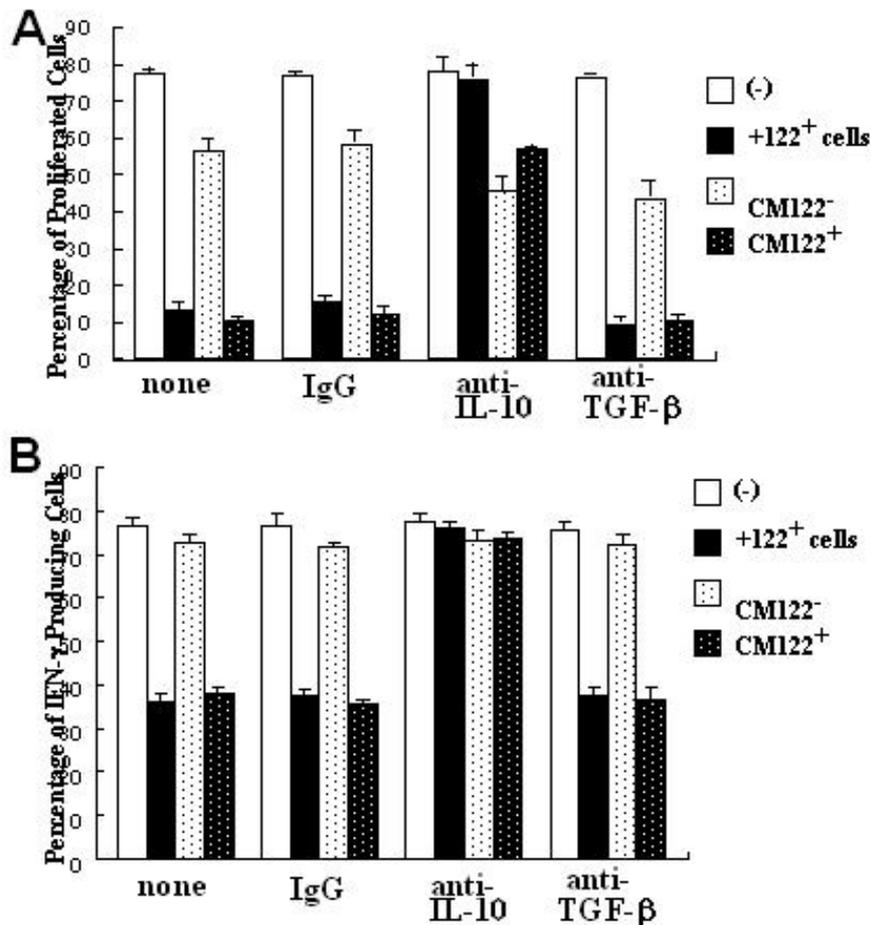
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Figure 1



(A) OT-1 T cells and APCs were cultured in a medium containing OVA peptide. 24 h later, CD8⁺CD122⁺T cells were added to the cultures. At the same time, anti-H-2K^b plus H-2D^b, anti-Qa-1^b, anti-I-A^b, anti-TCRβ, or anti-CD8α blocking antibodies were added and the culture was continued. After 48 h, cells were harvested and subjected to intracellular IFN-γ staining. The quantitative data of the percentages of cells producing IFN-γ are shown. (B) CD4⁺CD25⁻ cells were isolated from spleens of C57BL/6 (B6) mice or β2-microglobulin-KO (β2MKO) mice, and CD8⁺CD122⁺ cells were isolated from the C57BL/6 mice. CD4⁺CD25⁻ T cells were mixed with CD8⁺CD122⁺ cells and cultured under stimulation with plate-bound anti-CD3. After 48 h, cells were stained for intracellular IFN-γ. Data are shown as the histogram analysis of IFN-γ expression levels in CD4⁺ cells.

Figure 2



Regulatory activity of CD8⁺CD122⁺ T cells and conditioned medium is blocked by anti-IL-10 antibody but not by anti-TGFβ antibody. (A) CD8⁺CD122⁻ T cells were labeled with CFSE and cultured under stimulation with plate-bound anti-CD3 antibody for 48 h. CD8⁺CD122⁻ T cells were either cultured alone (-), co-cultured with one-fourth number of CD8⁺CD122⁺ T cells (+122⁺ cells), cultured in 50% conditioned medium obtained from the culture of CD8⁺CD122⁻ cells (CM122⁻), or cultured in 50% conditioned medium obtained from the culture of CD8⁺CD122⁺ cells (CM122⁺). Cells were cultured in a medium without the addition of antibody (none), with anti-IL-10 antibody (αIL-10), with anti-TGF-β (αTGF-β), or with rat isotype control IgG (IgG). Percentages of proliferated cells in CFSE-labeled cells. (B) CD8⁺CD122⁻ T cells were cultured as in A and were subjected to intra-cellular staining for IFN-γ.

- 2) Medical Sciences
- 2-1) Immune System

Studies on Immune Regulation by Interleukin (IL)-27, A New Member of The IL-12 Cytokine Family

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Introduction

IL-27 is a novel IL-6/IL-12 family cytokine that is considered to play a role in Th1 differentiation, whereas the exact role of IL-27 in Th1 differentiation and its molecular mechanism remain unclear. Here, we demonstrate a role for IL-27 in the early regulation of Th1 differentiation and its possible molecular mechanism.

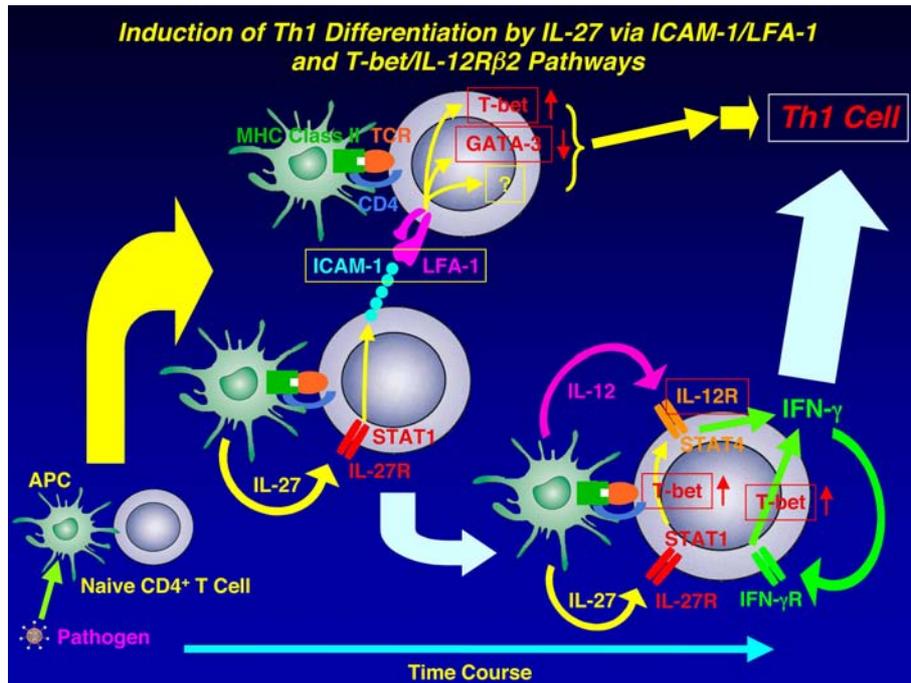
Results

To first elucidate the role of IL-12 in IL-27-induced Th1 differentiation *in vivo*, we examined whether IL-27 induces an antitumor activity even in IL-12 p40-deficient mice, and found that IL-27 can induce an antitumor activity by augmenting type 1 cell-mediated immunity *in vivo* in IL-12-independent manner. We then examined the effect of IL-27 on *in vitro* Th1 differentiation with various concentrations of IL-12. Naive CD4⁺ T cells were purified from spleen cells and stimulated *in vitro* by plate-coated anti-CD3 and anti-CD28 in Th1 polarizing conditions, neutral conditions, or Th2 polarizing conditions in the presence or absence of IL-27. These primed cells were expanded in IL-2-containing medium on day 3 and restimulated on day 6 by plated-coated anti-CD3 for 24 h. Resultant supernatants were analyzed for cytokine production by ELISA. Consistent with the *in vivo* results, IL-27 induced *in vitro* Th1 differentiation most efficiently in the absence of IL-12, and that the presence of higher concentration of IL-12 overruled the ability of IL-27 to induce Th1 differentiation. We then examined the requirement for STAT4, which is an essential signal-transducing molecule to IL-12/IL-12R signaling, in IL-27-induced Th1 differentiation using STAT4-deficient naive CD4⁺ T cells, whereas STAT4 was not required for IL-27-induced Th1 differentiation. Since IL-27 synergizes with IL-12 in primary IFN- γ production and IFN- γ is well known to play a critical role in Th1 differentiation through induction of T-bet and subsequent IL-12R β 2 expression, we next examined the requirement for IFN- γ in IL-27-induced Th1 differentiation using IFN- γ -deficient naive CD4⁺ T cells and neutralizing mAb against IFN- γ . However, IFN- γ was not required for IL-27-induced Th1 differentiation. Several previous studies

revealed that ICAM-1/LFA-1 interaction is important for Th1 polarization and that ICAM-1 expressed on T cells also can provide a costimulatory signal for T cell activation and Th1 cytokine production. More recently, it has been demonstrated that ICAM-1/LFA-1 interaction favors human Th1 development and this Th1 differentiation is overruled by IL-12 in a dose-dependent manner. Therefore, we next examined the cell surface expression of ICAM-1 and its counterpart LFA-1 on naive CD4⁺ T cells. IL-27 induced rapid and marked up-regulation of ICAM-1 expression on naive CD4⁺ T cells stimulated with plate-coated anti-CD3 and anti-CD28. Blocking experiments using anti-ICAM and/or anti-LFA-1 revealed that the ICAM-1/LFA-1 interaction plays an important role in the IL-27-induced Th1 differentiation. Since IL-27 induces expression of T-bet, we next examined the requirement for T-bet in IL-27-induced Th1 differentiation using T-bet-deficient mice. However, T-bet was revealed not to be essential to IL-27-induced rapid up-regulation of ICAM-1 expression and Th1 differentiation. We then investigated the role of STAT1 in IL-27-induced Th1 differentiation using STAT1^{-/-} and STAT1^{+/-} mice. In STAT1^{-/-} naive CD4⁺ T cells stimulated with plate-coated anti-CD3 and anti-CD28, induction of T-bet expression was almost abolished even in the presence of IL-27, IL-12 or both, although efficient induction of T-bet expression by IL-27 was observed in STAT1^{+/-} naive CD4⁺ T cells. Moreover, the absence of STAT1 markedly inhibited the Th1 differentiation induced by IL-27 as well as IL-12. These results suggest that STAT1 is required for IL-27-induced rapid up-regulation of ICAM-1 expression and Th1 differentiation. It was previously demonstrated that STAT1 activated by IFN- γ binds to the GAS located in the 5'-flanking region of ICMA-1 gene and confers IFN- γ responsiveness. To define the mechanism whereby IL-27 induces up-regulation of ICAM-1 expression, we finally performed a reporter assay and EMSA, and revealed that STAT1 directly mediates IL-27-induced transcriptional up-regulation of ICAM-1 gene expression.

Discussion & Summary

The present results suggest that the ability of IL-27 to induce Th1 differentiation is most prominent under Th1 polarizing conditions but without IL-12 and overruled by IL-12 dose-dependently. The IL-27-induced Th1 differentiation was mainly mediated by rapid and marked up-regulation of ICAM-1 expression on naive CD4⁺ T cells through ICAM-1/LFA-1 interaction in STAT1-dependent but T-bet-, IFN- γ - and STAT4-independent mechanism. Considering that IL-27 is produced from macrophages and DCs earlier than IL-12, the present results suggest that IL-27 may play a pivotal role in early efficient induction of Th1 differentiation until enough amounts of IL-12 are produced (Figure 1).



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- 2) Medical Sciences
- 2-2) Central Nervous System

The analysis of molecular mechanisms of schizophrenia and bipolar disorder caused by 22q11.2 deletion

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Introduction

Children with del 22q11.2 syndrome have learning difficulties, deficits of motor development, and attention deficit disorders¹. They have high risk for developing schizophrenia and bipolar disorder. Df1 is a mouse homologous region of human chromosome 22q11.2. Mice that are heterozygously deleted for Df1 region have deficits in pre-pulse inhibition, learning and memory².

