The NOVARTIS Foundation(Japan)
for the Promotion of Science

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Annual Report

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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

Printed media or electronic media

In the late 1960s, the time when I started my research career, I used to expect a large number of reprint requests by colleagues for my publication in leading international journals. It was my happiest moment if the reprint request cards accumulated more than 300. Of course I had to bear considerable amount of charge from the publisher. In today's academic society, even if you publish a superb paper in a leading journal with high impact factor, you never receive any reprint request. Also most of us place no reprint order to the publisher.

Many academic publications are moving from paper to electronic media. Referees, reviewers, and editors sitting in front of computer displays work together by exchanging texts and pictures in an electronic format. Use of electronic media reduces time for editing and printing as well as postage. Journals on medical and biological sciences are searched and retrieved easily by using PubMed and many other databases. It becomes important for university libraries to offer a good electronic access to magazines and journals rather than a vast selection of printed books or journals.

Recently NIH requests all NIH-funded investigators to make their peer-reviewed author's final manuscripts available to other researchers and the public at the NIH National Library of Medicine's PubMed Central (PMC) immediately after the final date of journal publication. Many medical journal publishers are against this NIH policy because it makes their publishing difficult. Researchers are generally in favor of the NIH policy because they naturally want to let the world know their work as soon as possible. NIH seems to reach a compromise that it waits for one year after journal publication.

NOVARTIS Foundation (Japan) introduced HTML version of Annual Report on web site this year and ceased the publication of printed version. If Annual Reports are published on our web page anyone interested in the research topic can read it and at the same time we can reduce the cost for publication. We may keep this policy if no problems arise.

However I recently realized that keeping printed matter as a part of archives is still very important. When I tried to find some materials describing NOVARTIS Foundation (Japan) at the time of its establishment, the first issue of the Annual Reports told me what I was wanting to know and I could read it without any difficulty of its retrieval.

Electronic media is useful only when retrieval means are available. Printed media has its history for more than a thousand years and is of vital importance. We NOVARTIS Foundation (Japan) are seriously reviewing how to store irreplaceable knowledge of human being.

Akimichi Kaneko, MD, PhD
Chairman, Board of Trustees
The NOVARTIS Foundation (Japan)

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Institute for Virus Research, Kyoto University
yshinkai@virus.kyoto-u.ac.jp

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Yamagata University faculty of Medicine
asao-h@med.id.yamagata-u.ac.jp

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Shigeki Higashiyama Ph.D.
Ehime University School of Medicine
Shigeki@m.ehime-u.ac.jp

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University of Tsukuba
inaba@kurofune.shimoda.tsukuba.ac.jp

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Kazunari K. Yokoyama
Gene Engineering Division, BioResouce Center, RIKEN
yokoyama@brc.riken.jp

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Hiroyuki Seimiya
Division of Molecular Biotherapy, Cancer Chemotherapy Center,
Japanese Foundation for Cancer Research
hseimiya@jfcr.or.jp

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Masaharu Noda
National Institute for Basic Biology
madon@nibb.ac.jp

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Graduate School of Life and Environmental Sciences, University of Tsukuba
kashiwa@sakura.cc.tsukuba.ac.jp

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Kazunori Nakajima
Department of Anatomy, Keio University School of Medicine
kazunori@sc.itc.keio.ac.jp

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Wataru Shoji
Institute of Development, Aging, and Cancer, Tohoku University
wshoji@idac.tohoku.ac.jp

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Keiko Ikeda
Jichi Medical School, Center for Molecular Medicine, Division of .Biology
kikeda@jichi.ac.jp

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Masato Hirata
Kyushu University, Faculty of Dental Scie
Hirata1@dent.kyushu-u.ac.jp

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Wataru Sakamoto
Research Institute for Bioresources, OkayamaUniversity
saka@rib.okayama-u.ac.jp

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Tomonao Matsushita
Department of Botany, Graduate School of Science, Kyoto University
matsushita@physiol.bot.kyoto-u.ac.jp

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Shibuya Akira
Department of Immunology, Institute of Basic Medical Sciences, University of Tsukuba
ashibuya@md.tsukuba.ac.jp

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Takayuki Sugiura
Faculty of Pharmaceutical Sciences, Teikyo University
sugiurat@pharm.teikyo-u.ac.jp

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Masato OGATA
Mie university graduate school of medicine
ogata@doc.medic.mie-u.ac.jp

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Toshiyuki Nakagawa
Gifu University Graduate School of Medicine, Department of Neurobiology
tnakagaw@cc.gifu-u.ac.jp

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Division of Anesthesiology, Niigata University Graduate School of Medical and Dental Science
kohno@med.niigata-u.ac.jp

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Hideo Yasukawa
Cardiovascular Research Institute, Kurume University, Fukuoka Japan
yahideo@med.kurume-u.ac.jp

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Masahisa Watarai
Obihiro University of Agriculture and Veterinary Medicine
watarai@obihiro.ac.jp

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Yoshiyuki Minegishi
Tokyo Medical and Dental University
Yminegishi.mbch@tmd.ac.jp

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Shinya MATSUURA
Research Institute for Radiation Biology and Medicine, Hiroshima University
shinya@hiroshima-u.ac.jp

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Toshirou Nishida
Osaka University Graduate School of Medicine
toshin@surg1.med.osaka-u.ac.jp

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MINEO KUROKAWA
Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo
kurokawa-tky@umin.ac.jp

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Noriyuki Nakajima
Biotechnology Research Center, Toyama Prefectural University
nori@pu-toyama.ac.jp

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Shigefumi Kuwahara
Graduate School of Agricultural Science, Tohoku University
skuwahar@biochem.tohoku.ac.jp

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Seisuke Hattori, Dsc.
Institute of Medical Scinece, The University of Tokyo
hattoris@ims.u-tokyo.ac.jp

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Kazuhiro Kondo
Graduate School of Pharmaceutical Sciences, Nagoya City University
kazuk@phar.nagoya-cu.ac.jp

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Yoshiki Morimoto
Graduate School of Science, Osaka City University
morimoto@sci.osaka-cu.ac.jp

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Masato Oikawa
Graduate School of Life Sciences, Tohoku University
mao@bios.tohoku.ac.jp

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Akira YANAGISAWA
Chiba University
ayanagi@scichem.s.chiba-u.ac.jp

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Toshiyuki INAZU
Department of Applied Chemistry, School of Engineering, Tokai University
inz@keyaki.cc.u-tokai.ac.jp

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Kenji Kuwasako
First Department of Internal Medicine, Miyazaki Medical College, University of Miyazaki
kuwasako@fc.miyazaki-med.ac.jp

Part Ⅱ:
Reports from the Recipients of Garants for International Meeting (Fiscal Year 2003)

Part I

Reports from the Recipients of Novartis Research Grants (Fiscal Year 2003)

1) Biological Sciences
1-1) Molecular Biology

The mechanism of $p16^{INK4a}$ gene induction by stress response

Naoko Ohtani
Division of Protein Information, Institute for Genome Research, The University of Tokushima
ohtani@genome.tokushima-u.ac.jp

Introduction

Oncogenic Ras/MAP kinase signalling provokes irreversible growth arrest in cultured human primary cells through the induced expression of $p16^{INK4a}$ tumour suppressor gene, which is an inhibitor of cyclin-dependent kinases (CDKs). This phenomenon is termed “premature senescence”, and is proposed to be a fail-safe mechanism, which protects normal cells from uncontrolled cell-proliferation and tumour formation. We have previously demonstrated that the activation of Ets2, which is a downstream mediator of Ras/MEK signalling cascade, is responsible for the up-regulation of $p16^{INK4a}$ in Ras-induced premature senescence. This finding was published in Nature (Ohtani et al. *Nature* 409, 1067-1070, 2001).

The phosphorylation of Ets2 takes place within several hours upon expression of oncogenic Ras. However, it takes about four to five days for $p16^{INK4a}$ induction. This time gap suggests that some other factors induced by Ets2 are necessary for $p16^{INK4a}$ induction.

Results

1 Ets2 induces JunB expression in human diploid fibrlblasts

Therefore, we searched for such factors by using micro-array technique. We found that a transcription factor JunB is induced by Ets2. Recently it was reported that the cells from JunB transgenic mice express higher level of $p16^{INK4a}$.

2 Ets2 and JunB cooperatively stimulate $p16^{INK4a}$ gene promoter activity

Therefore, we checked the possibility that Ets2 might cooperate with JunB to induce $p16^{INK4a}$ gene by reporter assays. As we anticipated, Ets2 and JunB cooperatively stimulated $p16^{INK4a}$ gene promoter activity by 16-fold in the presence of activated Ras/MEK signalling.

3 Ras/MEK signalling facilitates binding between Ets2 and JunB

Moreover, we found that the binding between Ets2 and JunB is strongly facilitated by activated Ras/MEK signalling in 293T cells. Also we were able to confirm that activated Ras/MEK signalling facilitates the binding between endogenous Ets2 and JunB in Ras infected human diploid fibroblasts.

4 Ets2 and JunB expression induces endogenous p16^{INK4a} gene expression

We confirmed the induction of endogenous p16^{INK4a} gene by Ets2 and JunB. Transfection into primary human fibroblasts is usually very difficult. Therefore, we used Nucleofector transfection system to transfect primary cells with Ets2 and JunB expression vectors, which reveals 95% transfection efficiency.

5 Endogenous Ets2 and JunB bind to the p16^{INK4a} gene promoter

We were also able to confirm that endogenous Ets2 and JunB bind to the p16^{INK4a} gene promoter in Ras-expressing primary human fibroblasts by chromatin immunoprecipitation (ChIP) assay using Ets2 and JunB antibodies.

Discussion & Summary

Oncogenic stress, such as activated Ras/MEK signalling, provokes irreversible growth arrest in cultured human primary cells through the induction of p16^{INK4a} tumour suppressor gene expression. We previously identified that one of the key transcription factor to induce p16^{INK4a} tumour suppressor gene upon oncogenic stress is Ets2 (Ohtani et al. *Nature* 409, 1067-1070, 2001). However, our previous results suggested that Ets2 needs other co-factors to transactivate p16^{INK4a} gene promoter.

Our current study suggests that one of the key factor to cooperate with Ets2 is JunB. Our results suggests that activated Ras/MEK signalling activates Ets2 activity and induces transcription factor JunB. These two transcription factors bind to p16^{INK4a} gene promoter and cooperatively induces p16^{INK4a} gene expression.

Figures & Tables

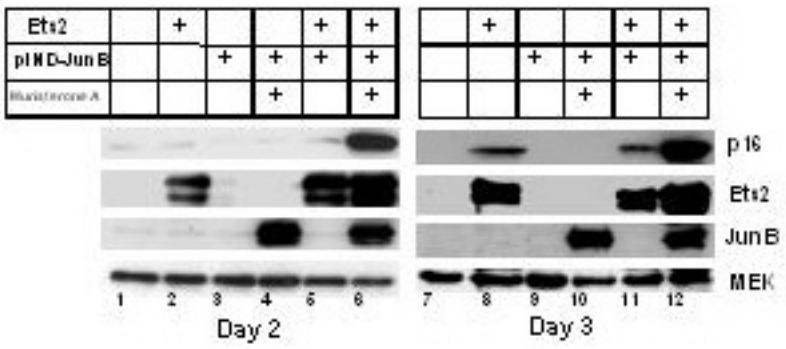

Figure 1. Ets2 induces p16INK4a expression cooperatively with JunB

A hormone (Muristerone A) -inducible expression vector encoding JunB(PIND-JunB) was

co-transfected with or without Ets2 expression vector into early passage HDFs using “Nucleofector transfection system” (Amaxa biosystems, Germany). Judging from the GFP signals driven by the transfected plasmids, nearly 100% of cells were successfully transfected. Although we were unable to see any significant increase of $p16^{INK4a}$ level by overexpression of either Ets2 alone or JunB alone, significant induction of $p16^{INK4a}$ was observed within 2 days if both Ets2 and JunB were coexpressed (lane6). As shown previously (Ohtani et al. Nature 2001), $p16^{INK4a}$ expression can be induced by Ets2 alone, 3 days after transfection (lanes 8 and 11), although the levels are still further enhanced by coexpression with JunB (lane 12).

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1) Biological Sciences
1-1) Molecular Biology

Regulation of NF-κB activation by PYPAF1/Cryopyrin

Takashi Suda
Kanazawa University, Cancer Research Institute
sudat@kenroku.kanazawa-u.ac.jp

Introduction

Cryopyrin/PYPAF1/NALP3 is an Apaf-1-like protein that induces activation of NF-κB and caspase-1 through an adaptor molecule ASC. The gene encoding cryopyrin has been identified as the gene mutated in the familial cold autoinflammatory syndrome and Muckle-Wells syndrome. Recently, we identified molecules (IPNs) that inhibit NF-κB activation induced by cryopyrin. IPN2 was a novel Apaf-1 like protein, which we named PYNOD (1). PYNOD also inhibited the cryopyrin-induced IL-1β secretion. Here, we investigated the inhibitory mechanism of PYNOD against NF-κB activation and IL-1β secretion. In addition, we have explored the mechanism of ASC-mediated NF-κB activaton.

Results

1) Inhibitory mechanism of PYNOD against crypyrin-induced NF-κB

Expression of cryopyrin in combination with ASC (cryopyrin + ASC) induces NF-κB activation in human embryonic kidney (HEK) 293 cells. In this experimental system, co-expression of PYNOD inhibited the cryopyrin + ASC-induced NF-κB activation. PYNOD also inhibited NF-κB activation induced by coexpression of CARD12 (another Apaf-1-like molecule that induces NF-κB activation using ASC, like cryopyrin) and ASC, or overexpression of ASC alone. In contrast, PYNOD failed to inhibit MEKK1-, Myd88- or TNF-α-induced NF-κB activation. In addition, PYNOD inhibited ASC-induced apoptosis without any effects on Fas- or Bid-induced apoptosis. PYNOD and ASC were coprecipitated with each other when either of them was immunoprecipitated from cell extracts containing both of them. There results indicate that PYNOD inhibits ASC-mediated NF-κB activation and apoptosis by physically interacting with ASC.

2) Inhibitory mechanism of PYNOD against crypyrin-induced IL-1β secretion.

Crypyrin induced caspase-1-mediated IL-1β processing and secretion when cryopyrin was coexpressed with ASC, caspase-1 and IL-1β in HEK293 cells. In this experimental system, coexpression of PYNOD inhibited the crypyrin-induced IL-1β secretion. PYNOD also inhibited

IL-1β secretion induced by caspase-1 overexpression in the absence of cryopyrin and ASC. When PYNOD was coexpressed with caspase-1, PYNOD inhibited autoprocessing of caspase-1. PYNOD and caspase-1 were co-precipitated with each other when either of them was immunoprecipitated. There results indicate that PYNOD physically interacts with caspase-1 and inhibits autoprocessing of caspase-1.

3) Establishment of an experimental system for the ASC-mediated NF-κB activation.