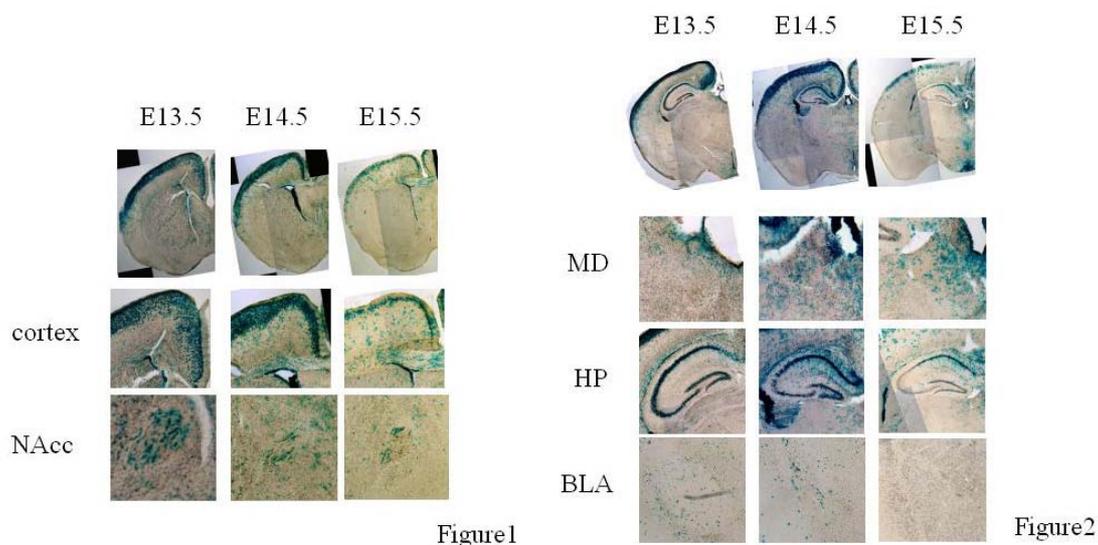
Results

Df1 region contains about 30 genes including COMT and PRODH. These genes are shown to be associated with schizophrenia and bipolar disease by genetical studies. Knockout mice of these genes show behavioral abnormalities such as hyper-locomotion and pre-pulse inhibition deficit, which are characteristic physiological disorders of patients with schizophrenia. Our aim is to elucidate which gene and which neural circuit is responsible for the psychiatric disorders of Df1-deleted mice. We are planning to use lenti virus and adeno virus mediated gene delivery to rescue these phenotypes of Df1 deleted mice. The neural circuit of pre-pulse inhibition includes hippocampus (HP), prefrontal cortex (PFC), ventral tegmental area (VTA), nucleus accumbens (NAc), ventral pallidum, basolateral amygdala (BLA), mediodorsal thalamus (MD) and pedunculopontine nucleus (PPTg), which modulates responses of the primary startle circuit composed of the auditory nerve, the cochlear root neurons, the nucleus reticularis pontis caudalis (PnC), and the spinal motor neuron³.

It has been reported that adenovirus injection to embryonic ventricles enables introduction of exogenous genes to specific layers of cerebral cortex⁴. However, It has not been known which neural circuit is affected by this adenovirus mediated gene delivery method, because adenovirus mediated gene introduction is only transient and introduced genes are lost by cell divisions after gene introduction. To identify all neural substrates effected by this adenovirus-mediated gene delivery at embryonic stage, we injected Cre-expressing adenovirus to embryonic ventricles of ROSA26 Cre reporter (R26R) mice⁵ at various developmental stages, E13.5, E14.5, and E15.5. The R26R consists

of a floxed PGK neo cassette with four polyadenylation sites and a β -galactosidase gene (β -Gal) downstream of the distal LoxP. In the absence of Cre the expression of β -Gal is blocked by the PGK neo polyadenylation sites. In the presence of Cre the LoxP sequences flanking the PGK neo cassette are deleted and β -Gal is expressed. With their β -Gal activity we can trace all progeny of adenovirus-infected cells. In brains infected at E13.5 or E14.5, β -Gal-positive cells were observed in cortex (layer IV and II/III), BLA, NAc, and CA1, CA2, CA3 and dentate gyrus of HP, and MD. In brains infected at E 15.5, lacZ-expressing cells were found only in cortex (layer II/III), NAc, and hippocampus CA1, CA2 region (Figure1,2).

We are also planning to search anatomical and developmental abnormalities of Df1-deleted mice. The migration pattern of differentiated neurons of early postnatal mice and the histology of hippocampus will be examined. This is because Disc1, which is known to be genetically associated with schizophrenia, is important for migration of differentiated neurons and damage to ventral hippocampus cause similar behavioral deficits.



Discussion & Summary

Our Results showed that adenovirus injection to embryonic ventricle can introduce exogenous genes not only to cerebral cortex but to BLA, NAc, and CA1, CA2, CA3 and dentate gyrus of HP, and MD, which play pivotal roles in PPI, though their expression is lost by cell divisions after birth. This results suggest potentials of this adenovirus-mediated method to modulate PPI neural circuit directly.

Gene delivery to mature neurons is very difficult. However, our method of virus-mediated gene delivery allowed us to elucidate which gene in which neural circuits are responsible for behavioral abnormalities of schizophrenia and bipolar disorder. This research can contribute to the exploitation of new remedy for schizophrenia and bipolar disease.

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- 2) Medical Sciences
- 2-2) Central Nervous System

Role of forkhead transcription factor Foxo1 in feeding behavior

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Introduction

Obesity should be a cause of type 2 diabetes, cardiovascular disease, hypertension and hyperlipidemia. Therefore, it is very important to elucidate molecular mechanism of obesity and to develop drugs for preventing obesity. Obesity is defined by both increased food intake and decreased energy expenditure. Now two important hormones are known well for regulating food intake. The first one is leptin, which is secreted from white adipose tissue, and the second one is insulin, which is secreted from pancreatic β -cell. Both hormones act through arcuate nucleus in hypothalamus. However, the molecular mechanism by which insulin regulates food intake, especially downstream of Irs-2, has not been known yet. Forkhead transcription factor FoxO1 is phosphorylated by insulin/IGF-1 through PI3-kinase-dependent manner and known to be a negative regulator of insulin signaling. Recently, we found out that FoxO1 is also expressed in the arcuate nucleus in hypothalamus. The purpose of this project is to clarify role of Foxo1 in feeding behavior. For this purpose, we construct knock-in mice of two kinds of mutant FoxO1 (3A; constitutively active type and Δ 256; dominant negative type) in ROSA26 genomic locus. Using Cre-loxP system, we will cross ROSA26/ mutant Foxo1 knock-in mice with Pomc Cre or Agrp Cre in order to overexpress mutant Foxo1 in arcuate nucleus-specific manner and analyze effects of overexpression of mutant Foxo1 on feeding behavior and glucose metabolism.

Results

1. Construction of Rosa26-mutant FoxO1 knock-in mice

In order to investigate roles of FoxO1 in arcuate nucleus, we tried to overexpress two kinds of mutant FoxO1s which include 3A (a constitutively active mutant FoxO1) and Δ 256 (a dominant negative FoxO1) in a neuron-specific manner using Cre recombinase transgenic mice (Figure 1a). During this research period, we established both Rosa26-3A and Rosa26- Δ 256 knock-in mice (Figure 1b).

2. Overexpression of mutant FoxO1s in Agrp/Npy neuron or Pomc neuron

For overexpression of mutant FoxO1s in a neuron-specific manner, we crossed Rosa26-FoxO1 knock-in mice with Agrp Cre or Pomc Cre transgenic mice.

In this report, we described data from Rosa26-3A FoxO1-Agrp Cre mice. About other knock-in mice of FoxO1, projects are going on and not finished.

3. Growth curve of Rosa26-3A FoxO1-Agrp Cre mice

In both male and female Rosa26-3A-Agrp Cre mice, we could not see any significant differences compared with other genotyped mice.

4. Food intake of Rosa26-3A-Agrp mice

There were any significant differences of food intake in Rosa26-3A-Agrp Cre mice compared with other genotyped mice.

5. Glucose metabolism of Rosa26-3A-Agrp Cre mice

1) IPGTT (intraperitoneal glucose tolerance test)

In both male and female Rosa26-3A-Agrp mice, glucose tolerance was better than in other genotyped mice (Figure 2a).

2) ITT (insulin tolerance test)

In male Rosa26-3A-Agrp Cre mice, we could observe better insulin sensitivity (Figure 2b).

6. Oxygen consumption (VO₂)

In male Rosa26-3A-Agrp Cre mice, increased oxygen consumption was observed.

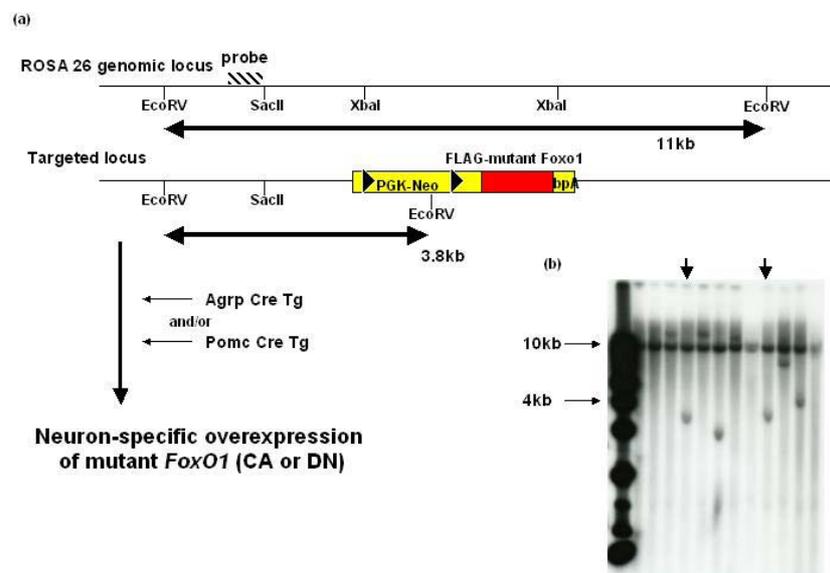


Figure 1. (a) Scheme of targeting vector of knock-in mice and (b) Southern blotting of positive clones

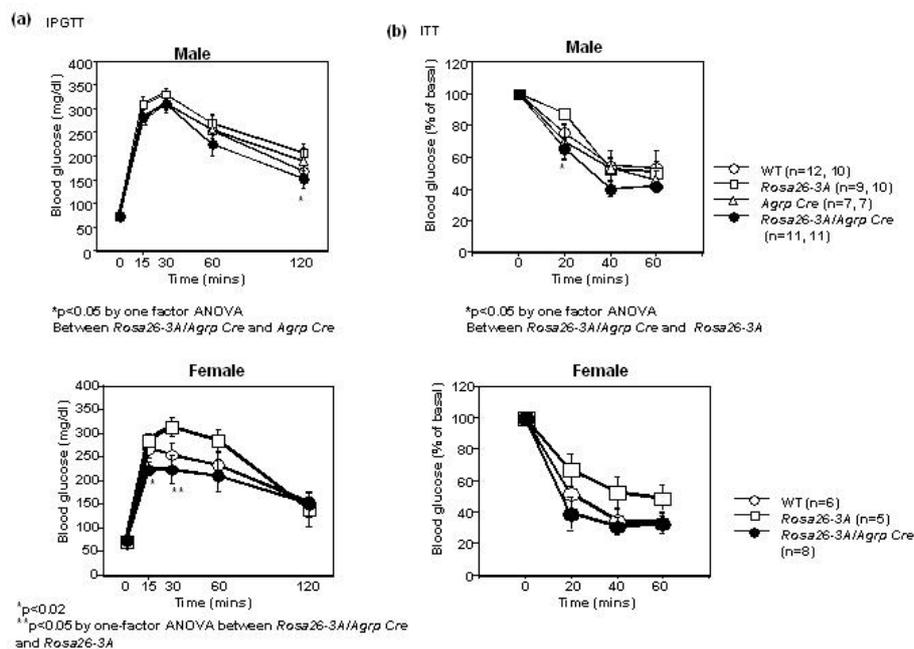


Figure 2 Overexpression of constitutively active mutant FoxO1 in *Agrp/Npy* neurons improves glucose tolerance at 2 months of age

Discussion & Summary

We established two kinds of *Rosa26*-mutant FoxO1 (3A and $\Delta 256$) knock-in mice. Using one (*Rosa26-3A* FoxO1) of them, we analyzed effects of overexpression of 3A FoxO1 in *Agrp/Npy* neurons on growth, food intake and glucose metabolism. Although there are no significant differences in body weight and food intake, mutant knock-in mice showed better glucose tolerance and insulin sensitivity. Furthermore, they showed increased oxygen consumption. The 3A FoxO1 is a constitutively nuclear mutant and reflects defect of insulin signaling. Therefore, these data suggest that insulin signaling in *Agrp/Npy* neuron worsens glucose metabolism and also transcriptional activation of FoxO1 may improve insulin sensitivity.

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- 2) Medical Sciences
- 2-4) Arthritis/Bone Metabolism

Cell cycle regulation therapy to treat rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial hyperplasia with massive infiltration of inflammatory cells in the affected joints. This leads to functional loss of the joints. Current treatments try to suppress inflammatory mediators including tumor necrosis alpha, but do not effective to all patients. We have been developing cell cycle regulation treatment since we showed that intraarticular gene transfer of cyclin-dependent kinase inhibitors (CDKIs) to suppress synovial hyperplasia has been effective in treating animal models of rheumatoid arthritis (RA) (1-3). CDKIs inhibit cell cycling by inhibition of cyclin-dependent kinases (CDKs) and also modulate immune function in a CDK-independent pathway. Therefore, we would like to see whether systemic administration of small-molecule (sm) CDK inhibiting compounds can ameliorate arthritis.

Results

The effects of smCDKIs, clinically tolerated flavopiridol and a newly synthesized CDK4/6-selective inhibitor, compound A, on synovial cell growth were determined using proliferation and cell cycle analyses. Mice were subjected to immunization with typeII collagen (CII) or serum transfer of K/BxN mice to develop arthritis. They were then treated with systemic administration of smCDKIs and the severity of arthritis was assessed clinically and histologically.

In vitro, smCDKIs inhibited proliferation of synovial fibroblasts without inducing apoptosis. Treatment of collagen-induced arthritis (CIA) with flavopiridol suppressed synovial hyperplasia and joint destruction. However, serum levels of anti-CII antibodies were preserved. The treated mice developed arthritis after termination of treatment. These facts argue that immune responses to CII were not impaired. The same treatment ameliorated K/BxN serum-induced arthritis elicited in lymphocytedeficient mice. Similarly, a CDK4/6-selective inhibitor suppressed CIA, and did so even when administered after the onset of clinical disease.

Figure 1 depicts Arthritis scores of CIA mice treated with flavopiridol. Mice with CIA were treated with intraperitoneal injection of 1mg/kg, 2.5 mg/kg, flavopiridol in 0.01 % DMSO, or DMSO saline for 10 consecutive days (a) or twice a week for 5 weeks (b). Treatment was started 25 (a) or 24 (b)

days after the initial immunization. Severity of arthritis was assessed with arthritis score. Shown are the mean score \pm SD of 7 mice per group. Another set of 2.5 mg/kg flavopiridol treatment was started concurrently, but was terminated after 10 days (b, open square). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ Figure 2 depicts Treatment of CIA mice with compound A. CIA mice were treated with compound A orally (200 mg/kg) every 12 hours for 7 days. Treatment was started at the day when arthritis became evident (24 days after the initial immunization). CIA mice were also therapeutically treated with compound A intraperitoneally (30 mg/kg) for 5 days and repeated again after a day interval. Control mice were treated with buffer including DMSO. Severity of arthritis was assessed by the arthritis score, ankle width, or paw thickness. Shown are the mean \pm SD of 8 mice per group. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

Discussion & Summary

We have demonstrated that systemic administration of smCDK inhibitors is anti-arthritic, with no apparent impairment of immune responses. In addition, the smCDK inhibitors were effective in treating lymphocyte-independent arthritis. Although we cannot formally exclude their effects on immune cells, these results argue that the effects of smCDK inhibitors on synovial fibroblasts primarily account for the therapeutic effects. Previous studies have shown that endogenous CDKs have an immunomodulatory effect, which does not depend on the kinase activity of CDKI, and thus could be responsible at least partly for the therapeutic effects of CDKI gene therapy. 16,17 However, the present study has determined that inhibition of CDK activity alone is sufficient to ameliorate animal models of RA. We propose that smCDKs be designated a new class of anti-rheumatic drugs, which inhibit a distinct phase in rheumatoid pathology.

Figure 1

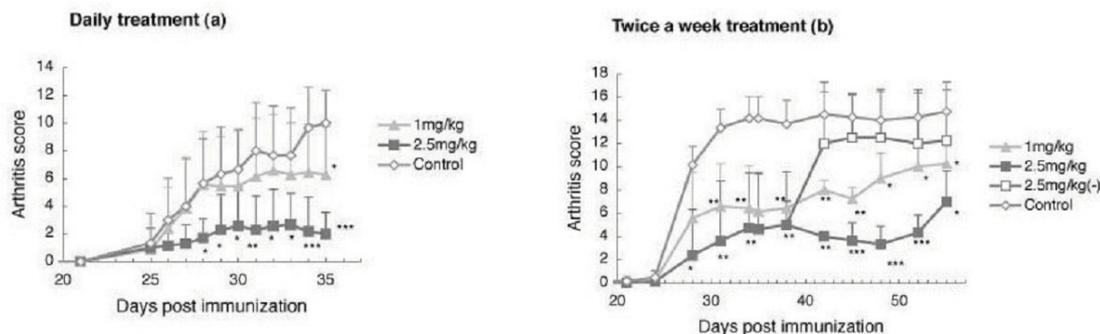
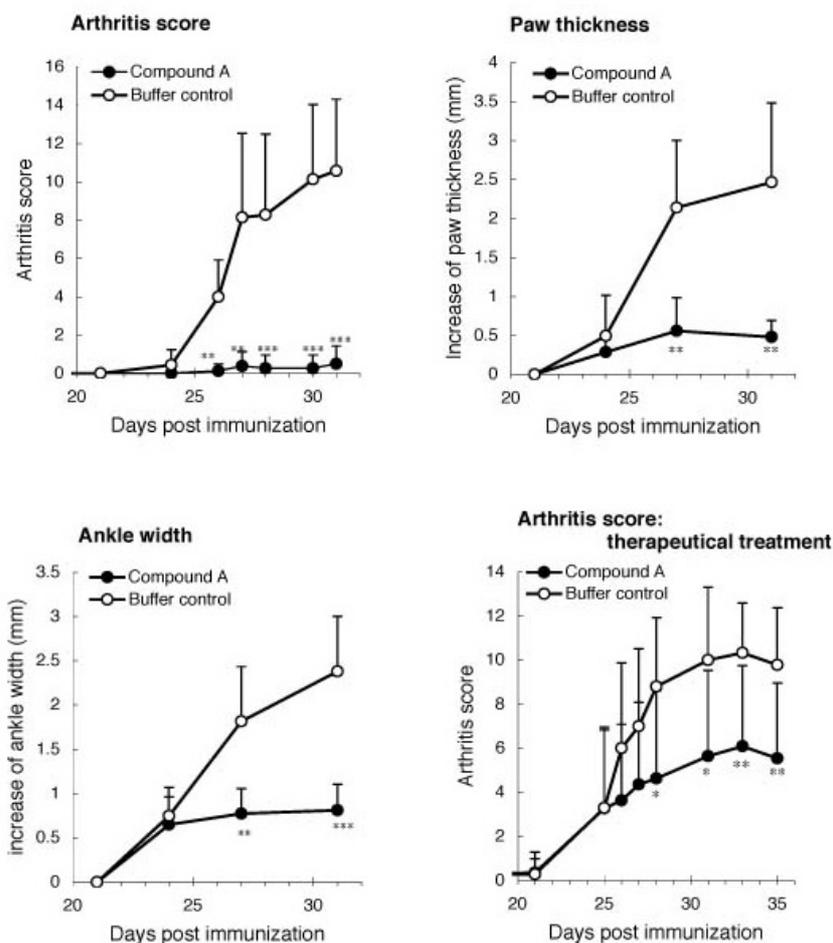


Figure 2



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- 2) Medical Sciences
- 2-5) Cardiovascular /Metabolic/Endocrine

Creation of knock-down transgenic rabbits using RNAi technology for the study of atherosclerosis

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Introduction

Rabbits are considered an excellent experimental model for the study of atherosclerosis. We laboratory has been using transgenic rabbits to investigate many gene functions and their relationship with atherosclerosis. However, compared to mice, rabbit ES cells are still unavailable, it is not possible to generate knock-out (KO) rabbits using gene target technology. Nuclear transfer may be used as another method to create KO rabbits, however, there are many technical problems which remain unsolved. In this study, we performed a pioneer study aiming at generating novel knock-down (KD) rabbit models using RNAi technology. Although the technology is still in the immature stage, it will provide a new avenue to create new rabbit models for biomedical research.

Results

In this study, we performed the following studies. First, we cloned rabbit lipoprotein lipase cDNA sequences, which will be used as a potential target for gene-knock-down. Second, we created a tRNA-promoter-directed construct for the gene transfer. This construct contains GFP sequence which will allow the detection of the target gene in transfected cells or tissues *in vivo*. The construct also has two copies of insulator, which keep the tissue-specific expression at the stable levels independent upon the copy number and location of the transgene. The efficacy of this construct was confirmed *in vitro* using cultured cells. The major difficulties in developing KD rabbits are in finding effective sequences used for gene silencing of the target gene and methods for the evaluation of gene-silencing ability. To explore a new method in this regard, we selected rabbit cholesteryl ester transfer protein (CETP), an important protein for the lipoprotein metabolism, which is not present in mice. Using the internet sequence information, we identified several sequences for rabbits CETP silencing sequences. After that, we attempted to find a hepatocyte cell line of rabbits. For this purpose, we received 16 hepatocyte cells lines of rabbit produced by Dr. Nakanishi's lab. This work is still under way and hopefully we can have such cell lines for the evaluation of any genes

expressed in the liver before we can start microinjection into rabbits.

Discussion & Summary

In this study, we have performed a pioneer study aiming at the creation of KD transgenic rabbits. Apparently, there are still several obstacles in technology, including the establishment of the easy method for the evaluation of gene-silencing efficacy *in vitro*, construct that can be used for microinjection, and germline transmission from founders to the offspring. To target the hepatic-specific gene, the hepatocyte lines are urgently needed. We also work out these procedures and hopefully we can find a new way to generate KD rabbits in future.

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- 2) Medical Sciences
- 2-6) Gastro-intestinal

Regulation of macrophage responses to commensal flora by the colonic mucosal microenvironment

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Introduction

In spite of enormous numbers of Gram-negative bacteria with outer membrane lipopolysaccharide (LPS) in the colon, inflammatory responses normally do not occur. Our previous study showed that macrophages in the large intestinal mucosal site are hyporesponsive to lipopolysaccharide (LPS) in terms of release of inflammatory cytokines, such as TNF- α . Since we found that various inflammatory cytokines and chemokines are constitutively produced in the colonic mucosa, we hypothesized that particular microenvironment in the colon comprised of these molecules rendered macrophages to be hyporesponsive to LPS in steady state.

Results

1. Murine bone-marrow (BM) derived macrophages or a macrophage cells line RAW264.7 were pretreated with various cytokines and chemokines known to be produced in colonic mucosa, including IL-4, IL-13, activin A, GM-CSF, MIF, lymphotactin, TGF- β , IL-1 β , and IL-6 for 12 hours and then stimulated with LPS to measure release of TNF- α . Among these cytokine and chemokine molecules, fractalkine (CX3CL1, FKN) was a potent inhibitor of TNF- α release induced by LPS stimulation, which was around 50 %. Dependence of this effect on IL-10 was limited because FKN inhibited TNF- α release by cells derived from IL-10 knockout mice, although less extent than as seen in cells from wild type mice. The levels of IFN- γ , IL-12, MCP-1, IL-10 or IL-6 release after LPS stimulation was not altered by pretreatment with FKN. FKN itself showed no effect on the TNF- α , IL-10 or other cytokine production tested in both BM-derived macrophages or RAW264.7 cells.

2. In immunohistological examination, we found that FKN was abundantly found in both small and large intestine of mouse, in epithelial cells and mesenchymal cells. Colonic tissue showed stronger staining of FKN than small intestine. We isolated myofibroblasts from the small and large intestine and established cell lines. Myofibroblasts derived from colonic tissue showed much higher expression of FKN than those from the small intestine, as determined by RT-PCR and

Western-blotting.

3. When BM-derived macrophages or RAW267.4 cells were pretreated with low concentration of FKN (1 ng/ml), expression of cell-surface Toll-like receptor 4 and MD-2 were decreased, determined as mean fluorescence intensity in flow cytometry. On the other hand, expression of CD14 did not change. The levels of mRNA of both molecules determined by RT-PCR also decreased after treatment with FKN when compared with cells without pretreatment.

4. MAP kinase activation was analyzed by western blotting using antibodies against phosphorylated ERK1 and ERK2. Six hours after LPS stimulation, MAP kinases were dramatically activated in RAW264.7 cells. In contrast, when cells were pretreated with FKN for 12 hours, such strong MAP kinase activation after LPS stimulation was not seen.

5. One hour after stimulation of RAW267.4 cells with LPS, nuclear translocation of NF- κ B subunits, p65/p50, was observed by EMSA; however, pretreatment with FKN prevented nuclear translocation of p50. We found that translocation of p65 was not inhibited but rather enhanced in FKN-treated cells using transfactor assay and immunostaining of cells analyzed with fluorescent microscopy. When cells were treated with high concentration of FKN (100 ng/ml), mild activation of NF- κ B was seen; however this activation was blocked in the presence of anti-FKN antibody in the culture. This indicated that effect of addition of FKN solution to the cells was not due to the contamination with endotoxin but FKN itself.