To characterize the signaling pathway mediated by ASC under physiologically relevant conditions, we generated a cDNA encoding a chimeric protein consisting of the signal transducing region of cryopyrin and the ligand binding domain of Nod2, whish is an Apaf-1-like molecule that recognizes muramyl dipeptide as a ligand and induces NF-κB activation using RICK as a downstream signaling molecule. We also constructed a cDNA encoding a similar chimeric protein from CARD12 and Nod2 (Fig. 1). Although both chimeric proteins induced NF-κB activation in response to muramyl dipeptide stimulation when they were expressed with ASC in cells, the CARD12-Nod2 chimera protein induced stronger response than the cryopyrin-Nod2 chimera protein. Therefore, we established stable cell lines (MAIL8 cells) expressing both the CARD12-Nod2 chimera protein and ASC. Successfully, stimulation by muramyl dipeptide induced an interaction between the CARD12-Nod2 chimera protein and ASC, and elicited cell-autonomous NF-κB activation in MAIL8 cells (3).

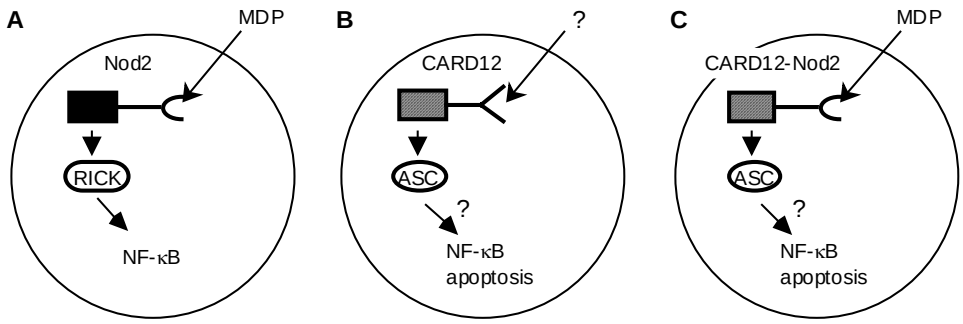

Fig. 1. Construction and function of a CARD12-Nod2 chimera protein
A.Nod 2 recognizes muramyl dipeptide (MDP) and activates NF-κB using RICK. B. CARD12 induces NF-κB activation and apoptosis using ASC, but its ligand is unknown. C. Our CARD12-Nod2 chimera protein induces NF-κB activation and apoptosis upon MDP stimulation.

4) Mechanism of ASC-induced NF-κB activation

Recently, it was reported that casapse-8 is involved in the ASC-induced apoptosis. On the other hand, we found that caspae-8 is involved in not only apoptosis but also NF-κB activation induced by Fas ligand (2). There results prompted us to investigate whether casapse-8 is involved in the ASC-mediated NF-κB activation (3). Small interfering RNA (siRNA) that attenuated caspase-8 expression inhibited the MDP-induced NF-κB activation in MAIL8 cells, whereas siRNAs against RICK, which mediates Nod2-induced NF-κB activation, did not do so. CLARP, a cellular caspase-8 inhibitor, as well as pan-caspase inhibitor z-VAD-fmk and caspase-8-specific inhibitor z-IETD-fmk but not granzyme B inhibitor z-AAD-fmk inhibited this response. These results indicate that caspase-8 and its catalytic activity is required for the ASC-mediated NF-κB activation. In contrast, siRNA against FADD and a dominant negative mutant of FADD did not influence the ASC-mediated NF-κB activation. ASC forms large aggregates called “speck” when it is activated. Consistent with above conclusions, ASC recruited caspase-8 and CLARP but not FADD and Nod2 to its speck-like aggregates when these proteins were overexpressed in cells. Furthermore, muramyl dipeptide stimulation induced formation of ASC-specks in MAIL8 cells and endogenous caspase-8 was colocalized with the specks. Finally, muramyl dipeptide induced IL-8 production in MAIL8 cells. These results are the first to indicate that caspase-8 plays an important role in the ASC-mediated NF-κB activation, and that the ASC-mediated NF-κB activation actually induces physiologically relevant gene expression..

Discussion & Summary

Mutation in cryopyrin causes systemic autoinflammatory syndromes. So far, pyrin, which is not a member of Apaf-1-like proteins, is known to inhibit cryopyrin-induced NF-κB activation and IL-1β secretion by interacting with ASC (Fig. 2). Importantly, mutation of the pyrin gene causes for another hereditary inflammatory disease, familial Mediterranean fever. Our results indicated that PYNOD has similar function with pyrin, suggesting that PYNOD may modulate the severity of these hereditary diseases. In addition, PYNOD directly interacts with caspase-1 and inhibits its activity (Fig. 2). We recently investigated the functions of two other Apaf-1-like proteins, PYPAF2 and PYPAF3, and demonstrated that PYPAF2 inhibits ASC and PYPAF3 inhibits caspase-1 (4). In addition PYPAF4 was reported to interact with IKKα and inhibits cytokine induced NF-κB activation (5). These results suggest that there is an anti-inflammatory subfamily among Apaf-1-like proteins. We demonstrated that caspase-8 is involved in the ASC-mediated NF-κB activation. Consistently, caspase-8 inhibitor CLARP inhibits ASC mediated NF-κB activation (Fig. 2). In contrast, CLARP does not inhibit ASC-induced IL-1β secretion, indicating that CLARP inhibits a part of cryopyrin function with a mode of action different form those of pyrin and PYNOD. Taken

together, our study revealed novel molecular mechanisms for the signal transduction and regulation of cryopyrin.

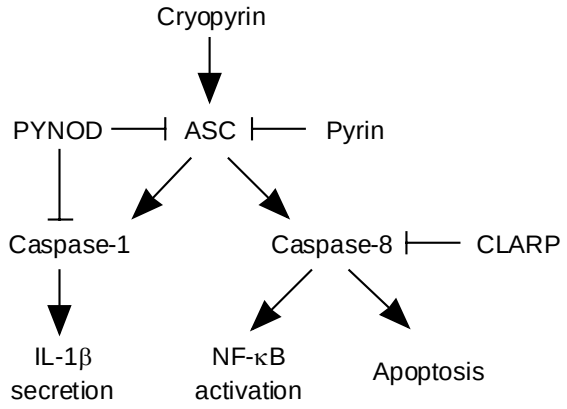

Fig. 2. Signal transduction and regulation of cryopyrin

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1) Biological Sciences
1-1) Molecular Biology

Identification of genes involved in neurodegenerative disorders and ER-stress by using a library of randomized ribozymes

Reiko Onuki
Graduate School of Pharmaceutical Sciences, the University of Tokyo
onuki@mol.f.u-tokyo.ac.jp

Introduction

Various types of stress, such as disruption of calcium homeostasis, inhibition of protein glycosylation and reduction of disulfide bonds, result in accumulation of misfolded proteins in the endoplasmic reticulum (ER). The initial cellular response involves removal of such proteins by the ER, but excessive and/or long-term stress results in apoptosis. Furthermore, recent studies indicate that this apoptosis is associated with neurodigenerative disorder. In this study, we used a randomized ribozyme (Rz) library and ER stress-mediated apoptosis (tunicamycin (Tm) -induced apoptosis) in SK-N-SH human neuroblastoma cells as a selective phenotype to identify factors involved in this process

Results

We isolated the candidate Rzs using three types of screening and performed the homology searching to identify the Rz-targeted proteins. Double-stranded RNA-dependent protein kinase (PKR) and SREBP cleavage-associated protein (SCAP) were suspected to be involved in this process. To confirm the effect of isolated Rzs on PKR, ribozyme stably expressing cells, named EB1-47 and EB2-68, were constructed. We examined the levels of Rz 47 and 68 in EB1-47 and EB2-68 cells and performed an analysis by RT–PCR with Rz-specific primers. The expression of each Rz was detectable in Rz-transfected cells. To confirm the activities of Rz 47 and Rz 68 against the PKR transcript, we also performed analysis by RT–PCR using primers specific for genes for PKR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The level of PKR mRNA in EB1-47 cells was 53% lower than that in empty vector-transfected cells and similar results were obtained in the case of Rz 68 (37% reduction). We also performed Western blotting and found that the level of PKR was reduced in EB1-47 and EB2-68. Thus, Rz 47 and Rz 68 specifically suppressed the expression of PKR at the post-transcriptional level.

To identify the role of PKR in vulnerability to ER stress, we prepared cytoplasmic and nuclear fractions from Tm-treated and untreated SK-N-SH cells and subjected them to Western blotting with anti-actin and anti-KDEL antibodies (this antibody interacts with GRP78 and 94). Levels of actin

remained unchanged but levels of GRP94 and GRP78/Bip in the cytoplasmic fraction increased after an 8-h incubation with Tm. Neither of these chaperones was detected in the nuclear fraction, suggesting that the nuclear fraction was not contaminated by cytoplasmic material. Western blotting analysis with anti-PKR and anti-phospho-PKR antibodies revealed that, when cells were stimulated with Tm for 8 h, the level of PKR rose in the nuclei (Figure 1) while the level of cytoplasmic PKR barely changed. Phosphorylation or cleavage of PKR is required for kinase activity. Using an anti-phospho-PKR antibody (which recognizes phosphorylation of Thr446), we detected phosphorylated PKR in the nuclear fraction, and the level of this form of PKR depended on the incubation time. The maximum level of phosphorylated PKR was detected 24 h after the start of treatment (Figure 1). When we examined the subcellular localization of PKR by immunofluorescence microscopy after SK-N-SH cells had been treated with Tm, we found most of the PKR in the cytoplasm of the untreated control. . After cells had been treated with Tm, levels of nuclear PKR increased and PKR was clearly localized in the nuclei, while levels of GRP 78 and 94 increased in the ER of treated cells. These results suggest that Tm-mediated ER stress promotes the expression of PKR in SK-N-SH cells.

We wanted to investigate whether phosphorylated PKR might affect Tm-induced cell death and so we constructed dominant negative (DN) PKR expressing cells and incubated them with Tm for 30 h. The level of cleaved caspase-3 (activated caspase-3) was examined and small amounts of cleaved caspase-3 were detected in these cells, while a large amount of cleaved caspase-3 was detected in the control cells. This result suggests that the expression of DN-PKR inhibits apoptosis.

Considerable evidence suggests that ER stress is closely related to genetic and/or neuronal degenerative diseases, such as Alzheimer's disease. Therefore, we examined the levels of PKR in the brains of sporadic AD patients obtained post-mortem. We prepared crude nuclear and cytoplasmic fractions (for seven disease-free controls and 17 patients with AD) for Western blotting with anti-phospho-PKR and anti-actin antibodies. Levels of phosphorylated PKR were significantly higher in crude nuclear fractions of AD tissues compared with controls (Figure 2).

Figure 1

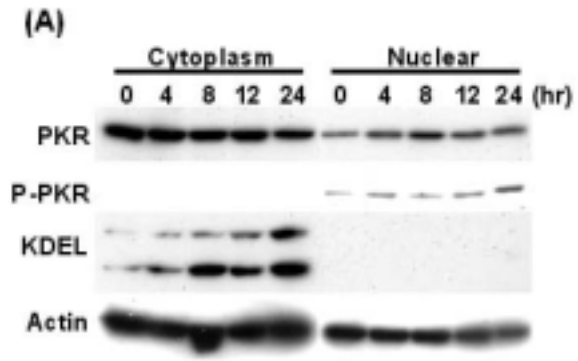
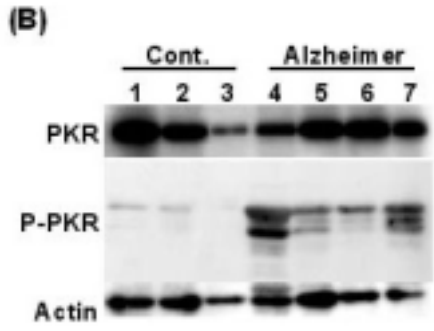


Figure 2



Discussion & Summary

In summary, we have identified PKR as a proapoptotic protein in Tm-induced apoptosis. During apoptosis, phosphorylation of PKR is increased and phosphorylated PKR is localized in the nuclei of SK-N-SH cells. In the nuclei in AD brains, similar results underscore the potential importance of phosphorylated PKR. Furthermore, we found aggregated PKR in the nuclei of samples from AD brains. Although many cells exhibited aggregation of PKR in their nuclei, histochemical investigations showed that these cells had not, apparently, undergone apoptosis. It is possible that the aggregation of PKR in the nuclei occurs at an early phase in the pathogenesis of AD. We also detected elevated levels of PKR in the brains of patients, at autopsy, who had died of Huntington's disease or Parkinson's disease. It is possible that PKR may be widely involved in a number of neuronal degenerative diseases.

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1) Biological Sciences
1-1) Molecular Biology

Role of small GTPase Rac in formation of neural circuit and synaptic plasticity in the central nervous system

Atsu Aiba
Kobe University Graduate School of Medicine
aiba@med.kobe-u.ac.jp

Introduction

The Rac is a member of the Rho family GTPases regulating actin reorganization. During development, Rho family GTPases integrate many extracellular signals to direct the outgrowth of axons and dendrites and the formation and dynamics of dendritic spines. To examine the roles of Rac in formation of neural circuit and synaptic plasticity in the central nervous system (CNS), we generated Rac conditional knockout mice.

Results

Of three mammalian Rac isoforms, Rac1 and Rac3 are expressed in the CNS. To gain an insight into a possible role of Rac in the CNS, we first examined the expression patterns of rac1 and rac3 mRNAs in the developing mouse brain. At embryonic day 15 (E15), postnatal day 7 (P7) and adult, mouse brain sections were prepared and analyzed by in situ hybridization. Rac1 mRNA is ubiquitously expressed in the mouse brain at E15, P7 and adult. On the other hand, rac3 mRNA is detected mainly hindbrain neurons at E15. At P7, rac3 mRNAs are prominently expressed in whole brain regions and rac3 mRNA expression declines in adult mouse brain. Overlapping but distinct expression pattern of rac1 and rac3 mRNAs in the CNS suggests distinct roles of the two Rac proteins in the CNS.

We previously showed that rac1 (-/-) embryos die before E9.5. Thus, we planed to generate Rac1 and Rac3 conditional knockout mice which lack Rac proteins in the specific neurons in order to examine the role of the Rac proteins in the CNS. We generated rac1 (flox/flox) mice in which the exon1 containing the initiation codon is flanked by two loxP sites in rac1 allele by gene targeting using embryonic stem (ES) cells. We also generated rac3 (+/flox-HcRed) mice in which loxP is inserted in the noncoding region upstream of the initiation codon of the exon1 and a loxP-HcRed, far-red fluorescent protein gene, is inserted downstream of the poly A addition signal in rac3 allele. rac1 (flox/flox) mice and L7 (+/cre) mice expressing Cre recombinase only on cerebellar Purkinje cells in the brain were crossed to generate rac1 (flox/flox) L7 (+/cre) mice. We confirmed the exon1 of the rac1 was deleted in the cerebellum of the rac1 (flox/flox) L7 (+/cre) mice (Purkinje

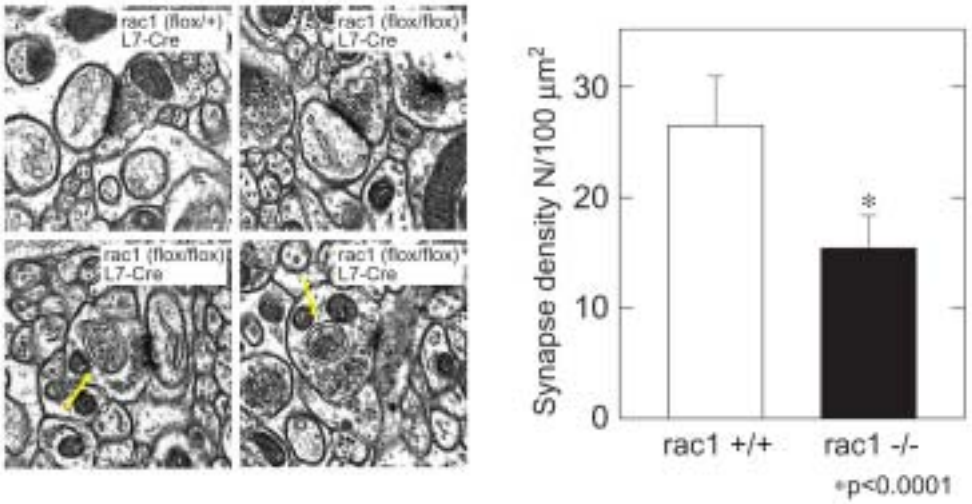
cell-specific Rac1 knockout mice) by PCR analysis of genomic DNAs. Purkinje cell-specific Rac1 knockout mice appeared normal and did not display ataxia. To further examine motor coordination, we used the rotating rod task. Purkinje cell-specific Rac1 knockout mice quickly learned how to keep themselves on the rod. There was no significant difference between the knockout and control mice, suggesting motor coordination of the Rac1 conditional knockout mice is normal. Next, we examined the structure of the cerebellum in the Purkinje cell-specific Rac1 knockout mice. Nissl staining showed that size and overall structure of the cerebellum of the knockout mice were normal as compared with controls. We further examined the dendrite structures of the Rac1-deficient Purkinje cells by immunohistochemical analysis using anti-calbindin antibody. We found that the Rac1-deficient Purkinje cells have more abnormal dendrites than those of wild-type mice. The mutant mice which lack GluR delta2, Purkinje specific ionotropic glutamate receptor, were reported to have reduced density of parallel fiber-Purkinje cell synapse. Thus we examined the synapse density of parallel fiber-Purkinje cell using an electron microscope and found that synapse density is significantly decreased in the Purkinje cells lacking Rac1. This result suggests that Rac1 is involved in synapse formation or synapse maintenance on the parallel fiber-Purkinje synapses.

Discussion & Summary

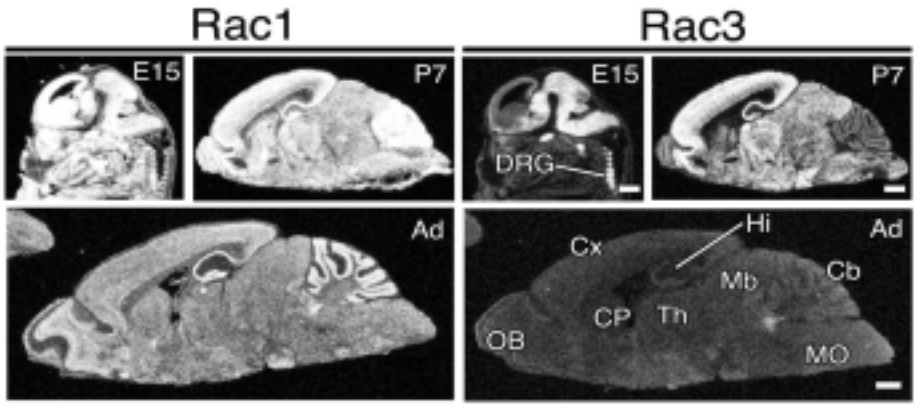
We generated the mice which have rac1 flox allele or rac3 flox allele by gene targeting using ES cells. Analysis of Purkinje cell-specific Rac1 knockout mice reveals that Rac1 plays an important role in formation or/and maintenance of parallel fiber-Purkinje cell synapses. Actin cytoskeleton whose reorganization is known to be regulated by Rac protein is implicated in regulation of synaptic transmission and plasticity. Further electrophysiological study using these knockout mice will show the role of Rac in these functions. Purkinje cell-specific Rac1 knockout mice showed apparent normal motor coordination in spite of reduced parallel fiber synapse density on the Purkinje cells. Associative leaning using Purkinje cell function such as eye-blink conditioning task may reveal the functional significance of the synapse density in the motor learning.

Figures & Tables

Decreased Synapse Density in Mice Lacking Rac1 in Purkinje Cells



in situ hybridyzation for *rac1* and *rac3* mRNAs



1) Biological Sciences
1-1) Molecular Biology

Molecular Analysis of Salivary Gland Branching Morphogenesis

Takayoshi Sakai
Osaka University Dental Hospital
sakai@dent.osaka-u.ac.jp

Introduction

Epithelial branching morphogenesis is essential for forming many organs such as exocrine glands, salivary gland, lung, and kidney. Repetitive epithelial cleft and endbud formation create the three-dimensional branching structures characteristic of many organs, yet the mechanisms are poorly understood. The purpose of this project is to identify molecules that are necessary for branching morphogenesis and to apply these molecules for regeneration of salivary gland. To identify molecules that are necessary for branching morphogenesis, laser microdissection (LMD) and analysis of gene expression by T7-SAGE (reference 1) were performed.

Results

1. Embryonic 12-day salivary glands were extracted from mouse embryonic body, and were cultured on filter membrane and cleft and endbud epithelia of frozen-sectioned salivary gland were laser-microdissected as previously described using LMD (laser microdissection) microscope system (reference 2). We realized that twelve micron frozen section was suited for our experiment. Figure 1 indicated the design for LMD and strategy for microanalysis of gene expression. Red area shows cleft epithelium and green area does endbud epithelium. Both of their tissues ware used for the production of T7-SAGE (serial analysis of gene expression, http://www.sagenet.org) libraries.

2. Conventional plastic slide was changed to new glass slide to avoid any static power on the membrane for LMD. Because previously plastic ones caused trouble to catch up salivary tissue using the cap of centrifuge tubes. Toluidine blue staining was performed to clearly identify epithelial cleft in salivary gland. Each 500 cells were collected to extract RNA.

3. Using column technique, total RNA was extracted from both cleft and end bud epithelia, and the cDNAs were synthesized after the RNAs were treated with DNase.

4. T7-SAGE was our modified technique of conventional SAGE that we previously established that was utilized T7-based RNA amplification method (reference 1). We have started the T7-SAGE method to make the gene expression profile from limited small amount of salivary epithelial cells.

5. Initial profiles of micro gene expression in salivary epithelium were identified.
Our T7-SAGE data showed that salivary gland epithelium expressed Fibronectin
and E-cadherin. Epithelial cleft expressed Fibronectin higher than epithelial end bud,
and epithelial cleft decreased E-cadherin.

6. T7-SAGE tags were quantitated using SAGE software (http://www.sagenet.org), and the pattern of gene expression including were determined. (Figure 1).
We started identifying the gene using NCBI database and SAGEmap database to identify some interesting gene expression.

Figure 1. Strategy for microanalysis of gene expression in organ development
The diagram shows the design of laser microdissection for RNA extraction from epithelial tissue of embryonic 13 day salivary gland.

We initially started confirming some data from our T7-SAGE database using quantitative real-time RT-PCR and in situ hybridization technique.
After we estimated the candidate genes that regulate the branching morphogenesis, we will perform functional knockdown technique, small interfering RNA (siRNA) to identify the role in organ development. Full length cDNA will be cloned using RACE technique for new or rare gene study.

Further study is necessary to complete our project after we complete the gene expression profile in embryonic epithelial cells of mouse salivary gland.

Finally we will identify the candidate gene which is required for glandular branching
morphogenesis, and apply the information to organ development and regeneration research. These

data will provide new possibilities for the guide of regeneration therapy in human organ, such as kidney and lung, salivary gland.

Discussion & Summary

T7-SAGE was performed to identify the profile of gene expression. Initial T7-SAGE data showed that salivary gland epithelium expressed upregulated fibronectin and downregulated E-cadherin. Our preliminary data was confirming our previous work (reference 2). Epithelial branching morphogenesis could be control by the interaction between cell-cell adhesion and cell-ECM (extra cellular matrix) adhesion.

Our analysis of gene expression using T7-SAGE method was very useful for microanalysis from limited small amount of sample such as laser microdissected tissue. Future works will be continued to identify the genes that regulate branching morphogenesis of some organs to regenerate important organ such as kidney and lung, prostate, pancreas, salivary gland.

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1) Biological Sciences
1-1) Molecular Biology

Structural manipulation of integrin ligand binding affinity and its use in the development of novel class of inhibitors

Junichi Takagi
Institute for Protein Research, Osaka University
takagi@protein.osaka-u.ac.jp

Introduction

Integrins are major metazoan adhesion receptors that play a fundamental role in cellular organization. One unique aspect of integrins is that the affinity of their extracellular domain for biological ligands can be rapidly upregulated by signals from within the cell(1). Rapid and precise control of integrin activation is particularly important for leukocytes and platelets. In order for screening anti-integrin drugs with minimum side-effects, we try to design integrin receptor which is fixed in its low-affinity or high-affinity state.

Results

Mutant $\alpha_{IIb}\beta_3$ integrin that has constitutively high affinity toward ligand has been designed(2). The design is based on the hypothesis that the angle between I-like domain and hybrid domain of integrin b subunit controls ligand affinity. The "glycan wedge" introduced at specific domain boundary activated integrin expressed on cell surface(3). We have proposed that the change in affinity at the ligand binding site in the I-like domain around its metal ion-dependent adhesion site (MIDAS) is communicated to the interface with the hybrid domain on the opposite end of the I-like domain by axial displacement in the C-terminal direction of the I-like domain α7-helix(4). We hypothesized that in both the unliganded and liganded $a_V\beta_3$ structures the C-terminal α7 helix of the β_3 I-like domain is in a position stabilizing a closed, low affinity conformation; therefore, these structures were used to design low affinity mutants. An open, high affinity conformation was modeled assuming that the α7-helix was displaced in the C-terminal axial direction by two α-helical turns. The distance between Cβ atoms of T329 and A347 in the unliganded and RGD-liganded $\alpha_V\beta_3$ structures is 4.4 Å and 4.9 Å, respectively, whereas it is 9.6 Å in the hypothesized high affinity model. Therefore, the mutant $b_3^{T329C/A347C}$ was expected to form a disulfide bond in the low but not the high affinity conformation, and to be stabilized in the low affinity, closed conformation. On the other hand, the distance between the Cβ atoms of V332 and M335 in the unliganded and RGD-liganded $\alpha_V\beta_3$ structures is 10.0 Å and 8.3 Å, respectively, whereas it is 3.7 Å in the hypothesized high affinity model. Therefore, the mutant $b_3^{V332C/M335C}$ was expected to form a

disulfide bond in the high but not the low affinity conformation, and to be stabilized in the high affinity, open conformation. Wild type and mutant β_3 subunits were co-transfected with wild type a_{IIb} in 293T cells, and subjected to immunostaining flow cytometry. Non-reducing SDS-PAGE of ^{35}S-labeled, immunoprecipitated receptors showed that the wild type and mutant α_{IIb} subunits migrated similarly, whereas the mutant $b_3^{T329C/A347C}$ and $b_3^{V332C/M335C}$ subunits migrated slightly faster than wild type b_3 . By contrast, all β_3 single cysteine mutants migrated similarly to wild-type β_3. In general, disulfide bonds increase the mobility of proteins in SDS-PAGE, and these results suggest that the cysteines introduced into the $\beta_3^{T329C/A347C}$ and $\beta_3^{V332C/M335C}$ mutants form a disulfide bond. Disulfide bond formation was confirmed by labeling free sulfhydryls with the maleimide-containing reagent, biotin-BMCC.

Binding to soluble fibrinogen was first examined using two-color flow cytometry in transiently transfected 293T cells, in which wild type $a_{IIb}b_3$ basally has low affinity for ligand. Wild type $\alpha_{IIb}\beta_3$ bound fibrinogen when stimulated with the activating mAb PT25-2, but not basally in Ca^{2+} (Fig. 1). Each of the four single cysteine mutants behaved similarly to the wild type receptor (Fig. 1). By contrast, the putative locked closed, double cysteine mutant $a_{IIb}b_3^{T329C/A347C}$ did not bind soluble fibrinogen even in the presence of PT25-2 (Fig. 1). Furthermore, the putative locked open mutant $a_{IIb}b_3^{V332C/M335C}$ bound soluble fibrinogen even in Ca^{2+}, and addition of PT25-2 mAb did not further increase binding (Fig. 1). Constitutive binding in Ca^{2+} by the $a_{IIb}b_3^{V332C/M335C}$ mutant was abolished by two blocking α_{IIb} mAbs HA5 and 10E5, but neither blocked nor further activated by the activating β_3 mAb AP5 (Fig. 2), confirming that the high affinity fibrinogen binding of the transfected cells was specific.

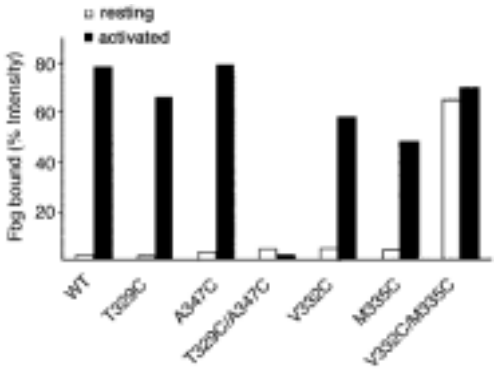

Figure 1

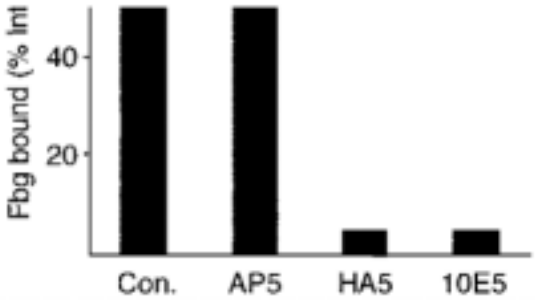

Figure 2

Discussion & Summary

The results with the $\alpha_{IIb}\beta_3^{T329C/A347C}$ and $\alpha_{IIb}\beta_3^{V332C/M335C}$ mutations strongly support the importance of β I-like domain α7-helix movement in integrin affinity regulation. Both hypothesis-driven mutations in the β I-like domain had the predicted effect. One, designed to displace the β6-α7 loop downward, indeed activated ligand binding. The other, designed to hold the β6-strand and α7-helix together near the end of the α7-helix, indeed maintained $\alpha_{IIb}\beta_3$ in the low affinity state and demonstrated that this relative arrangement of these two secondary structure elements, visualized in crystal structures, corresponds to the low affinity state.

The rational design of mutations that allosterically stabilize high affinity or low affinity conformations of integrins demonstrates marked advances in our understanding of the molecular basis of affinity regulation. These “hyperactive” or “inactive” mutant integrins can be used in the future as a tool for screening novel class of small molecule integrin inhibitors and/or monoclonal antibodies.