6. We stimulated RAW264.7 cells with LPS derived from different Gram negative bacteria strains (*Salmonella minnesota*, *Echerichia coli*, and *Bacteroides vulgatus*) as well as CpG-ODN, peptidoglycan and Pam3CSK4, after pretreatment with FKN. Of interest, inhibitory effect of FKN on TNF- α release was seen only when cells were stimulated with LPS, ligand for Toll-like receptor 4, irrespective of their origin and potential to induce TNF- α release. Peptidoglycan, Pam3CSK4, and CpG-ODN, which are ligands for Toll-like receptor 2 and 9, efficiently induced TNF- α secretion, which was comparable to the levels of LPS; however, it was not affected by pretreatment with FKN at all. These results indicated that FKN modulated the signaling pathway transduced by Toll-like receptor 4, but not Toll-like receptors 2 or 9.

Discussion & Summary

The treatment of macrophages with FKN attenuated the TNF- α production induced by LPS stimulation. FKN modified the signaling pathway and activation of nuclear transcriptional factor after LPS stimulation. Our findings, decrease of the expression of TLR4-MD-2 molecules, modulated NF- κ B activation and suppression of MAPKs phosphorylation, caused by pretreatment with fractalkine, all supported the suppression of TNF- α secretion after LPS stimulation. Since we found the constitutive production of fractalkine by myofibroblasts and epithelial cells in the colon,

FKN may contribute to hyporesponsiveness of colonic macrophages to LPS.

Possible mechanism of specific modification of signaling via Toll-like receptor 4 in the macrophage responses to LPS is under further investigation.

Figures and Tables

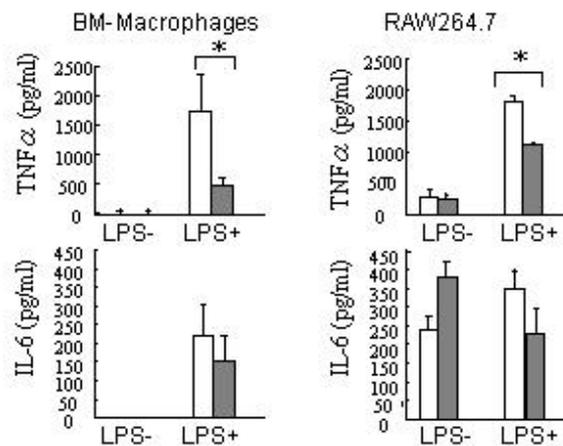


Figure 1. Pretreatment of macrophages with fractalkine attenuated the production of TNF- α but not IL-6 after stimulation with LPS. Bone marrow (BM) derived macrophages or a macrophage cell line RAW264.7 were cultured with (shaded columns) or without (blank columns) fractalkine for 12 hours, and then stimulated with LPS for 6 hours. Secretion of TNF- α or IL-6 in the culture medium was measured by ELISA. * Statistically significant differences ($P < 0.05$).

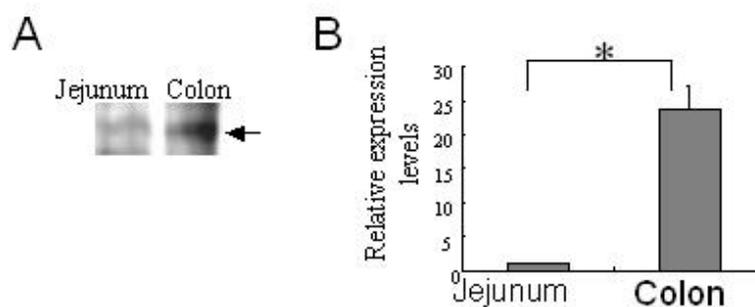


Figure 2. Constitutive expression of fractalkine in the colon is higher than in the jejunum. A, Western blotting of whole tissue extract. B, Quantitative RT-PCR for mRNA expression. * Statistically significant differences ($P < 0.05$).

- 2) Medical Sciences
2-10) Dermatology

A study of metastatic trait of malignant melanoma with expression
of MCM3-binding β -primase GANP correlated with
highly metastatic clinical cases

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Introduction

Radical treatment of malignant melanoma is hampered by the highly metastatic trait of cells. It is important to detect the nevus cells or melanoma cells that are potent to be transformed into highly metastatic property at the primary resection. Here, we found that MCM3-binding β -primase GANP is up-regulated in the severe stage malignant melanoma cells. Moreover, we hypothesized that the abnormal expression of the GANP is involved in transformation of malignant metastatic trait into melanoma cells.

Results

The department of dermatology has a largest collection of Japanese patients of malignant melanoma patients in the western area and has long records of clinical courses. We have studied the past clinical samples to evaluate our system of detecting a highly metastatic cell at a single cell level²⁾. First, we examined if melanoma cell lines also up-regulated the GANP expression compared to melanocytes and next observed the subcellular localization of GANP and P-GANP in the nucleus and cytoplasm. GANP and P-GANP were weakly expressed in normal melanocytes but they were up-regulated in melanoma cell lines (G361, HM3KO and MeWo). RT-PCR products of MCM3 and BCL-6 mRNAs were detected in human melanocytes and all three human melanoma cell lines. There was no significant difference in the level of MCM3 message. Second, we analyzed the expression of GANP and P-GANP in various melanocytic lesions. GANP and P-GANP were widely expressed at various levels both in benign and malignant melanocytic lesions. Most of the melanocytic cells co-expressed GANP and P-GANP, which was compatible to human melanoma cell lines. Intensity of GANP expression was not associated with disease progression. On the other hand, P-GANP in primary and metastatic melanoma lesions was significantly stronger than that of melanocytic nevus lesions ($p < 0.001$ and $p = 0.015$, respectively) (Table 1). Intracellular localization

of GANP and P-GANP in melanocytic nevus cells and melanoma cells was not significantly different. Third, we analyzed the levels of MCM3, BCL-6, and Ki67 expression in 10 melanocytic nevi, 11 primary melanoma, and 5 metastatic melanoma lesions. MCM3 and Ki67 were exclusively detected in primary and metastatic melanoma cells but not in melanocytic nevi cells. BCL-6 was only detected in 1 primary lesion with 20% positive cells, although BCL-6 was previously shown as a highly indicative marker for poor prognosis of melanoma patients. We compared the levels of MCM3 and/or Ki67 expression with GANP and P-GANP in 17 primary melanoma lesions. Higher expression of MCM3 and/or Ki67 (more than 20% of positive cells) was not associated with intensity of GANP and P-GANP expression in melanoma cells. In addition, cytoplasmic GANP and P-GANP expression showed a trend to higher MCM3 and Ki67 positive cells than those of nuclear localization, but this may not be significant, since the number of these lesions studied was too small for statistical analysis (Figure 1). These results suggested that MCM3 and GANP co-localizations are functionally related with cell cycle progression or cell proliferation in the melanoma cells. Finally, we established two kinds of mutant mice carrying *ganp*-gene in the melanocytes using tyrosinase promoter, because we recently reported lymphomagenesis in *ganp*-transgenic mice¹⁾. Although we observed melanocyte-specific *ganp*-transgenic of *ganp*-deficient mice for about one year, the mice did not show any abnormalities in skin cells. As it is very important to ask if the onset of malignant transformation is spontaneous or if it is affected by the various environmental stresses on the mice, we are now doing continuous observation of the phenotypes in these mice after exposure by ultraviolet and other chemical agents.

Figure 1

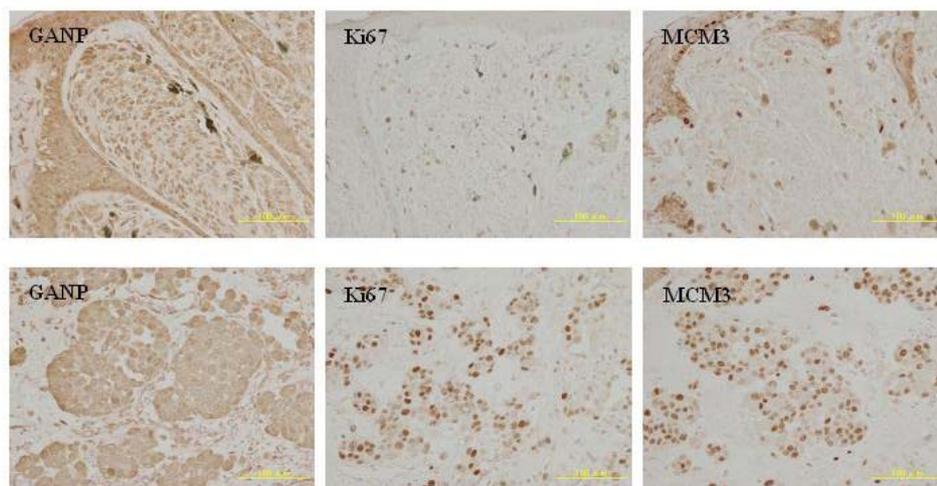


Table 1. Intensities of GANP and P-GANP expression in surgically removed melanocytic lesions

| | GANP | | | P-GANP | | | | |
|---------------------|------|-----------|-------------|---------|-----------|-------------|-------|------------------------|
| | # | Strong(%) | Moderate(%) | Weak(%) | Strong(%) | Moderate(%) | | Weak(%) |
| Melanocytic nevus | 14 | 0 | 10 (71) | 4 (29) | 0 | 8(57) | 6(43) | P = 0.001 P = 0.023 |
| Primary melanoma | 36 | 3(8) | 28(78) | 5(14) | 11(31) | 22(61) | 3(8) | |
| Metastatic melanoma | 9 | 2(22) | 6(67) | 1(3) | 3(33) | 5(56) | 1(11) | |

#: total number of lesions, %:Percentage of lesions

Discussion & Summary

The present study has demonstrated for the first time that GANP and P-GANP were up-regulated in human melanoma cell lines compared to human melanocytes. GANP and P-GANP were expressed in the nucleus of melanocytes but co-expressed both in the nucleus and slightly in the cytoplasm of melanoma cells. Intensity of P-GANP staining in melanoma cells showed stronger than that of benign melanocytic nevus cells. However, intensity of GANP staining in melanoma cells did not reach significant difference. These findings suggest that certain high level of GANP as well as P-GANP expression may be associated with malignant transformation through direct or indirect interactions with other factors that must play an important role in melanoma disease progression. Most of the melanocytic cells showed cytoplasmic and nuclear localizations of GANP and P-GANP, but nevus cells at lower portions of the dermis showed nuclear localization exclusively. On the other hand, cytoplasmic localizations of GANP and P-GANP in melanoma cells were associated with higher MCM3 and Ki67 expressions than those of nuclear localization suggesting that GANP expression is shuttled between the cytoplasm and the nucleus during cell cycle progression. These data were agreeable to those of cultured melanocytes and melanoma cells, since melanocytes showed only nuclear expression of GANP and P-GANP.

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2) Medical Sciences

2-12) Hamatology

Role of Polycomb-group complexes in Geminin degradation which is crucial for hematopoietic stem cell regulation

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Introduction

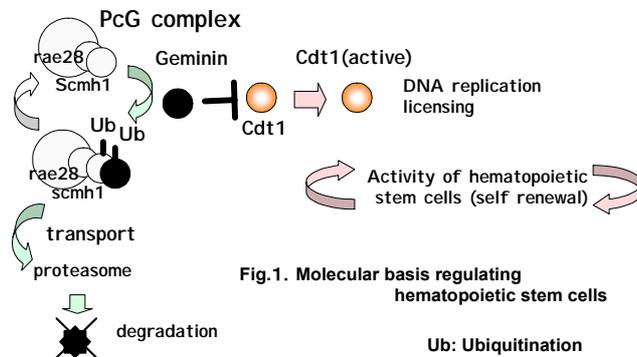
Hematopoietic stem cell (HSC) transplantation is now routinely performed in clinical practice for the treatment of patients with hematological malignancies and bone marrow failure. And new strategies with HSCs are developed for the treatment of patients with the other malignancies and vascular failure including myocardial infarction, attracting further attention to HSCs. However, molecular mechanisms regulating HSCs still remain unclear. In this study, focusing on Polycomb-group genes we attempted to uncover the role underlying stemness.

Results

Polycomb-group genes (PcG) were uncovered by *Drosophila* genetics as genes supporting the cellular memory system during development. We for the first time reported that PcG are required for sustaining HSC activity. Although PcG are recently reported to support stemness through the repression activity to *ink4A* locus encoding p16CDKI and p19ARF, so far the molecular bases underlying PcG functions supporting stemness are not fully understood. We found that licensing of DNA replication was impaired in mice lacking PcG. And PcG complexes interact with Geminin, an inhibitor of a DNA replication licensing factor Cdt1 through *Scmh1*, a member of PcG, which we originally identified based on the conserved protein structure in mammals. We also found *Hoxb4*, a well known factor regulating HSCs, also interacts with Geminin. Furthermore, by using retrovirus-mediated gene transfer method, we examined in detail the effect of affected Geminin regulation on HSCs. And we showed that PcG complexes regulate Geminin at the level of protein through ubiquitination and that Geminin regulation is pivotal in hematopoiesis and HSC regulation.