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1) Biological Sciences
1-1) Molecular Biology

Molecular mechanism of transcriptional regulation controlled by histone methylation

Yoichi Shinkai
Institute for Virus Research, Kyoto University
yshinkai@virus.kyoto-u.ac.jp

Introduction

Histone H3 Lys 9 (H3-K9) methylation is a crucial epigenetic mark for transcriptional silencing. G9a is the major mammalian H3-K9 methyltransferase that targets euchromatic regions and is essential for murine embryogenesis. There is a single G9a-related methyltransferase in mammals, called GLP/Eu-HMTase1. To elucidate in vivo function of GLP and functional interaction between G9a and GLP, we generated and characterized GLP deficient mouse and ES cells.

Results

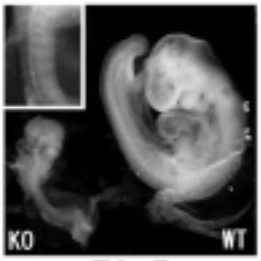
The human counterpart of GLP, Eu-HMTase1, has been shown to express H3-K9 HMTase activity *in vitro*. To investigate the substrate specificity and the enzymatic activity of GLP versus G9a, we generated GST-fusion proteins carrying their respective SET domains. The GST-fusion proteins were incubated with S-adenosyl methionine and several recombinant GST-histone H3 tail substrates. As reported previously, the GST-G9a SET protein preferentially methylated K9 and less efficiently K27 in our *in vitro* assay system. The GST-GLP SET protein also methylated K9 preferentially and K27 weakly Thus, the enzymatic activities of GLP were quite similar to those of G9a. To further explore the biological function(s) of GLP, we examined the intracellular localization profile of GLP as compared with G9a. Newly generated monoclonal antibodies specific for G9a or GLP were used for detection of the corresponding endogenous proteins using immunohistochemistry. G9a and GLP displayed similar intracellular localization profiles in mouse ES cells and embryonic fibroblasts. Both enzymes were detected exclusively in nuclei, but did not accumulate at DAPI-dense heterochromatin, and were excluded from nucleoli. We also examined RNA expression profiles of *G9a* and *GLP* by multi-tissue Northern blot hybridization in adult mice. *G9a* and *GLP* were ubiquitously expressed with some variations at steady-state mRNA levels when comparing different tissues. It was notable that the relative levels of *GLP* transcripts among the examined tissues were quite proportionate to those of *G9a*, except in testis, where *G9a* transcripts were elevated. Collectively, these results suggest that G9a and GLP may possess similar functions as H3-K9

HMTases *in vivo*. Next, to generate *GLP-/-* mice and cells by genetic mutation, we designed a targeting construct for replacement of exon 25 and a portion of exon 26 with the *neomycin* gene cassette. The replaced sequences encode the catalytic core region of the GLP SET domain. The targeting construct was introduced into TT2 mouse ES cells and selected by G418/Gnc. From the three hundred clones screened, we identified eight that carried a correctly targeted *GLP* allele and no extra copy of the construct. Three clones were injected into the morula stage of mouse embryos to generate chimeric animals. To establish GLP deficient ES cells, at first, we introduced a Flag-tagged *GLP* cDNA flanked with two loxP sites into *GLP*+/- ES cells and then generated *GLP-/-* ES cells expressing exogenous Flag-tagged GLP molecules. Finally, using Cre-recombinase treatment, we generated GLP-deficient ES cells. Analyses of GLP deficient mice and ES cells showed that GLP is also important for H3-K9 methylation of mouse euchromatin and murine embryogenesis. GLP-deficiency led to embryonic lethality around E9.5 (Figure 1), a severe reduction of H3-K9 mono- and dimethylation (Figure 2), the induction of *Mage-a* gene expression, and HP1 (HP1α, β and γ) relocalization in embryonic stem cells, all of which were phenotypes of G9a-deficiency.

Interestingly, *GLP*-deficiency caused a reduction in the steady-state levels of G9a protein to approximately ~1/3 of those observed in wild-type ES cell, TT2. RNA hybridization analysis of *GLP*-deficient ES cells demonstrated no decrease in *G9a*-transcript levels, indicating that the reduction in G9a protein occurred post-transcriptionally. Importantly, all phenotypes observed for the *GLP* deficiency, including G9a protein instability, loss of H3-K9 dimethylation, and de-repression of *Mage-a* transcripts, were rescued by the wild-type GLP expression, indicating that the observed *GLP-/-* phenotypes were intrinsic. Furthermore, we observed that G9a and GLP tightly formed a stoichiometric heteromeric complex in a wide variety of cell types. Biochemical analyses revealed that full-length G9a, GLP and their SET domains but not SET-domain deletion mutants formed complexes indicating the formation of the G9a/GLP complex is dependent on their enzymatic SET domains.

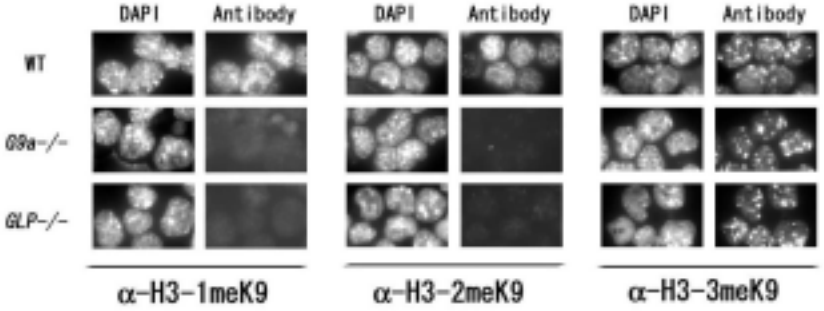
Figure1

GLP-/- embryos die around E9.5



E9.5

Figure 2
Severe reduction of H3-K9m1 and H3-K9m2 in GLP-/- ES cells



Discussion & Summary

In this study using G9a and GLP deficient mice and ES cells, we showed that GLP is also important for H3-K9 methylation of mouse euchromatin. Furthermore, the phenotypes observed for germline mutations of the *G9a* and *GLP* genes were identical in many respects, including embryonic lethality, drastic reduction of H3-K9 mono- and di-methylation, induction of *Mage-a* genes, and HP1 re-localization in ES cells. Moreover, we showed that G9a and GLP formed a stoichiometric heteromeric complex in a wide variety of cell types. Biochemical analyses revealed that formation of the G9a/GLP complex was dependent on their enzymatic SET domains. Taken together, our new findings revealed that G9a and GLP cooperatively exert H3-K9 methyltransferase function *in vivo*, likely through the formation of higher-order heteromeric complexes.

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1) Biological Sciences
1-3） Cell Biology

Analysis of the functional role of vacuolar protein sorting (Vps) protein, Hrs/Hgs in the regulation of TGFβ-induced signal transduction

HIRONOBU ASAO
Yamagata University faculty of Medicine
asao-h@med.id.yamagata-u.ac.jp

Introduction

We identified an IL-2-induced tyrosine-phosphorylated protein, Hrs/Hgs and STAMs in T cell, isolated a cDNA clone and then reported that Hrs and STAMs are associated to each other. Some reports showed that Hrs and STAMs were important molecules for vesicular transport from early to late endosomes.

TGFβ is a multifunctional cytokine activating Smad2/3 and has suppressive functions on immune system. We previously reported that Hrs formed a complex with Smad2/3 and TGFβ receptor and that the expression level of Hrs was diminished accompanied with T cell activation and then TGFβ signal was enhanced. In this study, we are going to ascertain the regulatory mechanism of the TGFβ signaling by Hrs.

Results

Recently we established cells that defect Hrs gene expression[1]. In the Hrs-deficient cells established from mouse embryonic fibroblast (MEF), TGFβ signal was tested. In the Hrs-deficient cells (HRSd), Smad2, which is a major signaling molecule of TGFβ signal transduction, was phosphorylated before TGFβ stimulation and Smad2 activation was enhanced and prolonged than that of wild type MEF (MEFw) (Figure 1). Translocation of Smad2 was studied by using transiently expressed GFP-Smad2 in these cells. In Hrs-deficient cells, Smad2 was located in the cytoplasmic region and also in the nucleus before TGFβ stimulation and then nuclear Smad2 was enhanced after TGFβ stimulation. On the other hand, in the wild type Hrs-introduced cells, Smad2 was located in the cytoplasmic and translocated into nucleus only after TGFβ stimulation. Introduction of wild-type Hrs (HRSw) or Hrs mutant with an intact STAM binding domain (Hrs-dFYVE) into the Hrs-deficient cells completely recuperated normal Smad2 activity while introduction of mutant Hrs with no STAM binding ability (Hrs-dC2, Hrs-dM) did not.

Then we investigated STAM that is an association molecule with Hrs in the Hrs-deficient cell lines. Surprisingly in the Hrs-deficient cells only trace amounts of STAM1 and STAM2 were detected

comparing with wild type MEF. While wild-type Hrs or Hrs-dFYVE mutant-introduced Hrs-deficient cells fully restored STAM1 and STAM2 expression, whereas Hrs-dC2, Hrs-dM mutants with no STAM binding ability failed to restored the expression of the STAMs[2].

This regulated control of STAM expression by Hrs was independent of transcription. Interestingly, STAM1 degradation was mediated by proteasomes and was partially dependent on the ubiquitin-interacting motif of STAM1. Revertant Hrs expression in the Hrs-deficient cells not only led to the accumulation of ubiquitinated proteins, including intracytoplasmic vesicles, but also restored STAM1 levels in early endosomes and eliminated the enlarged endosome phenotype caused by the absence of Hrs. These results suggest that Hrs is a master molecule that controls in part the degradation of STAM1 and the accumulation of ubiquitinated proteins.

Figure 1

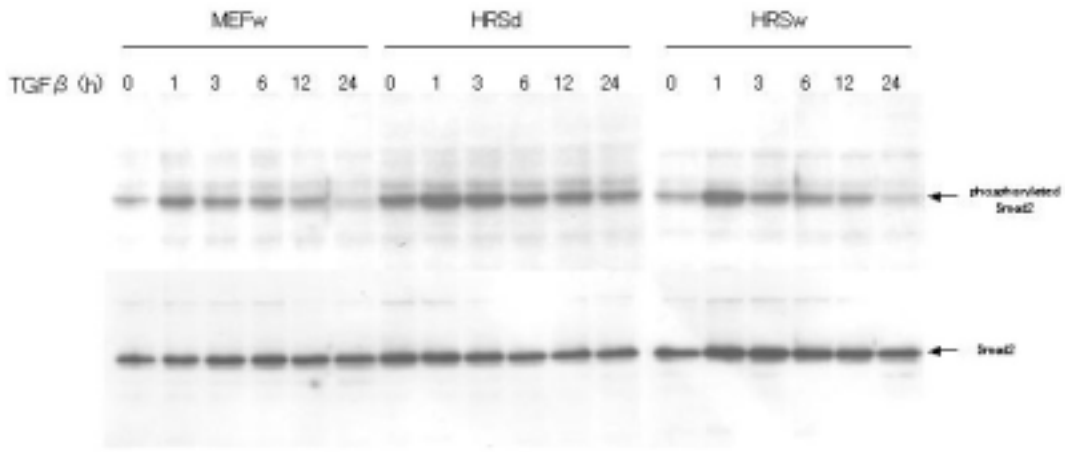

Figure 1 TGFβ-induced signal was enhanced in the Hrs- deficient cells
Cells were stimulated with 1ng/ml human TGFβ for the indicated periods.
Phosphorylated Smad2 (upper panel) or whole Smad2 (lower panel) was detected by immunoblot assay.

Discussion & Summary

In this study, we showed Hrs has an important role in regulating TGFβ signaling. Without Hrs, TGFβ-induced major signal transducing molecule, Smad2 is constitutively activated even without TGFβ. For the regulatory function of TGFβ signaling, STAM-binding domain of Hrs was indispensable suggesting that this regulatory mechanism required also STAM. We previously reported that epidermal growth factor receptor degradation is partially regulated by the Hrs-STAM complex. Degradation of TGFβ receptor, which is important negative regulatory mechanism, may also be regulated by the Hrs-STAM complex that are important molecules in the mammalian endosomal/vacuolar protein sorting pathway.

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1) Biological Sciences
1-3) Cell Biology

Comprehensive studies of gene regulation by posttranslational processing-mediated carboxy-terminal fragments of the EGF family members.

Shigeki Higashiyama Ph.D.
Ehime University School of Medicine
Shigeki@m.ehime-u.ac.jp

Introduction

Cleavage of membrane-anchored heparin-binding EGF-like growth factor (proHB-EGF) via metalloprotease activation yields amino- and carboxy-terminal regions (HB-EGF and HB-EGF-C), with HB-EGF widely recognized as a key element of epidermal growth factor receptor transactivation in G-protein coupled receptor signaling. Currently we have shown that subsequent to proteolytic cleavage of proHB-EGF, HB-EGF-C translocated from the plasma membrane into the nucleus. This translocation triggered nuclear export of the transcriptional repressor PLZF, which we identify as a HB-EGF-C binding protein. In this study, we focused on the functional analyses of C-terminal fragments of the EGF family members including HB-EGF as well.

Results

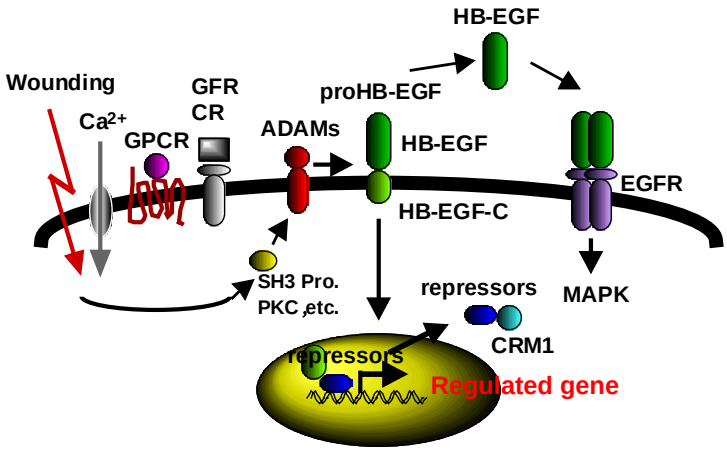
Based on the analysis of the HB-EGF-C binding domain of PLZF, we screened candidates as a binding partner of HB-EGF-C, and listed up 15 members of the PLZF family consisted of 75 members. Pull-down assay using GST-HB-EGF-C revealed at least additional 4 members, BCL-6, BAZF, Myoneulin and KIAA0441, of the PLZF family were able to interact with HB-EGF-C. In order to analyze the localization of C-terminal fragment of the EGF family members after their shedding, we carried out immunostining by their C-terminal fragment specific antibody, revealing that the C-terminal fragments of TGF-α, amphiregulin, epiregulin and epigen were tranlocated into nucleus. Their nuclear translocation effectively blocked by the addtion of a metalloprotease inhinibtor KB-R8301. We further confirmed that their C-terminal fragments were able to bind to PLZF. On the other hand, we have screened the genes which transcription are regulated by PLZF using DNA microarray, and identified Cycline A2 Pbx-1, tzfp and c-myc. We are currently investigating the target genes which regulates by BCL-6, BAZF, Myoneulin and KIAA0441.

Discussion & Summary

The C-terminal fragments on the EGF family members as well as HB-EGF-C translocate into nucleus after ectodomain shedding on cell surface. The The C-terminal fragments interact several transcriptional repressors including PLZF. Since the ectodomain shedding of the EGF family members is well known to be a crucial event of EGFR transactivation induced by various extracellular stimuli, the C-terminal fragment signaling of the EGF family members might be a fundamental event in cell growth and differentiation.

Figures & Tables

C-terminal fragment signaling of membrane-anchored growth factor HB-EGF



Taregeted repressors
PLZF, Bcl-6, BAZF, Myuneulin, KIAA0041

Regulated gene
Cyclin A2, Pbx-1, tzfp, c-myc

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1) Biological Sciences
1-3） Cell Biology

Molecular Network Underlying Sperm Motility

Kazuo Inaba
University of Tsukuba
inaba@kurofune.shimoda.tsukuba.ac.jp

Introduction

Activation of sperm motility is one of the dynamic changes after completion of spermatogenesis. This is a sophisticated process that many organisms have acquired during evolution and the abnormality in this process results in serious deficiency in fertilization, such as male infertility. This research was aimed to reveal the molecular interaction underlying the reception of extracellular signal, intracellular signal transduction and activation of motile apparatus of sperm flagella.

Results

To reveal the molecular network underlying the activation of sperm motility, the ascidian *Ciona intestinalis* was used as an experimental organism. *Ciona* sperm are immotile when they were spawned into seawater, but the motility was initiated and activated by an egg-derived substance SAAF (Yoshida et al., 2002). The activation process is easily observed *in vitro*. This animal provides not only a simple system for analysis of sperm activation but also genomic information that have been recently established: We have analyzed approximately 10,000 ESTs of testis-expressed genes in *C. intestinalis* (Inaba et al., 2002) and genome draft sequence has been determined (Dehal et al., 2002).

The research was started from the establishment of the analysis system for sperm proteins using peptide mass fingerprinting (PMF). The open reading frames of approximately 4,000 genes expressed in *C. intestinalis* testis were determined from EST data complemented with genome draft sequence. The accurate mass values of tryptic fragments derived from these testis-expressed genes were in silico calculated by the help of the program MS-Fit. These data was summarized as “MSCITS”, a database comprising peptide masses of 4,000 proteins expressed in *C. intestinalis* testis. Then a search program was devised for PMF analysis using Perl and was named as “PerMS”. To test the efficiency for identification of proteins by this system, we have randomly selected 50 proteins from *Ciona* sperm proteins separated by two-dimensional gel electrophoresis, treated with trypsin and analyzed by a matrix-assisted laser desorption ionization/time of flight/mass spectrometry (MALDI-TOF/MS). Approximately 80% of the proteins could be correctly identified,

suggesting that the MSCITS/PerMS is a efficient system for identification of *Ciona* sperm proteins (Hozumi et al., 2004).For comprehensive understanding of the signaling network for the activation of sperm motility, we carried out the analysis on 1) changes in proteins at activation of sperm proteins using two-dimensional gel electrophoresis, 2) molecular construction of outer and inner arm dyniens, 3) isolation of radial spokes and its molecular architecture, and 4) proteomic analysis of membrane/matrix fraction of sperm.

During the course of these study using MSCITS/PerMS system, we have identified a heat shock protein 40 in the radial spoke of flagellar axonemes, which is thought to be quite important in the signal transduction for the activation of dyneins (Satouh et al., 2005). Members of the heat-shock protein (HSP) 40 regulate the protein folding activity of HSP70 proteins and help the functional specialization of this molecular chaperone system in various types of cellular events, suggesting a correlation between Hsp40 related chaperone system and flagellar function. Furthermore we have found that *Ciona* 37-kDa Hsp40 is extracted from KCl-treated axonemes with 0.5 M KI solution and comigrates with radial spoke protein (RSP) 3 along with several proteins as a complex through gel filtration and ion exchange columns. PMF with MALDI-TOF/MS revealed that other proteins in the complex include a homolog of sea urchin spokehead protein (homolog of RSP4/6), a membrane occupation and recognition nexus repeat protein with sequence similarity with meichroacidin, and a functionally unknown 33-kDa protein. A spoke head protein, LRR37 (Padma et al., 2003), is not included in the complex, suggesting that the complex constructs the stalk of radial spoke. Immunoelectron microscopy indicates that Hsp40 is localized in the distal portion of spoke stalk, possibly at the junction between spoke head and the stalk (Figure 2).

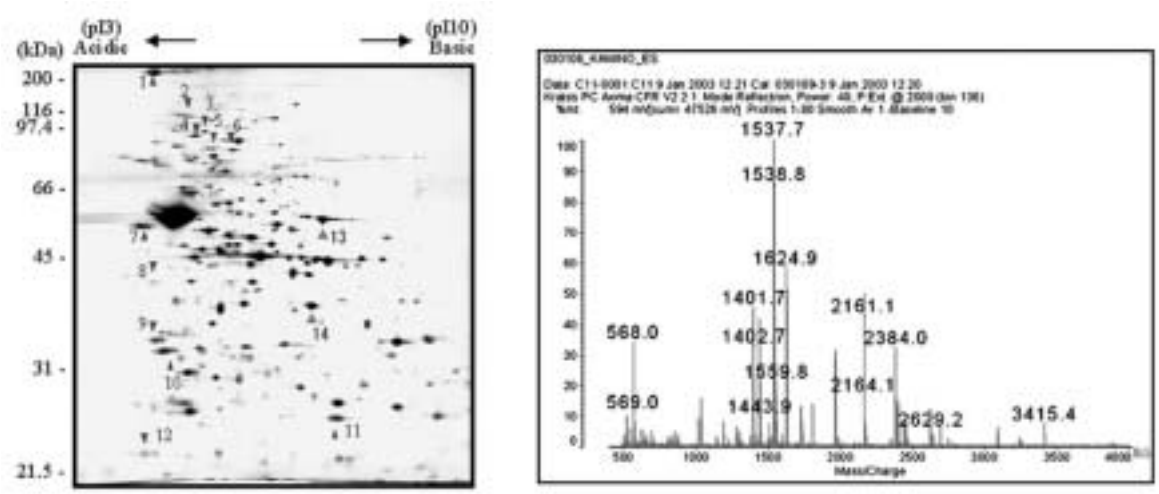

Figure 1. Two-dimentional gel electrophoresis of *Ciona* axonemal proteins (left) and MALDI-TOF/MS analysis of an axonemal proteins.

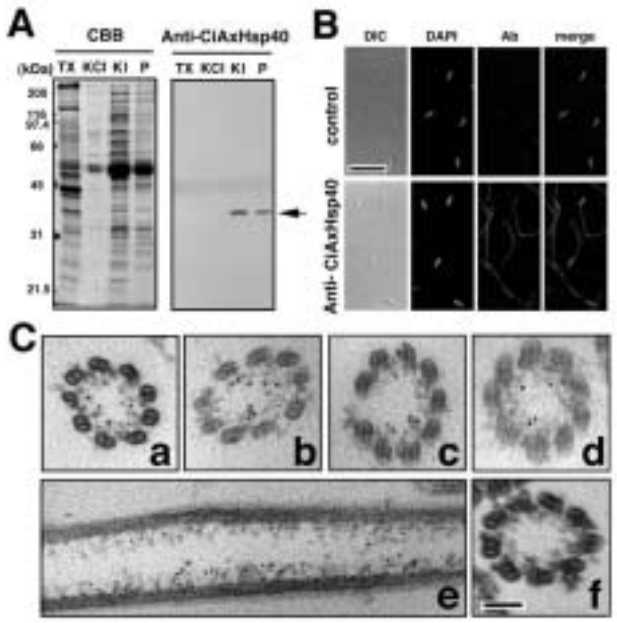

Figure 2. Immunological analyses of *Ciona* axonemal HSP40.A, western blot; B, immunofluorescence; C, immunogold localization.

Discussion & Summary

In order to perform a proteomic study on the molecular mechanism of sperm activation, we have constructed a MS database from *Ciona* testis ESTs and the genome draft sequence, along with a newly devised, perl-based search program PerMS for peptide mass fingerprinting. This system could identify more than 80% of *Ciona* sperm proteins, suggesting that it could be widely applied for proteomic analysis for a limited tissue with less genomic information. By using this system, we could identify HSP40, a homolog of sea urchin spokehead protein, a membrane occupation and recognition nexus repeat protein with sequence similarity with meichroacidin, and a functionally unknown 33-kDa protein in the radial spoke complex of flagellar axonemes.

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1) Biological Sciences
1-3) Cell Biology

Isolation of ES-like cells and production of cloned animals from genetically modified blastocysts derived from equine or bovine

Kazunari K. Yokoyama
Gene Engineering Division, BioResouce Center, RIKEN
yokoyama@brc.riken.jp

Introduction

One of the goals of animal biotechnology is the application of current techniques of genetic engineering to the production of farm animals with desired traits. At present, foreign genes can be induced into the murine genome via embryonic stem (ES) cell and the use of homologous recombination techniques. In this system, cultured murine ES cells can be used as a tool for the addition or deletion of genes at specific sites in the genome. With respect to species other than mice, human, sheep, pig, bovine, mice, mink, hamster, rat, monkey and equine have all been reported as source of putative ES cells. However, neither production of chimeric animals with traits that are transmitted through the germ line nor satisfactory demonstration of the essential traits of ES cells has yet been reported.

In attempt to generate that genetically modified ES (like) cells and cloned animals, we have examine gene.

The possibility of the generation of transgenic calves and equines from ES cells introduced by following genes,

(A) Japanese specific HLA class I and β2m genes;
(B) Human telomease genes.

Results

Capacity for self-renewal and pluripotency

Mouse ES cells can be maintained indefinitely in the presence of Leukemia inhibitory factor (LIF), and they express markers of the undifferentiated state that indicate the capacity for self-renewal and pluripotency, such as transcription factors Oct4, STAT-3, stage-specific embryonic antigen (SSEA-1), and alkaline phosphatase (AP). Upon removal of LIF, the cells cease to express markers such as Oct 4, rapidly losing the capacity for self-renewal and differentiating into a variety of cell types.

In addition to the mouse and man, the sheep, pig, rabbit, bovine, mink, hamster, rat and monkey have all been reported as sources of putative ES cells. However, in these otter animals, neither the

production of chimeric animals with traits that are transmitted through the germ line nor satisfactory clarification of the essential characteristics of ES cells has yet been reported.

Recently, we demonstrated the successful isolation of immortalized equine and bovine ES cells with a normal karyotype. We have cultured such cells for more than 50 passages in vitro with a minimum of 250 cell divisions. Strelchenko et al. reported that the limit for most non-immortalized cells is approximately 60 divisions. Therefore, we can distinguish immortalized cell lines from non-immortalized cell lines operationally on the basis of the ability of cells to undergo more than 60 divisions. Both our equine and our bovine ES cells exhibit the essential features of ES cells, expressing Oct4, STAT-3, SSEA-1 and AP (Fig. 1). We confirmed the pluripotency of these cells with respect to somatic differentiation in vitro to neural progenitor cells and to endothelial or hematopoietic lineages.

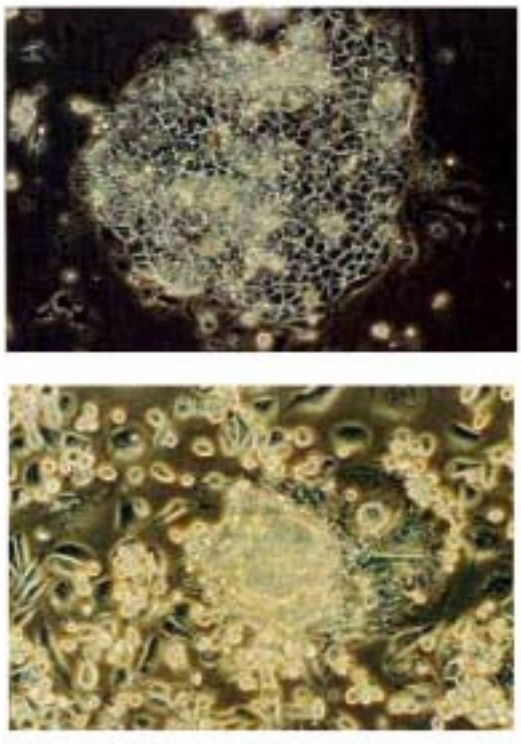

Fig.1:Morphology of equine (A) and bovine (B) ES cells.
Phase-contrast image of colonies of ES cells cultured for 5days in the absence of a feeder layer but in the presence of human LIF.
Diferentiated eqithelia-like,rounded and bi-polar shaped cells are visible among other cells in culture.
Magnification: A and B,×200;

By contrast to murine ES cells, equine ES cells differentiated to epithelial-like cells in the absence of a feeder layer, provided the medium was supplemented with LIF. Equine and bovine ES cells both required the support of a feeder layer for serial cultivation in the undifferentiated state. Furthermore, LIF was unable, by itself, to maintain self-renewal of murine ES, cells in serum-free medium. Hence, some other growth factors must be necessary, in combination with LIF to support the self-renewal capacity of ES cells.

Ying and colleagues showed recently that bone morphogenetic protein (BMP) can replace serum, defining the molecular requirements for the self-renewal of murine ES cells. These researchers demonstrated that, when ES cells were grown in serum-free medium, BMP4 and LIF acted synergistically to support the derivation and maintenance of ES cells, which are driven by the coordinated actions of BMP and STAT-3 signaling pathways, even when the starting material is single cells. Thus, it appears necessary to include BMP in the culture medium in the absence of a feeder layer for the consistent generation of ES cells, in which the full detail of the molecular basis of self-renewal remains to be clarified.

While it has proved difficult to identify the factors that are required for the maintenance of ES cells in the absence of a feeder layer, progress in establishing methods for inducing the differentiation of such cells. We developed a method for the induction of differentiation to neural precursors of equine and bovine ES cells, using a combination of basic fibroblast growth factor (bFGF; 10 ng/ml; Sigma), epidermal growth factor (EGF; 10 ng/ml; Sigma) and platelet-derived growth factor (PDGF; 10 ng/ml; Sigma) in serum-free MEMα medium.. We also developed a protocol for the differentiation of equine ES cells *in vitro* to hematopoietic and endothelial cell lineages in response to bFGF, stem cell factor (SCF) and oncostatin M.

Equine ES cells that were proliferating in two dimensions were able to differentiate to hematopoietic cells and, thus, it appeared that neither a three-dimensional ES structure nor a layer of feeder cells was required for this process, as was also the case for the differentiation of equine and bovine ES cells to neural precursors cells. Now, we need to develop methods that will allow us to pathways of differentiation of ES cells and the formation of tissues and organs from them *in vitro*.

Transgenesis of animal ES cells

Other research group, working on the isolation of porcine ES cells, determined conditions for maintaining such ES cells in an undifferentiated state for short periods of time. However, they failed to maintain the cell lines in an undifferentiated state for a long enough time to allow attempts at genetic modification. By contrast, our bovine ES cell lines can be maintained indefinitely *in vitro* and retain characteristics of pluripotency, as do mouse ES cells. Then, we have been able to achieve genetic transformation and the selection of transformants.