Discussion & Summary

These results indicate that DNA replication licensing is pivotal in sustaining HSC activity. And PcG complexes play a crucial role in regulation of Geminin. Now by using *in vitro* reconstituting system further detailed analysis of PcG complexes are in progress. These findings may help future progress in regenerative medicine with stem cells.



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- 3) Chemical Sciences
 3-1) Organic Chemistry

Design and Synthesis of Optically Active Supramolecular Capsules in Aqueous Solution and Their Application to Asymmetric Recognition.

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Introduction

Zinc(II) complex of 12-membered cyclic tetraamine **1** (ZnL^1) gives a stable complex with anion (X^-) in aqueous solution (Figure 1). We have reported that a 1:1 mixture of a tris(Zn^{2+} -cyclen) **2** (Zn_3L^2) and a trianion of trithiocyanuric acid (TCA^{3-}) yielded a 4:4 self-assembly complex, whose exterior is represented by a cuboctahedral framework, through Zn^{2+} - S^- coordination bond in aqueous solution at neutral pH. It was also discovered that the supramolecular complex has an inner cavity, in which hydrophobic guest molecules are encapsulated.

More interestingly, the supramolecular complex is a chiral molecule, since the three C-S- Zn^{2+} bonds around all of the four TCA^{3-} units in **3** are bent by 100° in a clockwise or counterclockwise fashion, as shown in **4** (Figure 1).

In this project, therefore, we have decided to synthesize optically active supramolecular cage for selective recognition of chiral guest molecules and asymmetric synthesis in aqueous solution.

Results

We have started to synthesize a chiral cyclen, (S,S,S,S)-2,5,8,11-tetramethyl-1,4,7,10-tetraazacyclododecane) **5** (L^3) as an important unit for an optically active tris(Zn^{2+} -cyclen) **6** (Zn_3L^4) (Figure 2). First, L-alanine was reduced to L-alaninol, which was N-alkylated by reductive amination with benzaldehyde to give N-benzyl-L-alaninol. N-benzyl-L-alaninol was reacted under the conditions for Mitsunobu reaction to yield an N-benzyl-1-(S)-methylaziridine. N-Benzyl-1-(S)-methylaziridine was reacted with acid to yield **5** (L^3), whose three nitrogens were protected with $(\text{Boc})_2\text{O}$ to give 3Boc- L^3 .

Discussion & Summary

As described above, we have succeeded to synthesize 3Boc- L^3 . So, we are now reacting 3Boc- L^3 with 1,3,5-tris(bromomethyl)benzene followed by deprotection to obtain an optically active tris(cyclen) (L^4), which will be converted to trimeric Zn^{2+} complex **6** (Zn_3L^4). The attempts to

construct optically active supramolecular capsule 7 will be made.

Fig. 1 Supramolecular capsule formed from Zn_3L^2 and TCA^{3-}

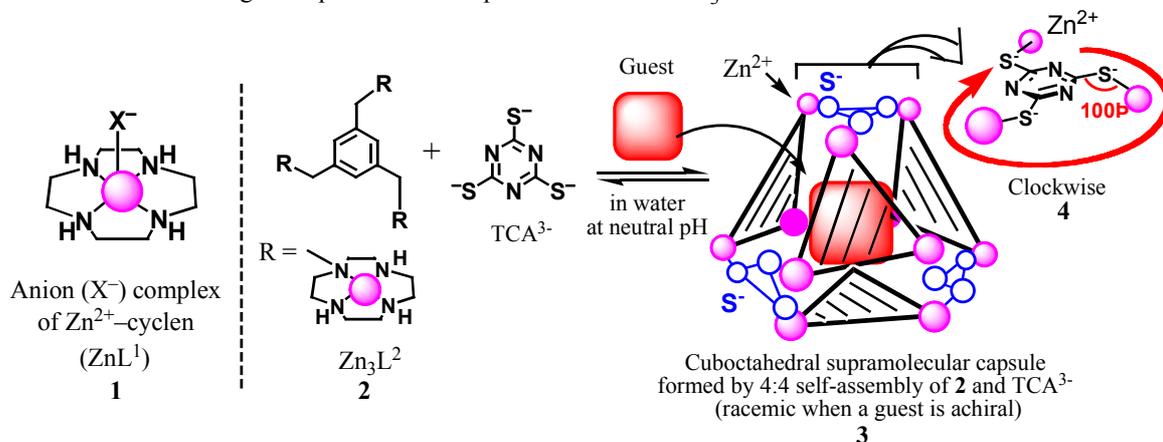
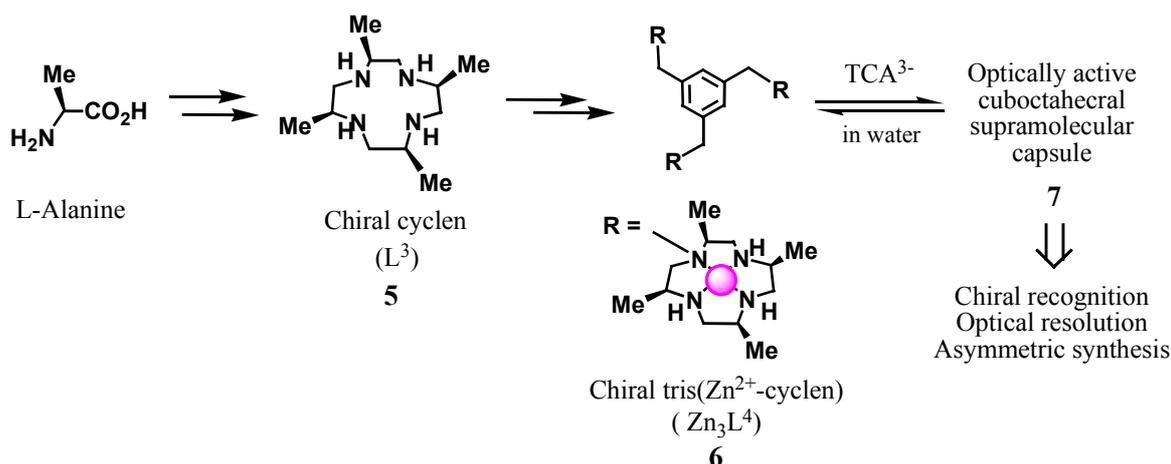


Fig. 2 Design and synthesis of optically active supramolecular capsule



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- 3) Chemical Sciences
3-1) Organic Chemistry

Development of New Oxidation Reactions and Synthesis of Bio-active Compounds

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Introduction

1. Oxidation of N-carboalkoxy amines

N-carboalkoxy ketimines should be attractive electrophilic targets for creating chiral quaternary carbon centers; however, only a few N-carboalkoxy ketimines have been prepared, and most did not have acidic α -protons. We expected that such N-carboalkoxy ketimines would be synthesized under mild conditions by N-t-butylbenzenesulfinimidoyl chloride(**1**)-mediated oxidation of N-carboalkoxy amines.

2. Stereoselective glycosylation

For an efficient O-glycosyl bond formation, it is desirable that glycosyl donors should be prepared conveniently, and thus-prepared glycosyl donors should be activated under mild conditions to realize stereoselective glycosylation.

Results

1. Oxidation of N-carboalkoxy amines

Oxidation of N-carbobenxyloxy(Cbz)-amines **2** to the corresponding N-Cbz ketimine **3** was performed by the following procedure: **2** was treated with n-BuLi in THF at -78 °C and then **1**¹ was added at the same temperature. As expected, the **1**-mediated oxidation of **2** proceeded rapidly at -78 °C, and the desired product **3** was obtained in high yields. It was noted that ene carbamate **3'** was not obtained by this procedure (Scheme 1).

It was also found that yields of N-Cbz aryl ketimines **3** depended strongly on substituents of the phenyl ring. In the presence of an electron-attracting group, such as chloride, ketimine was isolated in 96% yield as a sole product, while a mixture of ketimine and ene carbamate was obtained in the presence of electron-donating substituents such as methyl and methoxy groups. It was thought that protonation to the nitrogen atom of the ketimine took place more easily when electron-donating groups were attached on the phenyl ring.

It should be noted that only one isomer of ketimine was obtained by the present procedure. We speculated that anti-N-Cbz ketimines **3** were selectively formed, judging from ^{13}C NMR chemical shift differences between the α -carbons of N-Cbz ketimines and the corresponding ketones.

Cyclic and acyclic dialkyl ketimines were generated by the above-mentioned method and MeOH-addition products were obtained in good to high yields by trapping ketimines with MeOH.

Kugelrohr distillation of the MeOH-addition product gave ene carbamate as a sole product. Therefore, oxidation of N-Cbz amine with **1**, followed by addition of MeOH and successive distillation would give a useful method for preparation of ene carbamates.

2. Stereoselective glycosylation

N-Trichloroacetylcarbamate donor **5** was prepared from 2,3,4,6-tetra-O-benzyl-D-glucopyranose **4** ($\alpha/\beta = 86/14$) by the reaction of 1.1 equivalents of trichloroacetyl isocyanate in dry CH_2Cl_2 at room temperature (Scheme 2). The reaction completed within 30 min, and evaporation of CH_2Cl_2 in vacuo gave **5** quantitatively as a mixture of anomers ($\alpha/\beta = 89/11$, determined by ^1H NMR).

It was found that 1.2 equivalents of Me_3SiOTf smoothly catalyzed the glycosylation with **5** at 0°C to afford a disaccharide **6** in 95% yield as a mixture of anomers ($\alpha/\beta = 61/39$). The same reaction proceeded slowly by using other Lewis acids such as $\text{Mg}(\text{ClO}_4)_2$ and $\text{Cu}(\text{OTf})_2$ even at room temperature, and $\text{Zn}(\text{OTf})_2$ did not activate the donor **5**.

As in glycosylation with other glycosyl donors, such as glycosyl fluorides, good α -selectivity ($\alpha/\beta = 87/13$) was observed in the Me_3SiOTf -catalyzed glycosylation of **5** in Et_2O . The α -selectivity was further improved to a ratio of $\alpha/\beta = 93/7$ by changing the activator Me_3SiOTf to $\text{Me}_3\text{SiClO}_4$.² Interestingly, the present catalytic glycosylation had to be carried out in the presence of MS5A because the $\text{Me}_3\text{SiClO}_4$ -catalyzed glycosylation did not proceed in the presence of MS4A or MS3A.

β -Selective glycosylation of **5** was performed by using MeCN^3 as a solvent and a stoichiometric or catalytic amount of Me_3SiOTf .

Glycosyl acceptors having primary, secondary, and even tertiary hydroxy groups readily reacted with the glycosyl donor **5** in Et_2O to give α -glycosides in high yields and with high α -selectivity by using 20 mol% of $\text{Me}_3\text{SiClO}_4$. On the other hand, though high β -selectivity was observed in Me_3SiOTf -catalyzed glycosylation in EtCN with glycosyl acceptors having a primary hydroxy group, moderate β -selectivities were observed in the cases of acceptors having secondary and tertiary hydroxy groups.

Stoichiometric one-pot dehydrative glycosylation of **4** proceeded smoothly to afford α - or β -glycoside, respectively, in high yields by preparing glycosyl donor **5** in situ with trichloroacetyl isocyanate, followed by activating **5** with 1.5 equivalents of $\text{Me}_3\text{SiClO}_4$ in Et_2O or Me_3SiOTf in EtCN . Also, catalytic one-pot dehydrative glycosylation proceeded stereoselectively in the

presence of MS5A.

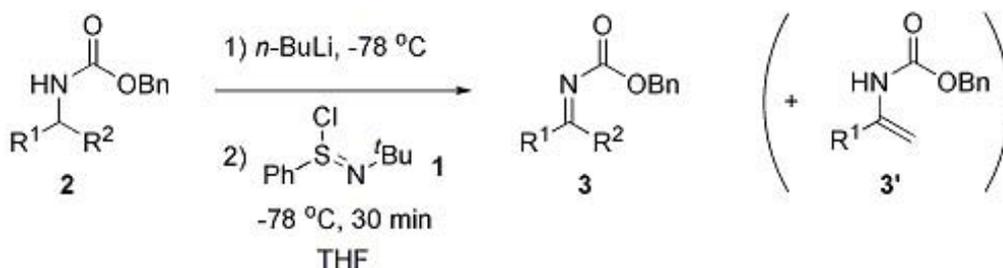
Discussion & Summary

1. Oxidation of *N*-carboalkoxy amines

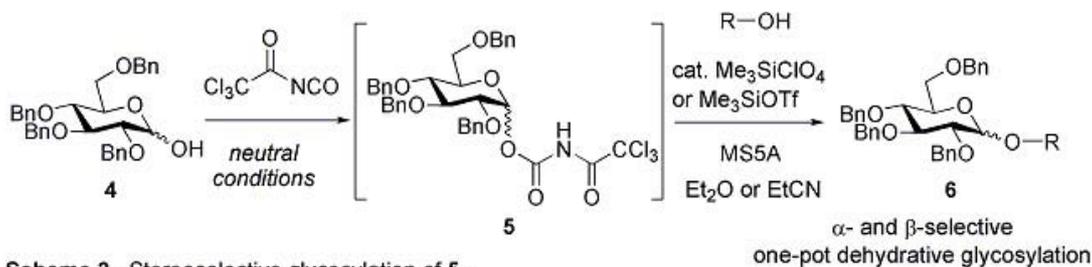
Since a variety of *N*-Cbz ketimines are now available and *anti*-isomers of ketimines were selectively formed by the present oxidation, it is expected that the *N*-Cbz ketimines would be useful synthetic intermediates in organic synthesis.