Bovine ES cells were transfected by a lipid-based reagent (Effectene, Qiagen, Tokyo, Japan) with

DNA fragments derived from pCX-Neo-EGFP and incubated for 3 days. Then, we selected colonies that expressed fluorescent GFP protein by exposing of cells to the antibodies G418 (200-400 ug/mg). Recipient embryos produced by in vitro fertilization (IVF; day 4 embryos) were injected with 10 to 15 transgenic ES cells per embryo.

After 5 days in culture, more than 40% of the chimeric embryos (17 out of 41) included contributions due to the injected GFP-expressing stem cells. Fluorescence due to GFP was detectable both in the ICM and in trophectodermal cells of the injected blastocysts. Our data showed clearly that the transfected bovine ES cells were able to participate in the formation of chimeras after injection into embryos at the 8- to 16- cell stage, while similarly transfected fibroblast did not have this ability. Our observations indicated that the procedures for genetic transformation and selection had not interfered with the ability of the ES cells to participate in the formation of chimeras.

Human leukocyte antigen-β(HLA-B) are useful, therapeutic agents but, because they are available only from human donors, their supply and, thus, their applications are limited. Transgenic animals carrying a gene for human leukocyte antigen (HLA) might be a good source of HLA-B. Therefore, we recently established lined of bovine ES cells that express HLA plus GFP (HLA-B-GFP) or human telomerase reverse transcriptase plus GFP (HTERT-GFP). These genes were efficiently introduced using FuGene 6 (Roche, Tokyo, Japan) or FluoroGene (ImaRx, Tucson, AR, USA) in combination with an Enhancer A (Nepagene, Chiba, Japan) as transfection reagents. The efficiency of transfection using these reagents was apprpximately 50%, as determined 72h after the start of the transfection. Consequently, the expression of GFP in bovine ES cells was 5 to 10 times higher than that obtained with other reagents such as LipofectAMINE (Life Technologies, Rockville, USA), Effectene (Quiagen) and Escort (Sigma).

Nuclear transfer (NT) from murine ES cells allows generation of mice with DNA insertions without necessity of generating chimeric mice, as is required when convential gene-targeting techniques are used because animals produced by NT are entirely transgenic. Thus, with improved efficiency, NT with ES cells should shorten the time required for the generation of transgenic animals. Furthermore, it might be possible to insert genes at a single site in multiple lines of animals to generate homozygous lines of animals rapidly while avoiding inbreeding. The realistic prospect of gene targeting in large domestic animals opens up a vast range of novel applications and research possibilities.

Nuclear transfer of animal ES cells

Nuclear transfer (NT) was accomplished first in frogs to determine whether nuclei from differentiated cells remain genetically totipotent. Unsuccessful results from cloning experiments with adult cells suggested that the nuclei from differentiated frog cells could not support the development of an entire frog. However, the generation of Dolly, the sheep from an adult

mammary gland cell suggested the nuclear totipotency of at least some cells within an adult animal. It remains to be determined whether cloned animals are derived selectively and for the most part, from the nuclei of less-differentiated adult cells, such as adult stem cells, rather than from the nuclei of fully differentiated adult cells. The generation of cloned mice, sheep and bovine derived from pluripotent blastomeres or ES cells suggests that the nuclei of less-differentiated cells are more useful.

For example, mouse blastocysts derived from 2- to 8- cell blastomeres and from morula stage nuclei developed to term with an efficiency of 20-40%. Similarly, Willadsen et al. reported a success rate of 36% for live cloned calves derived from morula-stage nuclei by NT in cattle. Furthermore, in mice blastocysts cloned from nuclei of cultured ES cell developed to term at an efficiency (10-30%) that was 3 to 10 times greater than in the case of blastocysts cloned from the nuclei of somatic cells (1-3%).

We performed NT experiments using bovine ES cells, that had been purified by flow cytometry in a cell sorter (FACScan, Becton Dickinson, Mountain View, CA, USA) with SSEA-1- specific antibodies. Cloned blastocysts derived from cultured ES cells yielded a pregnancy rate of 71% by ultrasonography 50 days after transfer, and calves developed to term at a frequency of 43%. These values are higher than those for early pregnancies (55%) and term pregnancies (8-14%) that have been reported after transfer of nuclei from differentiated somatic cells. Wakayama et al. demonstrated that live offspring cannot be generated after direct transfer of nuclei from terminally differentiated lymphocytes to early embryos. Their observations suggest that reprogramming of a terminally differentiated cell might be extremely inefficient and, thus, the development of cloned embryos is likely to fail. Indeed, the genome might undergo not only epigenetic but also genetic changes during differentiations to, for example, terminally differentiated lymphocytes.

Compared to differentiated somatic cells, embryonic cells require less-reprogramming of their genome, perhaps because many genes critical for early development are already active in the donor cells and do not need to be reactivated. Thus, it might prove possible to generate cloned animals from the nuclei of adult stem cells but not from those of terminally differentiated cells. Adult stem cells and ES cells might exist in a similar epigenetic state that allows them to differentiate to multiple lineages.

As a next step towards our goal of applicable ES cell technologies, we are now attempting the generation of transgenic clonal calves that harbor an HLA gene from cultured ES cells to produce human leukocyte antibodies against HLA.

Discussion & Summary

It is important to note that NT often results in problems, such as respiratory distress, kidney and liver defects, and abnormal growth of the placenta and/or fetus. In our experiments, one of cloned calves died two days after birth as a result of abdominal bleeding. Experiments on mice indicate that, most cloned animals suffer from abnormalities that range from, subtle to severe, as confirmed by aberrant patterns of gene expression. Many of the observed abnormalities in cloned animals might be caused by the inefficient and incomplete reprogramming of post-zygotic epigenetic modifications that were imposed on the genome during differentiation. Such epigenetic modifications, include DNA methylation, histone modifications and the expression of development-related genes.

We understand very little about reprogramming and ways in which it can be achieved. A better understanding of the molecular basis of reprogramming might allow us to improve the efficiency of NT and to facilitate its application to gene and cell therapy.

Studies of NT may help us to clarify the contribution of epigenetic and genetic modification to cellular differentiation and transformation.

Figures & Tables

Table 1: Cloning efficiencies of various cell types

	Source of donor nucleus	Species	Efficiency (%) (transferred blastocysts / term births)
Differentiation	Zygote	Mouse	20-50%
	Embryonic blastomeres	Mouse	20-40%
		Sheep	5%
		Bovine	19-36%
	Cultured ICM cells	Bovine	12%
	ES cells	Mouse	10-30%
		Bovine	40%
	Adult stem cells	——	no data
	Differentiated: cumulus cells,	Mouse	1-3%
	fibroblasts,	Sheep	3-8%
	mammary gland cells,	Bovine	8-14%
	Terminally differentiated lymphocytes	Mouse	0%

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1) Biological Sciences
1-3) Cell Biology

Physiological functions and regulation mechanisms for tankyrase and TAB182

Hiroyuki Seimiya
Division of Molecular Biotherapy, Cancer Chemotherapy Center,
Japanese Foundation for Cancer Research
hseimiya@jfcr.or.jp

Introduction

Tankyrase 1, a member of poly(ADP-ribose) polymerases, binds to a telomeric protein, TRF1, and poly(ADP-ribosyl)ates it. This post-translational modification inhibits the ability of TRF1 to bind telomere DNA and the TRF1-unbound telomeres allow access of telomerase, a telomere-synthesizing enzyme. Thus, tankyrase 1 is a positive regulator for telomere length (Refs. 1 & 2). TAB182 is another tankyrase 1-binding protein that is poly(ADP-ribosyl)ated by tankyrase 1 (Ref. 3). Tankyrase 1 is present not only at telomeres but also at Golgi apparatus and mitotic centrosomes, suggesting that this enzyme has additional non-telomeric functions. In this study, we focused on physiological significance of the tankyrase 1-TAB182 interaction (especially, functions of TAB182).

Results

(1) Generation of TAB182 knockout mice.

After cloning mouse *TAB182* genomic DNA clones by the lambda phage screening, we constructed two types of the *TAB182* gene targeting vectors (conventional and conditional). These vector plasmids were linearlized by *Not* I digestion and then transfected into mouse ES cells by electroporation. As for conventional targeting, 9 homologous recombinants (*TAB182*$^{+/-}$) were successfully isolated from 215 G418-resistant ES cell clones (Figure 1). In the targeted allele, *TAB182* exon 2 was disrupted by in-frame insertion of a *lacZ* gene. The downstream portion of the exon 2 and whole exon 3 were also deleted in the targeted allele, which would abolish the production of *TAB182* transcripts. Patterns for genomic integration of the vector were verified by Southern blot analysis with four different probes and PCR. Representative ES cell clones have just been injected into blastocysts to obtain chimeric mice. As for the Cre recombinase/loxP-based conditional targeting of the gene, 5 homologous recombinants (*TAB182*$^{+/flox}$) were isolated from 190 G418-resistant ES cell clones. However, none of these clones retained the critical 3rd loxP sequence

because one side of homologous recombination occurred at the locus between the 3rd loxP sequence and the *neo* gene cassette of the vector. Thus, for conditional targeting of the gene, we still need to add the 3rd loxP sequence to the floxed allele of the recombinants in the future.

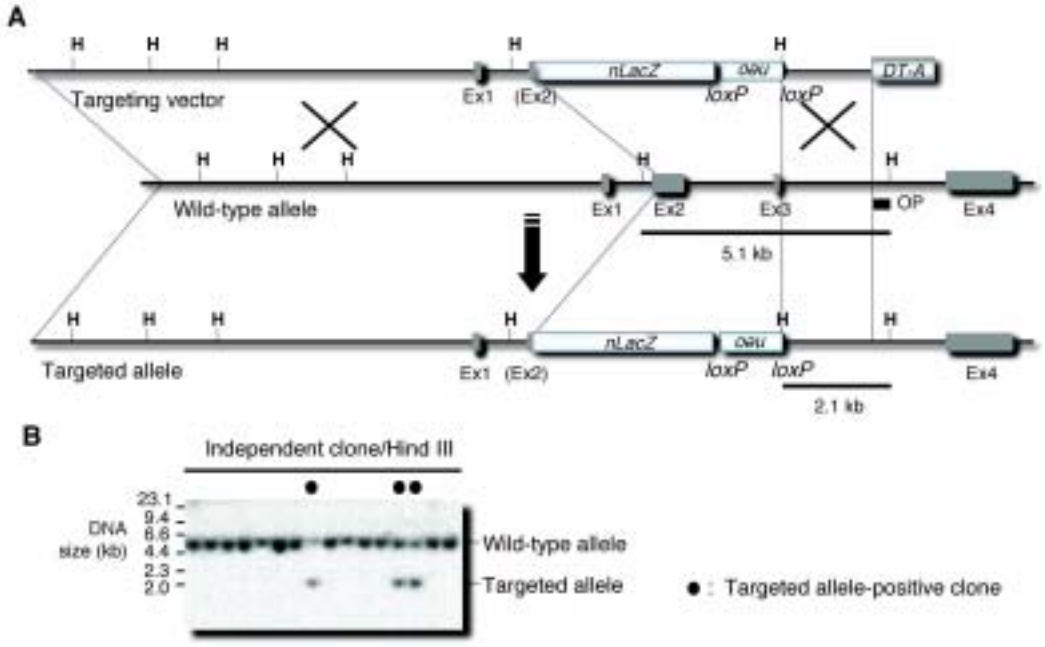

Figure 1. Generation of mouse TAB182$^{+/-}$ ES cells. A, targeting scheme. OP, outside probe for primary screen; Ex, exon; H, Hind III. **B**, representative result of Southern blot analysis. Genomic DNA from each clone was digested with Hind III and analyzed with the outside probe.

(2) Functional analysis of TAB182 by using RNA interference.

Human fibrosacoma HTC75 cells were transfected with several kinds of small interfering RNA (siRNA) to knockdown TAB182 protein expression in the cells. After verifying the effect of each siRNA on TAB182 protein level by western blot analysis, retroviruses that produced respective short hairpin RNA (shRNA) were constructed with the same target sequences. Then, the cells were infected with the viruses to establish cell lines in which the amount of TAB182 protein was stably down-regulated. Of note, the pilot study with siRNAs did not predict the extent of knockdown effect of shRNAs even with the same target sequences. The cell lines with decreased TAB182 expression grew at comparable rates to that of parental cells. After prolonged cultivation for 100 population doublings, these cells did not exhibit any change in mean telomere length as compared to that of parental cells.

(3) Estimation of the tankyrase 1-TRF1 interaction in mouse cells.

Human TRF1 has a tankyrase 1-binding motif, RGCADG, at the amino terminal acidic domain

whereas mouse TRF1 does not have such a motif. By using a pull-down assay with recombinant glutathione-S-transferase fusion proteins and *in vitro* translated proteins, we confirmed that neither human or mouse tankyrase 1 bound mouse TRF1 *in vitro*. On the other hand, both human and mouse tankyrase 1 bound human TRF1 *in vitro* (Figure 2). Consistently, transient overexpression of mouse tankyrase 1 in human HeLaI.2.11 cells released TRF1 from telomeres whereas that of human tankyrase 1 in mouse NIH3T3 cells had no effect on telomeric localization of TRF1, which was assessed by indirect immunofluorescence staining. Meanwhile, both human and mouse tankyrase 1 bound TAB182, which had a well-conserved tankyrase 1-binding motif, RPQPDG. Chromatin immunoprecipitation assay showed that telomere DNA was pulled down by anti-TRF1 but not by anti-TAB182 antisera. Consistent with this observation, affinity-purified anti-TAB182 antibody did not stain telomeres.

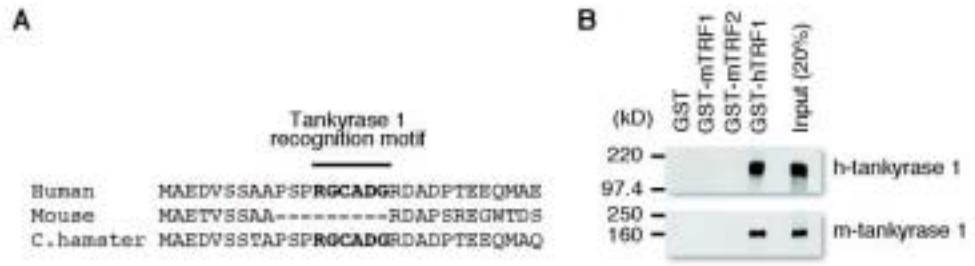

Figure 2. Tankyrase 1 recognizes human but not mouse TRF1. A, alignment of partial amino acid sequences for TRF1 N-termini. C.hamster, Chinese hamster. **B**, *in vitro* pull-down assay. *In vitro*-translated human or mouse tankyrase 1 proteins were incubated with the bead-bound GST-fusion proteins as listed. After extensive washing, bead-bound fractions were detected by western blot analysis with anti-tankyrase antibody. h, human; m, mouse.

Discussion & Summary

Inability of tankyrase 1 to recognize TRF1 in mouse cells strongly suggests that telomere length regulation by tankyrase 1 could be a species-restricted phenomenon. This also implies that tankyrase 1, especially in mice, would play a role for non-telomeric events through interaction with other binding partners, such as TAB182. In this study, so far, our observations have not shown any linkage between TAB182 and telomere dynamics. We have generated *TAB182*$^{+/-}$ ES cells and are expecting the chimeric mice. In the near future, we would like to confirm germ line transmission of the targeted allele and obtain *TAB182*$^{+/-}$ F1 and *TAB182*$^{-/-}$ F2 mice. We expect that these knockout mice would tell us the physiological importance of TAB182. Recently, we have reported that pharmacological targeting of tankyrase 1 enhances the anti-tumor impact of a telomerase inhibitor on human cancer cells (Ref. 1). Before assuming that tankyrase 1 is a good anticancer molecular target,

we still need to investigate whether tankyrase 1 inhibition disrupts its crucial physiological functions at other loci than telomeres. Functional analyses on TAB182 would give further insight into expanding network of tankyrase 1-mediated biological events.

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1) Biological Sciences
1-3) Cell Biology

Receptor protein tyrosine phosphatase ζ (Ptprz) signaling in gastric ulcer induction

Masaharu Noda
National Institute for Basic Biology
madon@nibb.ac.jp

Introduction

Protein tyrosine phosphorylation plays crucial roles in various aspects of cellular function. The level of tyrosine phosphorylation is determined by the balance between the activities of protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP). Ptprz (PTPz/RPTPb) is a member of the receptor-like PTP family and predominantly expressed in the brain. Recently, we found that Ptprz is expressed in the stomach, and that the mice deficient in Ptprz gene do not show mucosal damage by vacuolating cytotoxin, VacA, secreted from *Helicobacter pylori*. We revealed that VacA behaves as a ligand for Ptprz. In this study, we aimed to elucidate Ptprz-signaling cascade underlying the gastric ulcer induction.

Results

We here developed a genetic method to screen for PTP substrates which we have named the "yeast substrate-trapping system" (Fig. 1). This method is based on the yeast two-hybrid system with two essential modifications: the conditional expression of a PTK to tyrosine-phosphorylate the prey protein, and screening using a substrate-trap PTP mutant as bait. In our screening of substrates for Ptprz, we isolated four substrate candidates and 11 putative continuously interacting molecules (Fig. 2).

The four substrate candidates are Git1, p190, PIST and MAGI-1. Git1 is a GTPase-activating protein (GAP) for ADP-ribosylation factor (ARF) family of GTPases and believed to function in the wide range of cellular events including vesicle trafficking, cell adhesion, and cytoskeletal organization. p190 is a GAP for the Rho family of GTPases and known to participate in the cytoskeletal organization through the regulation of Rho activity. Rho family GTPases act as the main regulator of actin cytoskeletal dynamics in a variety of situations including neurite extension, cell migration, cell adhesion, and cell morphological changes. PIST was first identified as a PDZ domain-contining protein which interacts with TC10, a member of the Rho family of GTPases and

most closely related to cdc42. MAGI-1 contains a guanylate kinase domain, two WW motifs, and five PDZ domains, and is believed to function as a molecular scaffold in the formation of multiprotein complexes on the cytoplasmic surface of the cell membrane as is PSD-95. Thus, Ptprz likely regulates the cytoskeletal dynamics and cell adhesion through the control of Rho family GTPase activity.

Furthermore, we demonstrated that all of the four candidates can be tyrosine phosphorylated by v-src in mammalian cells, and dephosphorylated by GST-Ptprz in vitro. As the next step, we need to analyse the tyrosine phosphorylation of these molecules upon VacA treatments to know which molecule is involved in gastric ulcer induction. This is highly intriguing because they are given common functional features as described above.

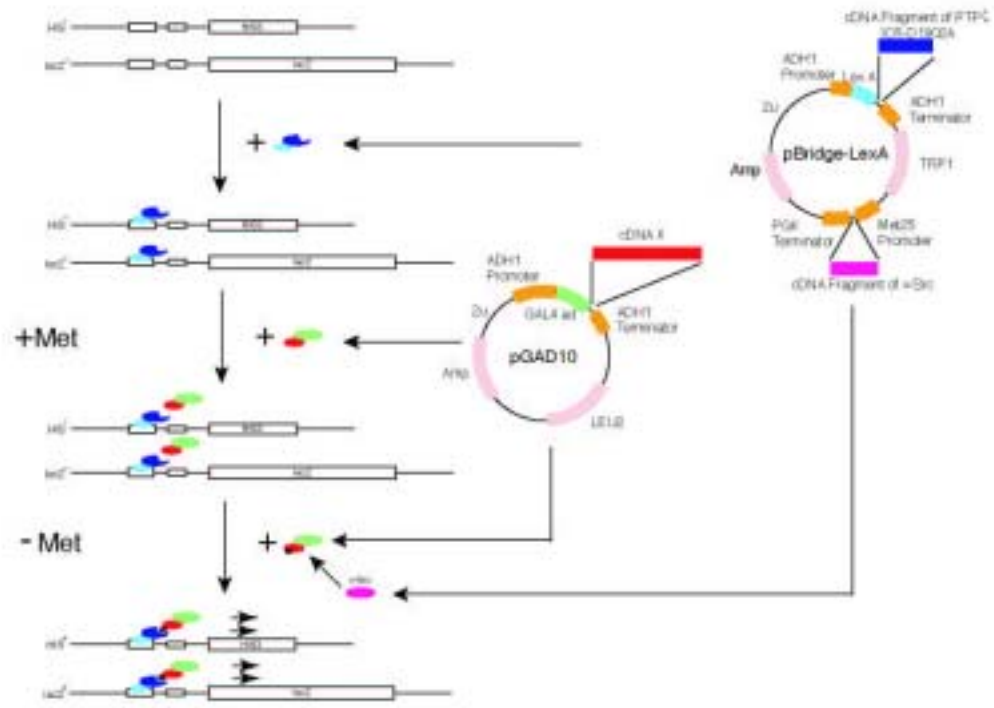

Fig. 1 Schematic diagram of the Yeast substrate-trapping system

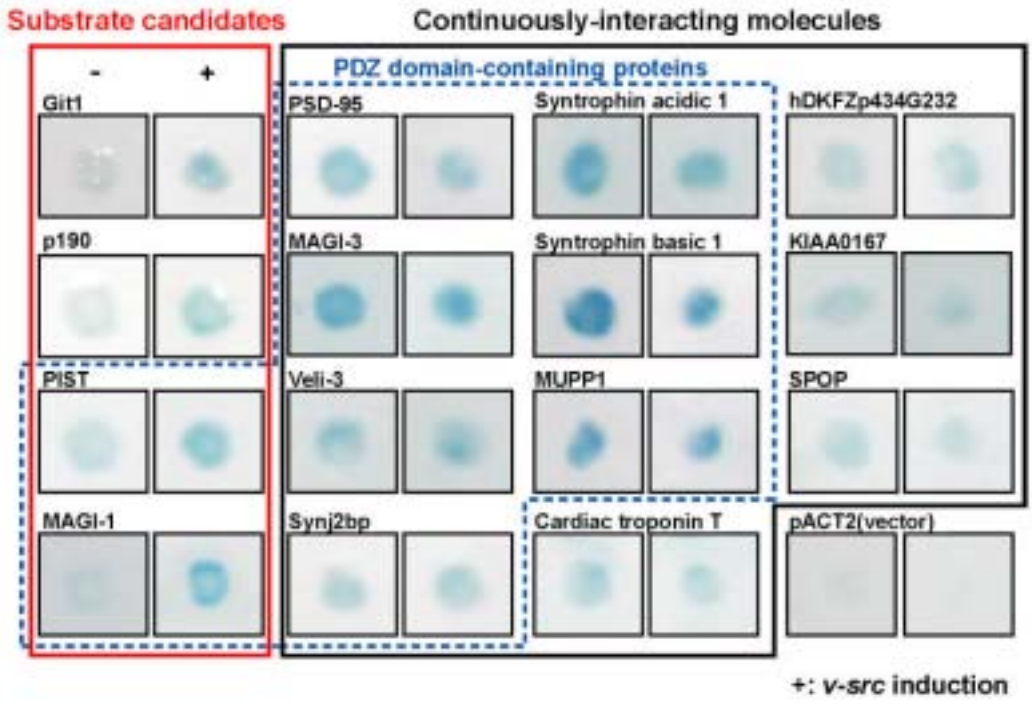

Fig. 2 Colony-lift filter assay

Discussion & Summary

In contrast to PTKs, our understanding of the biological functions of PTPs has been limited due to a lack of information concerning the physiological substrates for most PTPs. To fully understand the biological functions of PTPs, the identification of each of their substrates is essential. The yeast substrate-trapping system can be applied to all members of the PTP family and identify substrate candidates together with continuously interacting molecules. The identification of PTP substrates and elucidation of cellular phenotypes correlated with the tyrosine phosphorylation levels of substrates will lead to a full understanding of the physiological roles of individual PTPs. We believe that the clones isolated by the yeast substrate-trapping system will be promising candidates for substrates and that they will shed light on the Ptprz-signaling cascade in gastric cells underlying the gastric ulcer induction.

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1) Biological Sciences
1-4) Developmental Biology

Regulation of spermatogenesis by cytoplasmic polyadenylation of mRNAs in male haploid germ cells

Shin-ichi Kashiwabara
Graduate School of Life and Environmental Sciences, University of Tsukuba
kashiwa@sakura.cc.tsukuba.ac.jp

Introduction

A testis-specific cytoplasmic poly(A) polymerase, TPAP, is involved in poly(A) tail extension of specific mRNAs, including TRF2, TFIIAγ, and TAF10, in the cytoplasm of haploid, round spermatids. Mice lacking TPAP resulted in spermatogenesis arrest at the round spermatid stage due to the severely reduced expression of haploid-specific genes required for morphogenesis of germ cells[1]. The purpose of this study is to elucidate the molecular basis of spermatogenesis arrest caused by TPAP deficiency.

Results

1. Transgenic expression of TPAP in the wild-type and TPAP-deficient mice[2] --- Transgenic mice overexpressing TPAP showed normal spermatogenesis and fertility. The poly(A) tail length of substrate mRNAs was also unaffected. Introduction of TPAP transgenes in the TPAP-deficient mice complemented both the incomplete elongation of the poly(A) tails of substrate mRNAs, and reduced expression of haploid-specific genes (Fig. 1A), resulting in the resumption of normal spermatogenesis and fertility (Fig. 1B).

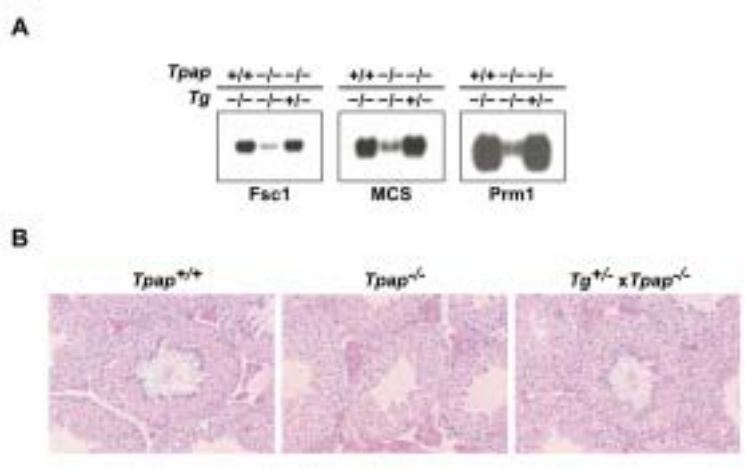

Fig. 1. Transgenic expression of TPAP in TPAP-deficient mice.

2. Mechanism of poly(A) tail extension of specific mRNAs in the cytoplasm of round spermatids --- CPEB2, a paralogue of CPEB1, which is implicated in cytoplasmic polyadenylation of maternal mRNAs during oocyte maturation, and CPSF160 showed similar expression patterns with TPAP during spermatogenesis. In addition, GST-pull down assay revealed that TPAP interacts with CPEB2 through CPSF160.

3. Nuclear transport of TAF10 in round spermatids --- In testes lacking TPAP, TAF10 was insufficiently transported into the nucleus of germ cells. Thus, mRNA encoding a transporter protein of TAF10 may be a true substrate of TPAP. Immunoprecipitation of testicular extracts by anti-TAF10 antibodies identified KIF2β and KIF2γ, which are splicing isoforms of motor protein KIF2A. The mRNAs encoding KIF2β and KIF2γ were overexpressed in testis. Although the expression levels of these two mRNAs in mice lacking TPAP were the same as those in the wild-type mice, the poly(A) tail extension of the mRNAs was incomplete, and translational efficiency was reduced.

4. Profiling of proteins affected by TPAP deficiency --- When protein extracts of round spermatids of the wild-type and TPAP-deficient mice were compared by two dimentional gel electrophoresis, at least two proteins, sorbitol dehydrogenase and adenylate kinase, were reduced in round spermatids of mice lacking TPAP. However, the mRNA levels of the two proteins were the same between the wild-type and mutant mice, suggesting that poly(A) tail extension of some mRNAs in round spermatids is required for normal translation.

Discussion & Summary

Transgenic expression of TPAP in the wild-type testes implys the presence of a regulatory mechanism(s) defining the extent and specificity of the cytoplasmic mRNA polyadenylation. Resumption of normal spermatogenesis and fertility in TPAP-deficient mice with TPAP transgenes conclusively demonstrates the essential role of TPAP in spermatogegesis. The mechanism of poly(A) tail extension by TPAP is suggested to be similar to that in oocyte maturation. The reduced expression of haploid-specific genes due to inefficient nuclear transport of TAF10 in the mutant mice may be attributed to incomplete poly(A) tail extension of mRNAs for KIF2A splicing isoforms. By proteomic approach, it is also suggested that proper translation of some mRNAs in round spermatids requires poly(A) tail elongation.

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1) Biological Sciences
1-4) Developmental Biology

Mechanism of the functional neuronal histogenesis in the developing brain

Kazunori Nakajima
Department of Anatomy, Keio University School of Medicine
kazunori@sc.itc.keio.ac.jp

Introduction

In the developing mouse cerebral cortex, gamma-aminobutyric acid (GABA)ergic neurons and non-GABAergic neurons arise in distinct places and migrate into the cortical plate via different pathways. Although the "inside-out" alignment of projection neurons in the cortex has been thoroughly analyzed, the pattern of interneuron alignment is not well understood.

Results

To investigate the birth-date dependent alignment of GABAergic neurons in the mouse visual cortex, we initially analyzed the birth-date dependent alignment of GABA+/BrdU+ cells quantitatively and compared the distributions of GABA+/BrdU+ cells to those of GABA-/BrdU+ cells (projection neurons).

The distributions of GABA+/BrdU+ cells and GABA-/BrdU+ cells were analyzed by dividing the cortex into 10 bins positioned parallel to the brain surface. The correspondence between each bin and the cortical layers was determined by staining the sections with anti-NF and anti-NeuN. Bin 10 nearly corresponded to layer I, while bin 1 contained the lowest part of layer VI and the white matter.