2. Stereoselective glycosylation

α - or β -Selective glycosylation of using glycosyl *N*-trichloroacetylcarbamate **5** as a glycosyl donor proceeded efficiently by activating the donor with a catalytic amount of $\text{Me}_3\text{SiClO}_4$ in Et_2O or Me_3SiOTf in EtCN , respectively, in the presence of MS5A. Preparation of **5** from a 1-hydroxy carbohydrate **4** and successive stereoselective and catalytic glycosylation were also realized in a one-pot manner, and the desired α - or β -glycoside was obtained directly from **4**. The easy operation of the present glycosylation, especially one-pot dehydrative glycosylation, would be useful in the synthesis of oligosaccharides or bioactive compounds having carbohydrate parts.



Scheme 1. Oxidation of *N*-Cbz amines **2** with **1** to the corresponding *N*-Cbz ketimines **3**.



Scheme 2. Stereoselective glycosylation of **5**.

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- 3) Chemical Sciences
3-1) Organic Chemistry

Development of enantioselective reactions based on organocatalysis directing at the total synthesis of biologically active compounds

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Introduction

The development of asymmetric organocatalysis (catalytic asymmetric reaction utilizing a metal-free organic small molecule a catalyst) is one of the most important and challenging topics in recent organic chemistry because of their environmentally benign nature. Considering that highly nucleophilic Lewis-bases form hypervalent silicates with trichlorosilyl compounds to afford the aldol and allylation adducts, the development of novel organocatalyses based on chiral Lewis bases should be a significant addition of a new type of catalyst to the field of asymmetric synthesis.

Results

Phosphine oxides are highly polar to form complexes with various compounds, however, examples of applications of chiral phosphine oxides to asymmetric organocatalyses are still limited. BINAPO is a precursor of BINAP which is the most common chiral phosphine ligand. We have developed the enantioselective allylation, aldol reactions and ring opening reaction with trichlorosilyl compounds promoted by a chiral phosphine oxide as an organocatalyst.

The asymmetric allylation of carbonyl compounds to generate two successive stereogenic centers has been the subject of investigation in recent years. We found that enantioselective allylations of aldehydes with allyltrichlorosilanes in the presence of 10 mol % of BINAPO, gave the allylated adducts in good enantioselectivities, where the use of the combination of diisopropylethylamine and tetrabutylammonium iodide as additives was crucial for the acceleration of the catalytic cycle. The best enantioselectivity was obtained in the reaction of 3,5-dimethylbenzaldehyde and methallyltrichlorosilane (equation 1).¹ γ -Allylated *syn*-homoallylic alcohol was obtained from (*Z*)-crotyltrichlorosilane, while the corresponding *anti*-alcohol was produced from (*E*)-crotyltrichlorosilane. These results suggest that these allylations mediated by BINAPO proceed *via* cyclic chair-like transition structures that involve hypervalent silicates.

The asymmetric aldol reaction is one of the most powerful methods for constructing two successive chiral carbon centers. BINAPO was found to promote the enantioselective aldol reactions of

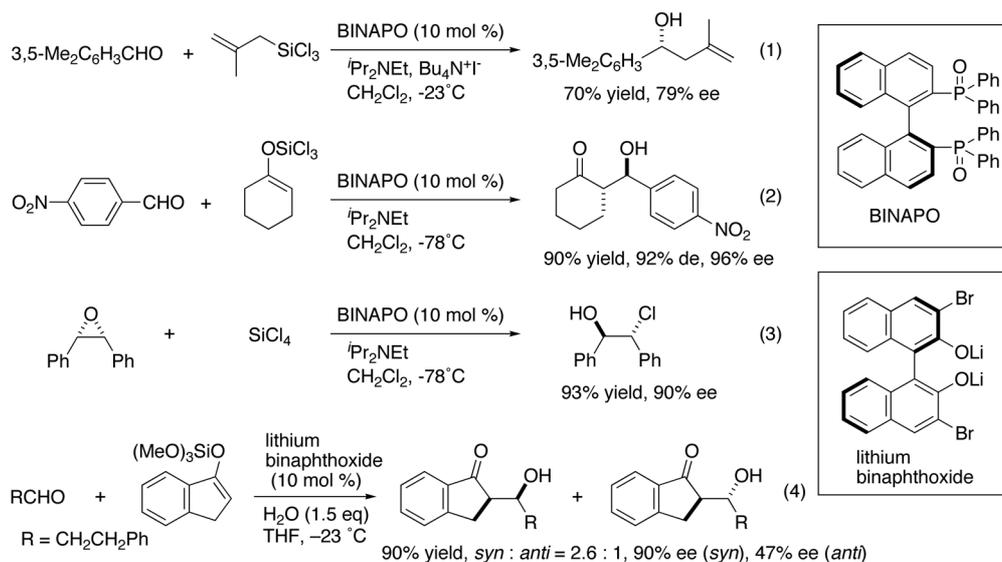
aldehydes with trichlorosilyl enol ethers in the presence of diisopropylethylamine as an additive, affording the corresponding aldol adducts in high diastereo- and enantioselectivities. It is noteworthy that *anti*-adducts were predominantly produced from the *E*-enol ethers and the *syn*-adduct was predominantly produced from the *Z*-enol ether, suggesting that the reaction proceeded via a chair-like six-membered transition state. The best result was obtained in the reaction of 4-nitrobenzaldehyde and the trichlorosilyl enol ether derived from cyclohexanone (equation 2).²

BINAPO also promoted the enantioselective ring opening of *meso*-epoxides with tetrachlorosilane in the presence of diisopropylethylamine, affording the corresponding chlorohydrins in high enantioselectivities. For example, the reaction of *cis*-stilbene oxide and tetrachlorosilane in the presence of diisopropylethylamine and BINAPO as a catalyst afforded the corresponding chlorohydrin in 90 % ee (equation 3).³ This result provides a new methodology of desymmetrization of *meso*-epoxide by the use of an organocatalyst.

To explore novel silyl enol ethers, which are more stable than trichlorosilyl enol ethers, we examined aldol reactions of trimethoxysilyl enol ethers with aldehydes. Trimethoxysilyl enol ethers, which are easily prepared from the corresponding lithium enolate with chlorotrimethoxysilane or enone with trimethoxysilane, are so stable that they survive the aqueous work-up or silica gel column chromatography. However, examples of base-catalyzed aldol reactions of trimethoxysilyl enol ether have yet to be reported and the reactivity of hypervalent silicate derived from the trimethoxysilyl enol ether remains unknown. We found that addition of trimethoxysilyl enol ether derived from cyclohexanone to benzaldehyde with 10 mol % of lithium binaphthoxide as a catalyst in THF afforded the corresponding *anti*-adduct predominantly in high chemical yield. To our surprise, the above aldol reaction in the presence of H₂O (1.5 equiv) afforded *syn*-adduct predominantly. The best enantioselectivity was obtained in the reaction of trimethoxysilyl enol ether derived from indanone to hydrocinnamaldehyde (equation 4).⁴

Discussion & Summary

The enantioselective reactions catalyzed by BINAPO is the first examples of the use of a chiral phosphine oxide as an organocatalyst for the asymmetric reactions, which demonstrated the novel importance of chiral phosphine oxides in synthetic organic chemistry. The aldol reaction of trimethoxysilyl enol ether is the first example of base-catalyzed aldol reactions of trimethoxysilyl enol ethers with carbonyl compounds, which exhibit the high potentiality of trimethoxysilyl enol ethers as enol equivalents, one of the most valuable reactive intermediate. These results will exploit a new chemistry in asymmetric synthesis, which will receive much attention of the researchers in the field of organic chemistry.



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- 3) Chemical Sciences
3-3) Protein Chemistry

Development of real-time sensors to simultaneously assess in vivo productions of two different second messengers

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Introduction

Intracellular second messengers regulate many critical cellular functions. The fluorescent sensor would be an ideal tool for the convenient measurements of these second messengers. An attractive framework for the design of highly efficient biosensors is the natural protein domain with high affinity and selectivity to the second messenger. An intracellular second messenger inositol-1,4,5-trisphosphate (IP₃) is a key biological signaling molecule that controls the cellular calcium concentration. We have designed a protein-based sensor for IP₃ by exploring the selective IP₃ binding properties of pleckstrin homology (PH) domain. Fluorescent signal transduction is imparted to the protein by mutating proximal residues to cysteine and then alkylation of the active site by various fluorophore derivatives. This creates functionalized proteins that show micromolar affinity for IP₃, reasonably strong fluorescence emission and wavelength changes in the fluorophore and selectivity higher than the original PH domain among different inositol phosphate derivatives in vitro (J. Am. Chem. Soc. **2002**, 124, 1139) and in vivo (EMBO J. **2003**, 22, 4677).

Results

In order to understand further signaling mechanisms related to calcium ion concentration changes and productions of inositol phosphates, it is necessary to assess simultaneously productions of other inositol phosphates, such as inositol-1,3,4,5-tetrakisphosphate (IP₄). We have focused on development of fluorescent sensors that enable simultaneous detection and quantitation of two different inositol phosphates derivatives.

Tailoring IP₄ sensors.

We have developed fluorescent inositol-1,3,4,5-tetrakisphosphate (IP₄) sensors that emit at various wavelengths upon binding to IP₄. Receptors for IP₄ have been made from a GRP1 PH domain according to our previously published method for fluorescent IP₃-sensors (J. Am. Chem. Soc. **2002**, 124, 1139). An amino acid sequence of five arginine residues (R5) was added at the C-terminus of

the GRP1 PH domain to help the sensor permeabilize the cell membrane. Two amino acid residues that located near the binding pocket and did not interact with IP_4 directly, Val15 and Glu82, was chosen to mutate into a Cys residue (V15C, E82C) so that a selective modification with fluorophores could be possible (Figure 1). Four different IP_4 sensors were constructed with different fluorophores and mutated GRP1 PH domains. The mutant GRP1 PH domains were modified at the Cys residue with thiol reactive fluorophores, 6-iodoacetamidofluorescein (6F) and 6-bromoacetyl-2-dimethyl-aminonaphthalene (DAN), at each of these positions, and successive purification with a gel filtration followed by a cation exchange chromatography gave four different fluorophore-labeled PH domains. CD spectra of these fluorophore-labeled GRP1 PH domains, V15F, E82F, V15DAN, and E82DAN, were indistinguishable from that of the wild type, indicating that the secondary structure was not perturbed by the introduction of fluorophore.

Each fluorophore-labeled PH domain was tested for IP_4 binding by monitoring the fluorescent emission spectra of a 100 nM solution. Distinct changes of emission spectra were observed for all the sensors, V15F, E82F, V15DAN, and E82DAN. Though the affinity of sensors to IP_4 varied both with the labeled position and with fluorophore, the equilibrium dissociation constants obtained by the fluorescent titrations were comparable to that reported for the parent GRP1 PH domain. These results suggest that a combinatorial approach with various fluorophore and with attachment positions is necessary to optimize the sensitivity of the biosensor. The selectivity of biosensor to various inositol phosphate derivatives was next analyzed. All the IP_4 sensors efficiently discriminated IP_4 from other inositol phosphate derivatives.

Proper placement of an environmentally sensitive fluorophore within the PH domain successfully afforded a variety of IP_4 biosensors that enables to monitor the IP_4 concentration at various wavelength. The sensor targets IP_4 with a submicromolar dissociation constant and with a selectivity to IP_4 over other inositol derivatives.

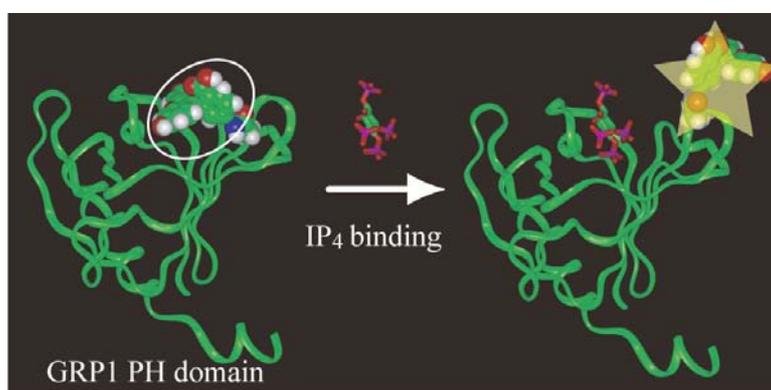


Figure 1. A schematic illustration shows design of the IP_4 sensor.

Sensing of IP₃ and IP₄ in the cell.

Cellular functions are regulated by intracellular second messengers, such as Ca²⁺ and inositol phosphates. However, metabolizing pathways of these second messengers are not completely understood. We have succeeded quantitatively assess *in vivo* regulation of inositol 1,4,5-trisphosphate production in intact cells. The IP₄ sensor obtained in this study was incorporated into HeLa cells by electroporation. We next analyzed the intracellular production of inositol-1,3,4,5-tetrakisphosphate by using the IP₄ sensor. The fluorescent intensity of the IP₄ sensor incorporated HeLa cells increased when stimulated by histamine, suggesting that the IP₄ sensor detected the concentration changes of IP₄.

Discussion & Summary

We have succeeded in constructing optical IP₄ sensors from a naturally existing PH domain which already has high affinity and selectivity to IP₄. It is possible to incorporate these sensors into mammalian cells and detect the concentration change of IP₄. These IP₄ sensors are expected to be utilized to clarify the behavior of IP₄ during signal transduction.

The fluorescent IP₄-sensor obtained in this study do not interfere the emission of IP₃ sensor. We will covalently connect the IP₃-sensor and the fluorescent peptide portion of IP₄-sensor by a disulfide bond or a chemical ligation reaction. This allows us to obtain a dual sensor that contains both IP₃- and IP₄-sensors within the same body. Introduction of the dual sensor into a cell would be tested in the conditions used for our *in vivo* IP₃ sensor to access IP₃ and IP₄ concentrations at the same location in the cell.

References

In preparation

- 3) Chemical Sciences
- 3-4) Analytical Chemistry

Role of chymase in the development of aortic aneurysm

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Introduction

Chymase contributes not only angiotensin II (AII) formation but also pro-matrix metalloproteinase activation and its function may play an important role in the development of aortic aneurysm. In this study, we clarified the role of chymase in the development of A II-induced abdominal aortic aneurysm in mice.

Results

Twenty male apolipoprotein E (apo E)-deficient mice on C57BL/6 background were obtained from Jackson Laboratories. 10-week-old Apo E-deficient mice (n = 10) were infused with A II (1000 ng/kg per min) for 4 weeks. The remaining mice were used as control mice. At 10 weeks (before A II infusion), 14 weeks (just after A II infusion), 20 weeks, 30 weeks and 40 weeks of age, we measured the blood pressure and the luminal diameter of abdominal aorta using ultrasound imaging system. At 14 weeks of age, the blood pressure in the AII-infused apo E-deficient mice was significantly higher than that in the control mice, but it was not observed at the other point. At over 30 weeks of age, the luminal diameter of abdominal aorta was significantly larger in the AII-infused apo E-deficient mice than in the age-matched control mice (Figure 1). At 40 weeks of age, mice were sacrificed and directly measured the diameters of abdominal aorta. The diameters of supra- and infra-renal abdominal aortas were significantly larger in the in the AII-infused apo E-deficient mice than in the control mice (Figure 2). Aortic chymase and matrixmetalloproteinase-9 levels were significantly higher in the A II-infused apo E-deficient mice than in the control mice.

Discussion & Summary

We found the upregulation of chymase in the A II-induced abdominal aortic aneurysm in mice, and this finding suggest that chymase may play an important role in the development of the aortic aneurysm. Furthermore, in this study, we established the method which could evaluate the measurement of aortic aneurysm time-dependently. This method may be useful for evaluating the regressive effects of drugs.

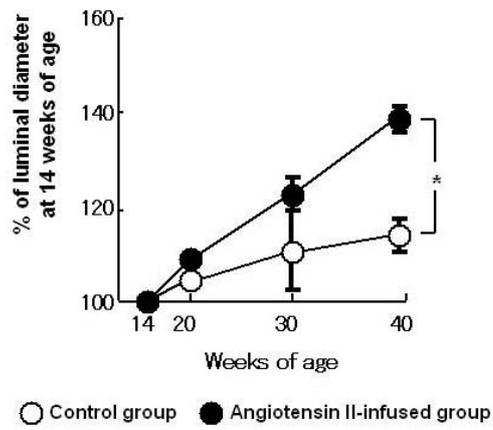


Figure 1 Time course of luminal diameter of abdominal aorta using ultrasound imaging. * $p < 0.05$ vs. control group.

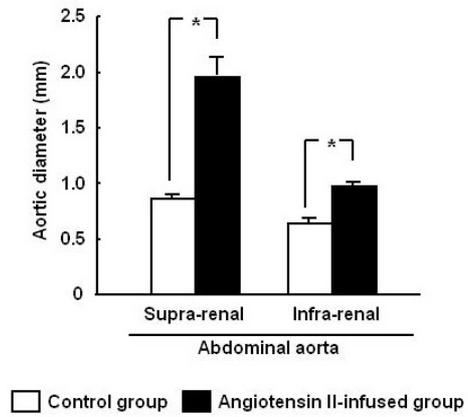


Figure 2. Aortic diameters at 40 weeks of age in mice. * $p < 0.05$ vs. control group.

Part II

Reports from the Recipients of Garants
for International Meetings
(Fiscal Year 2004)

4th European-Japanese Bioorganic Conference (EJBC-4)

1. Representative

Tetsuo Toraya, the chairman of the organizing committee

2. Date and location

March 15-19, 2005

Hotel Limani (Conference room: Olympus), Ushimado, Setouchi,

Okayama-Pref., Japan

3. Number of participants

Approximately 75 persons (including 20 from abroad)

4. Total cost:

4,168,498 yen

5. Main use of subsidy

As a part of the rent for the Conference rooms

6. Results and impression

The recent development of chemical biology is amazing. The chemical biology in European countries is at a top level of the world. On the other hand, the bioorganic chemistry in Japan is also at a high level. Based on such backgrounds, this European-Japanese Bioorganic Conference (EJBC-4) was held with bioorganic chemistry and chemical biology as central themes.

Twenty European speakers from 5 countries (one of them was absent because of illness) and 17 Japanese speakers from 9 universities gave the invited lectures. The invited speakers are listed below. In the two poster sessions, 31 posters were presented by European and Japanese participants. In addition, there were a get-together party, excursion, and banquet as well in the meeting. The total number of the participants were approximately 75.

In the meeting, European and Japanese invited speakers presented newest results and insights of their works, followed by very active discussion. The aim of the meeting to promote the progress of both fields of increasing importance was achieved by exchanging new results and new insights. From close interactions between European and Japanese participants, it can be expected

to stimulate fruitful collaborations between Europe and Japan in the future. Relatively young Japanese and European researchers presented their results in the poster sessions. They were stimulated by the discussion with famous scientists from overseas countries and Japan, which was also an important fruit of the meeting from the viewpoint of growing young scientists of Japan.

After the meeting, many European participants sent me “thank you” e-mails. They indicated that European people were also satisfied with the high quality of the science in the meeting. One of them wrote to me that this meeting was the most successful EJBC ever held. I proposed in the meeting that the title of the meeting (EJBC) is changed to EJCB (European-Japanese Chemical Biology) in the future. This proposal was accepted by the co-organizers. It was decided that the next “EJCB” meeting will be held in Munich in 2007.

7. Additional description

The invited speakers were: Prof. B. T. Golding (Newcastle upon Tyne, UK); Prof. R. J. Griffin (Newcastle upon Tyne, UK); Prof. C. J. Schofield (Oxford, UK); Prof. N. J. Turner (Edinburgh, UK); Prof. T. D. H. Bugg (Warwick, UK); Prof. C. Abell (Cambridge, UK); Dr. W. P. Watson (Cheshire, UK); Dr. P. L. Roach (Southampton, UK); Prof. W. Buckel (Marburg, Germany); Prof. M. Marahiel (Marburg, Germany); Prof. T. Carell (Muenchen, Germany); Prof. H. Dobbek (Bayreuth, Germany); Dr. A. J. Pierik (Marburg, Germany); Dr. B. M. Martins (Bayreuth, Germany); Dr. T. Selmer (Marburg, Germany); Dr. M. Boll (Freiburg, Germany); Prof. A. M. Papini (Firenze, Italy); Dr. M. Toernqvist (Stockholm, Sweden); Dr. M. Sahlin (Stockholm, Sweden); Prof. M. Shionoya (Univ. of Tokyo); Dr. H. Shimada (Keio Univ.); Prof. N. Esaki (Kyoto Univ.); Prof. H. Kato (Kyoto Univ.); Prof. K. Tanizawa (Osaka Univ.); Prof. H. Hayashi (Osaka Med. Coll.); Prof. S. Itoh (Osaka City Univ.); Prof. J. Kikuchi (NAIST); Prof. Y. Hisaeda (Kyushu Univ.); Prof. M. Sisido (Okayama Univ.); Prof. K. Uneyama (Okayama Univ.); Prof. K. Takai (Okayama Univ.); Prof. H. Tanaka (Okayama Univ.); Prof. Y. Wataya (Okayama Univ.); Prof. T. Sakai (Okayama Univ.); Prof. T. Toraya (Okayama Univ.).

6th Asian-Pacific Conference on Medical and Biological Engineering (APCMBE 2005)

1. Representative (Chair of the meeting)

Name: Katsuhiko Tsujioka

Institution: Kawasaki Medical School

Position: Professor

2. Venue of the Meeting: Tsukuba International Congress Center

Date: April 24-27, 2005

3. Number of Participants

376 (including 128 from abroad)

4. Total cost

¥13,599,000

5. Use of the Grant:

1. personnel expenses: a part-time job, PC operator
2. travel expenses: staff travel expenses
3. conference hall: Epochal Tsukuba
4. borrowing expenses: PC projector

6. Result and impression of the meeting:

Asian-Pacific Conference on Medical and Biological Engineering (APCMBE) has been playing a leading role as an international meeting in Asian-Pacific region on medical and biological engineering research, and is held every 3 years as a regional meeting of International Federation for Medical and Biological Engineering (IFMBE) since 1990. IFMBE is an international scientific organization for research of human function and clinical application. The scope of IFMBE resides in development of diagnostic and therapeutic equipment.

The present meeting (6th APCMBE) was successfully held in Tsukuba International Congress Center in Tsukuba city, Ibaraki, from April 24 to 27, 2005. Nearly 400 researchers (including about 130 from abroad) from 19 countries got together to present and discuss about diagnostic and therapeutic equipment including Cardiovascular Bioengineering, Molecular - Cell - Tissue engineering, Biorheology, Biomechanics (Bone, Musculo-skeletal, Vasculature, etc.), Dental Bioengineering, Electromagnetic Radiation and Ultrasound, Bio-micro-nanotechnology, Drug

Delivery, Artificial Organs, Biomedical Engineering Education, Bioinformatics, Healthcare, Rehabilitation Engineering, Elderly Persons Assist Technology, Virtual Reality, Biomedical Instrumentation, Neural Engineering, Computational Bioengineering, In Silico Medicine, Medical Imaging, Computer-Aided Surgery, etc. This meeting greatly contributed to a progress of medical and biological engineering research in this area.

On each day, nearly 100 posters were presented, and a total of about 300 posters in three days were discussed. As a highlight of the meeting, 3 special lectures, chairperson's lecture, 8 invited lectures, 1 evening session, 6 symposia (32 presentations) by foreign and domestic leading scientists were presented as follows, and discussed actively with the audience.

Special Lecture

1. Robert M. Nerem (USA): Bioengineered Tissues and Organs: The Emergence of Cell-Based Therapies
2. P. Åke Öberg (Sweden): Bio-Optical Sensors: Diagnostic and Monitoring Possibilities in Health Care
3. Joachim H. Nagel (Germany): Perspectives for Advanced Therapeutic Applications of Ultrasound

Chairperson's Lecture

Katsuhiko Tsujioka (Japan): Micromechanics of Endothelial Cells

Invited Lecture

1. Takeyoshi Dohi (Japan): Computer Aided Surgery
2. Jun Miyake (Japan): Tissue and Cell Technologies Directing to Regenerative Medicine
3. Kenji Sunagawa (Japan): Bionic Cardiology: New Therapeutic Modality in the 21st Century
4. MyoungHo Lee (Korea): Current Status and Future Perspectives on e-Health in Korea
5. Xiaochuan Pan (USA): Imaging Sciences and Their Medical Applications
6. Kunio Awazu (Japan): Free Electron Laser as New Diagnosis and Therapy for Bio-Molecules
7. Si-Shen Feng (Singapore): Nanoparticle Technology for New-Concept Chemotherapy
8. Koji Ikuta (Japan): Optical-Driven Nanorobotics for Minimally Invasive Microsurgery of Intra-Cell

Evening Session

Alfred Dolan (Canada): Risk Management for Medical Devices -Ensuring Safety and Efficacy

Symposium

Symposium I: IAMBE (International Academy for Medical and Biological Engineering) symposium: Nanotechnology and Physiome (Coordinator: Fumihiko Kajiya (Japan), Chairpersons: Fumihiko Kajiya (Japan), Niilo Saranummi (Finland))

Symposium II: Neural and Rehabilitation Engineering (Coordinator: Toshiyo Tamura (Japan), Metin Akay (USA), Chairpersons: Toshiyo Tamura (Japan), Metin Akay (USA))

Symposium III: Promotion of Biomedical Engineering in Asian-Pacific Region -Academic, Clinical, and Industry's Perspective (Coordinator: Makoto Kikuchi (Japan), Chairpersons: Makoto Kikuchi (Japan), Joachim H. Nagel (Germany))

Symposium IV: Mechanobiology of the Vascular System (Coordinator: Joji Ando (Japan), Chairpersons: Joji Ando (Japan), Xiaoqiang Yao (Hong Kong))

Symposium V: Cellular Biomechanics (Coordinator: Masaaki Sato (Japan), Chairperson: Masaaki Sato (Japan))

Symposium VI: Past, Present and Future of MBE in Asian-Pacific Region (Coordinator: Katsuhiko Tsujioka (Japan), Chairpersons: Masatsugu Hori (Japan), Walter H. Chang (China-Taipei))

7. Topics of the meeting

In the meeting, Young Investigator's Award (YIA) Competition was held for encouraging young scientists in the field in Asian-Pacific countries. Over 50 papers were applied to YIA, and 8 papers were selected in advance by the program committee to present orally at the conference. Finally the two best papers were selected and awarded as the YIA best presentation award.

The 58th Meeting of the Japan Society for Cell Biology

1. Representative

Keiji Tanaka, the president of the annual meeting

2. Opening period and place

June 15-17, 2005

3. Number of participants

703 persons (42 persons from foreign countries)

4. The total cost

Twenty-seven million yen

5. Main use of the subsidy

Support for travel expenses of invited speakers

6. Result and Impression

The 58th Annual Meeting of the Japan Society for Cell Biology was held to encourage the international and inter-generational interaction of the scientists working in various fields of the cell biology.

For further development of the Society, the annual meeting last year has adopted a variety of new attempts. For example, English is now used as the official language and many distinguished scientists have been invited from overseas and Japan to provide an overview of topics from all fields of cell biology. Furthermore, many young scientists, who were recommended by outstanding researchers from overseas, have been invited to present posters and to discuss the topics with Japanese scientists. All invited presenters are requested to participate in all to the sessions so as to provide for a more stimulating meeting.

In addition to these innovations, Computer Based Presentation has newly adopted at the poster session this year to allow presenters to use visual data and movies. To concentrate on the sessions in a relaxed atmosphere, we announced the participants to dress casual to attend the meeting, just like the other meetings in the U.S. and Europe. Any formal or business wear with a tie was not recommended.

I think that all participants discussed their ideas extensively with many scientists from overseas and Japan, and created an international relationship during the meeting. Symposia had been organized to cover all fields of cell biology and no more than two symposia will run simultaneously. So, the participants could attend all symposia and obtained current information of all fields of cell biology.

The scientific program consisted of 7 symposia and poster presentations. 341 contributed papers including invited ones were accepted for poster presentation and 35 distinguished speakers for symposia have been invited from Japan and overseas. These played a prominent role in the exchange of ideas and provided us with new research results on various topics in cell biology. Particularly, the main focus of the Meeting in this year was to discuss the basic mechanisms, pathophysiological roles, and technological developments of the modern biology in the post-genome era. In addition, the Meeting aimed that the young Japanese scientists take the opportunity to make friends with leading scientists from overseas and exchange their opinions on their work. On the second day of the meeting, a mixer reception was held at a low price and most invited presenters participated. I believe this meeting was the most informative one in cell biology and related scientific fields.

7. Additional description

Number of participating countries : 9

International Symposium on Ran and the Cell Cycle

1. Representative

Yoshihiro Yoneda, the chairperson of organizing committee

2. Opening Period and Place

October 2 (Sunday) ~ October 4 (Tuesday), 2005

Awaji Yumebutai International Conference Center (Hyogo, Japan)

3. Number of participants

111 persons (7 persons from foreign countries)

4. The total cost

4,612,350 yen

5. Main use of the subsidy

Support for travel expenses and hotel accommodations of invited speakers

6. Results and Impression

A remarkable range of insights into nuclear structure and function has resulted from the establishment by Nishimoto et al. (1978) of the temperature-sensitive mutant tsBN2 cell line that rapidly undergoes mitosis during S phase without completion of DNA replication. Because of the rapid and inappropriate nature of chromosome condensation under non-permissive temperature, the end result was a striking chromosomal fragmentation, resulting in pulverized mitotic chromosomes and the formation of micronuclei upon mitotic exit. These intriguing observations have developed the Ran GTPase field, which has become a key interface between the control of mitotic progression, nuclear-cytoplasmic trafficking, the formation of the interface nuclear envelope and the mitotic spindle. More than one hundred former lab members, collaborators and other scientific colleagues from around the world gathered October 2-4, 2005 to celebrate the career and scientific achievements of Professor Takeharu Nishimoto. The International Symposium on Ran and the Cell Cycle, held in his honor at the Awaji Island, Japan, was organized by Yoshihiro Yoneda (Osaka University) and Mary Dasso (National Institutes of Health) into sessions reflecting major areas under investigation: cell cycle and chromosomes; nuclear import and export of proteins and RNA; nuclear envelope and the nuclear pore complex; and RCC1 and chromatin. In addition to the many excellent scientific presentations (too many to discuss individually) and reminiscences about and tributes to Dr. Nishimoto from the

participants, highlights of the meeting included a special lecture by Dr. Nishimoto on his career and accomplishments (see below) and a guitar performance by his son Takeshi Nishimoto, who is a professional musician. We feel that Dr. Nishimoto's retirement from Kyushu University is a perfect time to look back at the milestones of his career, to assess the current state of the field, and to discuss his scientific legacy and the challenges that remain in order to unravel the complexities of the Ran GTPase system. Thus, at this symposium, the participants discussed the progress of the intervening 27 years and revealed the broad significance of Nishimoto's original finding. In addition, many of the talks at this meeting and the formal and informal discussions among participants implicitly or explicitly raised many interesting questions and issues that remain to be addressed in the future.

7. Additional description:

In a special lecture, Professor Takeharu Nishimoto provided both a historical perspective on the development of the Ran field and a current outlook of the unresolved questions that have arisen from genetic studies conducted within his laboratory. In the 1980s, Nishimoto and colleagues accomplished the impressive feat of cloning the gene that was mutated in tsBN2 cells by complementation. They found that tsBN2 phenotype was rescued by a protein named RCC1 (Regulator of Chromosome Condensation 1). This chromatin-associated protein was rapidly proteolyzed upon temperature shift, suggesting that tsBN2 cells were functionally null for RCC1 activity at their restrictive temperature. After the discovery that RCC1 acts as exchange factor for the Ran GTPase, Nishimoto and colleagues identified many now well-known Ran pathway components, including Yrb2p in yeast and RanBP2 in vertebrate cells. They also identified a large number of components whose roles have not been fully established. In their recent studies on one of these interactions, Nishimoto and colleagues have examined the genetic relationships between the *S. pombe* homologue of RanGAP1, Rna1, and the histone H3 K9 methyltransferase Clr4. On the basis of in vitro studies and chromatin immunoprecipitation, Nishimoto proposed that a small amount of Rna1 within nuclei works to activate Clr4 and promote the formation of silenced chromatin near centromeres. This model is particularly intriguing with respect to the previous finding that RanGAP1 mutants show altered responses to centromeric heterochromatin, disrupting the accurate packaging of sperm chromosomes in *Drosophila*. Indeed, this model would provide another novel function for the Ran pathway within eukaryotic nuclei.

International Interdisciplinary Conference on Vitamins, Coenzymes, and Biofactors 2005

1. Representative

Hiroyuki Kagamiyama, the president of the conference and the chairman of the organizing committee.

2. Opening period and place

6–11 November, 2005, Awaji Yumebutai International Conference Center, Hyogo, Japan

3. Number of participants

217 persons (46 persons from foreign countries)

4. The total cost

Eighteen million yen

5. Main use of the subsidy

Support for the local expenses of the excellent young researchers (7 persons)

6. Result and Impression

This conference is the first joint meeting of the 4th International Symposium on Vitamin B₆, PQQ, Carbonyl Catalysis and Quinoproteins, and the 4th International Congress on Vitamins and Related Biofactors, both of which have been held every 3 to 4 years.

The recent advances in genomic science, structural biology, and biomedical applications have enhanced mutual communications between the individual studies on vitamins, coenzymes, and biofactors. It is, therefore, one of the best times to hold an interdisciplinary meeting on the biologically important small molecules. These molecules include vitamin A, D, E, K, ubiquinone, essential fatty acids, carotenoids, vitamin B₁, B₂, B₆, B₁₂, niacin, biotin, folic acid, pantothenic acid, vitamin C, PQQ, quinoproteins, and biofactors.

To take the full advantage of the joint meeting, some closely related areas were combined, and many sessions included contributions from both sides. Accordingly, sessions were divided by technical aspects. These include enzyme science, chemical aspects, metabolic aspects, molecular and cellular biology, nutritional aspects, pharmaceutical aspects, medical aspects, and biotechnology. Sixty-three invited speakers and 14 persons selected from poster presentation lectured on these topics. One-hundred-one posters were presented.

The largest group was enzyme science, which occupied nearly half of the both oral and poster

presentations. This certainly reflects the rapid technical advancement in the field of structural biology. The enzyme actions are now discussed in detail based on the structural data of the wild-type and mutant enzymes, and reaction intermediates. Even the transient species could be structurally analyzed by kinetic crystallography. Combined with the further advancement in the “classical” analytical method, such as the global analysis of the spectral change using the singular value deconvolution and mass spectrometry, the detailed reaction mechanism has been elucidated for many enzymes presented at the conference. These enzymes are mainly pyridoxal 5'-phosphate (PLP)-dependent enzymes and PQQ/quinoprotein enzymes. Actually, the reaction mechanisms of these enzymes have been the main topic of the former 3 meetings of the “International Symposium of Vitamin B₆, PQQ, Carbonyl Catalysis and Quinoproteins”, and the antecedent 8 meetings “the Vitamin B₆ Meeting” which has been held since 1962. However, this is the first meeting in which the research papers on PLP (B₆) enzyme were presented and discussed together with those on flavins (B₂) and NAD (niacin). There were many active discussions between the research groups, both inside and outside the meeting room.

Because of the number of the oral presentations (62) and the fact that half of the titles belonged to the area of enzyme science, the speeches were presented at two meeting rooms, Main Hall was for enzyme science and Reception Hall was for other fields. In the area other than enzyme science, biomedical aspects of small molecules have been discussed. It should be pointed out that, also in these fields, the research is growing to be based on structural biology. That is, biological and medical processes of small molecules such as vitamins and biofactors are now understood in terms of the interaction with macromolecules, especially proteins.

This trend has much relevance to the debate on the issue that whether or not PQQ is a vitamin, which emerged since the publication of the paper in *Nature* (2003 Apr 24;422(6934):832) that claims PQQ is a vitamin involved in the lysine degradation pathway. These authors used “bioinformatics” as a tool in addition to nutritional studies and proposed that α -amino adipate δ -semialdehyde dehydrogenase is a PQQ enzyme. However, the proposal lacked structural evidence. In this conference, two groups, one is a structural biology group and the other a nutritional group, gave oral presentations on this problem. Both groups addressed remarks that there is no evidence for the proposal that PQQ is a vitamin and pointed out the possible flaws in the *Nature's* report. This is a good example to show that such biomedical studies must be based on exact molecules, and detailed analyses on the biomolecules are important not only in enzyme science but also in all the fields of bioscience. This also indicates that too much reliance on today's “bioinformatics” sometimes leads to the erroneous conclusions.

The two meeting rooms are adjacent to each other on the same floor, and coffee breaks are held at the foyer between the two rooms. The participants at each meetings rooms could communicate at the coffee break. Breakfast was also served at the same place.

The poster session was held at the corridor connecting the conference center and the hotel. This was highly appreciated by the participants. "In most meetings, posters are being displayed at some places that are not easy to access. In this meeting, we could see many interesting contributions from young researches on the way to and back from the meeting room." a participant from UK said.

Have done travel expense help to 6 overseas (5 countries) and 1 domestic young participants. This was especially important for those scientists who succeed in continuously doing good science under quite difficult circumstances.

It was decided that the next conference is held at Athens, Georgia, USA in 2008, by the initiative of Prof. Robert S. Phillips, University of Georgia, Athens.

7. Additional description

8. Participating countries (in alphabetical order)

Bangladesh, China, Finland, France, Germany, Greece, Israel, Italy, Japan, Korea, Pakistan, Russia, Slovenia, Switzerland, UK, USA (16 countries)

9. Approvals

Approved by the International Union of Biochemistry and Molecular Biology (IUBMB) as the IUBMB Symposium No. 344

10. Satellite symposium

After the conference, a satellite symposium on redox bioscience (organized by Prof. Etsuo Niki, AIST, Japan) was held at the same conference center.