The distribution of E12.5 GABA+/BrdU+ cells (n=2321 cells, 5 animals) showed two peaks around bins 4 and 9; the following significant differences were obtained between the bins: 1-2 ($p=0.0007$), 2-3 ($p=0.011$), 3-4 ($p=0.02$), 4-5 ($p=0.0099$), 5-6 ($p=0.001$), 7-8 ($p=0.03$), 8-9 ($p=0.006$), and 9-10 ($p=0.008$). The distribution of E12.5 GABA-/BrdU+ cells (n=1229 cells, 5 animals) showed only one peak around bin 2; the following significant differences were obtained between the bins: 1-2 ($p=0.0000007$), 2-3 ($p=0.02$), 5-6 ($p=0.006$), 6-7 ($p=0.02$), 7-8 ($p=0.01$), and 8-9 ($p=0.02$). Thus, in P9.5 mice, GABAergic neurons born on E12.5 were distributed in the cortex around two peak locations, a major peak near layer V and a minor peak near layer II/III. On the other hand, non-GABAergic neurons from the same day were only distributed around one peak location, in layer VI. Interestingly, the major peak location of the GABA+/BrdU+ cells was always slightly above the

peak location of the GABA-/BrdU+ cells in all 5 animals analyzed in this study. In the most superficial quarter of the cortex, the proportion of GABA+/BrdU+ cells among all BrdU-positive cells was 98.9±0.68 % (n=537 cells, 5 animals). This result indicates that most of the BrdU-positive cells in this area are GABAergic neurons.

In contrast, the E15.5 GABA+/BrdU+ cells (n=1526 cells, 3 animals) exhibited only one peak location around bin 9, with the following significant differences between bins: 6-7 ($p=0.04$), 7-8 ($p=0.03$), and 9-10 ($p=0.003$). E15.5 GABA-/BrdU+ cells (n=595 cells, 3 animals) also showed only one peak location around bin 9, with the following significant differences obtained between bins: 6-7 ($p=0.03$), 7-8 ($p=0.03$), and 9-10 ($p=0.002$). These results indicate that both GABAergic and non-GABAergic neurons born on E15.5 are aligned around a common, single peak location near layer II/III in the cortex.

To investigate whether the two peak locations of GABA+/BrdU+ cells born on E12.5 still exist in the mature visual cortex, we performed double-immunostaining with anti-GABA and anti-BrdU in P30 mice and displayed the results as a histogram. The distributions of E12.5 GABA+/BrdU+ cells in P30 mice (n=478 cells, 3 animals) showed a two-peak pattern, which was very similar to that observed in P9.5 mice

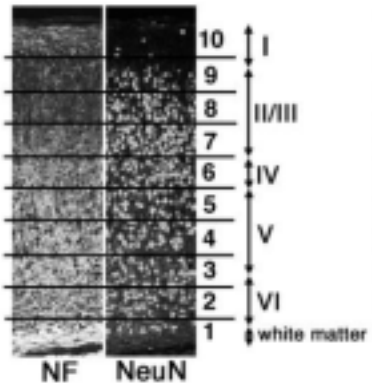
To investigate whether the two peak locations of E12.5 GABAergic neurons differ according to their GABAergic neuron subtypes, we then analyzed the subtypes of E12.5 GABAergic neurons. The calretinin-positive cells were located preferentially at the minor peak location near layer II/III, whereas somatostatin-positive cells were located preferentially at the major peak location near layer V.

Discussion & Summary

In conclusion, the present results show that, in the mouse visual cortex, 1) E12.5 GABAergic neurons are distributed around two peak locations, while non-GABAergic E12.5 neurons are distributed around only one peak location, 2) The major peak location of GABAergic E12.5 neurons was always slightly above the peak location of the non-GABAergic E12.5 neurons, 3) Calretinin-positive E12.5 cells did not distribute similarly to the projection neurons with the same time of birth. These results suggest that the birth-date dependent alignment of GABAergic neurons occurs in a different pattern from that of non-GABAergic neurons in the mouse visual cortex. Further analysis of how the termination of interneuron migration is regulated is essential to understanding cortical histogenesis.

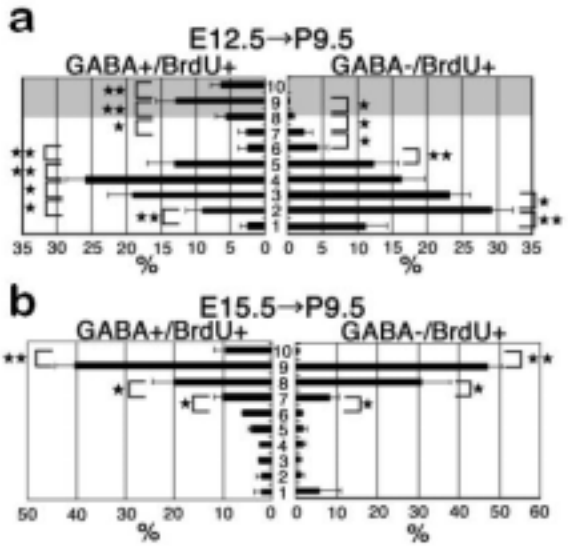
Figures & Tables

Quantitative analysis



The visual cortex in coronal sections obtained approximately 500 μm caudal to the caudal end of the corpus callosum was equally divided into 10 bins positioned parallel to the brain surface (from the ventricular surface to the pial surface); the number of cells in each bin was then counted. The correspondence between each bin and the cortical layers was determined by staining P9.5 coronal sections with anti-NF and anti-NeuN.Data were analyzed statistically using the t-test.

Histograms of the distributions of GABA+/BrdU+ cells and GABA-/BrdU+ cells in P9.5 mice



Coronal sections from P9.5 mice that had received a single pulse of BrdU on E12.5 (a) or E15.5 (b) were double-immunostained with anti-GABA and anti-BrdU. In both histograms, the x-axis depicts the percentage of cells in each bin among all counted cells from bin 1 through 10 and the y-axis depicts the bin number. Significant differences between the bins are indicated by one star ($p<0.05$) or two stars ($p<0.01$).

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Birth-date dependent alignment of GABAergic neurons occurs in a different pattern from that of non-GABAergic neurons in the developing mouse visual cortex. Masato Yozu, Hidenori Tabata, and Kazunori Nakajima. ***Neurosci. Res.*** 49 (4), 395-403 (2004).

1) Biological Sciences
1-4) Developmental Biology

Development of the neural network and the blood vessels

Wataru Shoji
Institute of Development, Aging, and Cancer, Tohoku University
wshoji@idac.tohoku.ac.jp

Introduction

During early embryogenesis, development of nervous system and blood vessels suggest a variety of interaction. In some cases, they run together with close contact, whereas in other cases, they develop exclusively. By chemical mutagenesis for zebrafish and following F3 screening, we obtained a mutant that exhibits defect in both nervous and blood vessel network.

Results

Zebrafish mutant *mujina* was initially identified with missing posterior lateral line ganglion at 3 dpf (days post-fertilization). The lateral line nerve plays an important role in the escape reflex circuit by transmitting sensory information from the neuromasts on the surface of the fish larva. The neuromasts are equipped with hair cells to detect water vibration that implies approaching predators. Interestingly, development of the posterior lateral line nerve and neuromasts are intimately associated (Figure). They were born together as cranial ganglia just posterior to the otocyst, and differentiate as neurons and neuromast primordium. When peripheral axons begin extension from the neurons of the ganglion, the neuromast primordium migrates with the growth cones, the leading edge of the axons. The primordium migrates from the head to the tail and periodically deposits group of cells that give rise to the neuromasts. In corresponding to the deposition, some axons cease extension to innervate the neuromasts, which ensures rigid association between axons and their targets.

In *mujina* mutant, the lateral line neurons are missing at 3 dpf. We examined developmental process of the neurons and the primordium in the mutant. The course described above, from cranial ganglia to deposition of the neuromast occurred normally. The lateral line axons extended in a normal pattern until at least 1.5 dpf. Defect was seen first on 2 dpf when hair cells should differentiate in the center of the neuromast. In the mutant embryos, the lateral line neurons begin to degenerate and the hair cells do not appear in the neuromast. We are now investigating relation between the hair cell defect and the neuronal degeneration by cellular and molecular analysis.

In addition to the defect in the nervous system, we found that blood vessel system also decay in the

mutant embryos. The blood circulation starts on 1 dpf and appeared normal by 2 dpf. Subsequently at 3 dpf, the mutant suffers with brain infarction by emborization at the primordial midbrain channel and middle cerebral vein. We are now investigating more closely for the blood vessel defects.
Thus, identification of mutated gene for *mujina* may elucidate new developmental process governing both neural and blood vessel development. Our intentional effort for positional cloning recently picked up closest microsatellite marker within 0.4 cM.

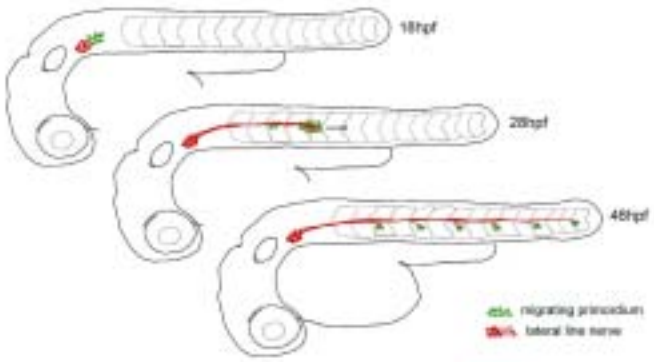

Figure: Development of the lateral line organ

Discussion & Summary

We characterized a new zebrafish mutant, *mujina,* which exhibits defects in neurons and blood vessels.
In *mujina* embryos, lateral line neuron develops once normally but dies when its target hair cells failed to differentiate. In other vertebrates, it is known that peripheral sensory axon requires trophic support from its target organs. Based upon this idea, we are hypothesizing and examining if hair cells may support lateral line axons or vice versa. Interestingly, blood vessels and circulation are once established in normal, then decays later. This may imply a common defect in neurons and blood vessel development in the mutant. Cloning of the responsible gene should be next step of this study.

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1) Biological Sciences
1-5) Physioiogy

Involvement of Na,K-ATPase in Respiratory Rhythm Generation during Perinatal Period.

Keiko Ikeda
Jichi Medical School, Center for Molecular Medicine, Division of .Biology
kikeda@jichi.ac.jp

Introduction

The sodium pump is the enzyme responsible for the maintenance of Na^+ and K^+ gradient across the cell membrane. Among four isoforms of the catalytic a subunits, α2 subunit gene (*Atp1a2*) is specifically expressed in excitable tissues. Mice homozygous for the α2 mutation survive when they are born under spontaneous labor, but died just after birth when they are born through cesarean section. The aim of this research is to clarify why *Atp1a2* knockout homozygotes survive when born under spontaneous labor, by comparing respiratory neural network activities between the wild-type and the cesarean delivered neonatal mice.

Results

The α2 subunit is abundantly expressed in neurons in the brainstem, including respiratory center. The neural network for respiratory rhythm is genetically programmed and developed *in utero*, and newborn mammals breathe immediately after birth. This neural circuit is located rostrocaudally in the ventrolateral medulla (VLM) of the lower brainstem. It produces rhythmic activity, which is transmitted to spinal motoneurons to generate a periodic contraction of respiratory muscles, such as diaphragm. As a measure of respiratory output, we recorded the respiratory activity from the C4, one of phrenic nerve motoneurons, in an *en bloc* brainstem-spinal cord preparation (figure 1) isolated from E18.5-E19 mice through cesarean section. During the recording, the brainstem-spinal cord preparation was superfused with oxygenated modified Krebs' solution at 26 ° C. Spontaneous rhythmic discharges related to inspiration were recorded in the wild-type and heterozygous mice (n = 5), but were completely absent in the homozygous mice (n = 3). We also recorded C4 in the brainstem-spinal cord preparation from fetuses, which were born under spontaneous labor (within 9 hours after birth). In this case, we observed C4 spontaneous discharges not only in the wild -type and heterozygous mice (n = 3), but also in the homozygous mice (n = 3). These results were consistent with the observation that the homozygous mice spontaneously breathe under spontaneous labor, but completely lacked breathing through cesarean section, leading their immediate death after birth.

It has been known that some neuromodulators are released in the fetal brain during passing through the mother's birth canal. Therefore, we applied various neuromodulators to the brainstem-spinal cord preparation by adding them in the superfusate. We found that adrenaline and substance-P induced more regular and strong respiratory rhythmic discharges in the case of the wild-type/heterozygous mice. These chemicals also induced regular inspiratory bursts in the homozygous mice. The results strongly indicated that exposure of neuromodulators to the respiratory rhythm generator neurons during spontaneous delivery are important (but not essential in the wild-type/heterozygous mice) for the establishment and maturation of autonomous respiratory rhythm generation in the central nervous system.

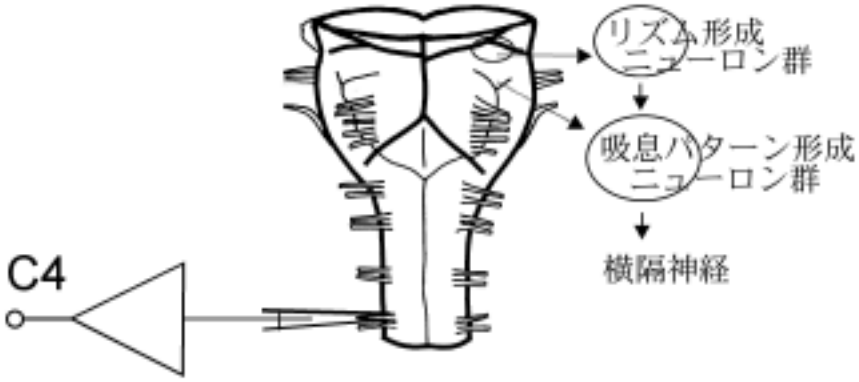
To obtain insights into the underlying defects in the homozygous rhythm generator neurons, we performed optical recordings to visualize respiratory neural activity from the ventral surface using voltage-sensitive dye (50 μg/ml Di-2-ANEPEQ; Molecular Probes, Eugene, OR) and the system of MiCAM01 (Brain Vision Inc.)(Ref.1). We compared the optical signals during adrenaline application between the wild-type and homozygous mice born through cesarean section. Strong fluorescence changes at two regions of medulla (one is rhythm generator neuron complex and the other is pattern generator neuron complex=preBoetinger. complex), that indicate electrical burst, were detected close to the peak of C4 inspiratory activity. The fluorescence decreased (i.e., depolarization) in the wild-type (n = 3), heterozygous (n = 6), and homozygous (n = 5) mice in the pattern generator neuron complex. We observed an increase in the fluorescence (i.e., hyperpolarization) in the wild-type and heterozygous rhythm generator neuron complex. In contrast, we observed a decrease in the fluorescence (i.e., depolarization) in the homozygous mice. These results suggest impairment of the inhibitory neural activity in the ventral medulla and its transformation to excitatory activity in the homozygous mice. To get insight into the molecular basis for the transformation in the homozygous mice, we measured intracellular chloride ion concentration in the rhythm generator neurons using gramicidin patch clamp techniques. We found that higher intracellular chloride ion concentration in the homozygous mice neurons when born through cesarean section than in the wild-type mice.

Discussion & Summary

Using an en bloc brainstem-spinal cord preparation, we observed that Atp1a2 knockout homozygous mice showed spontaneous respiratory rhythm neural activity when they are born under spontaneous labor, but lacked through cesarean section. We found that adrenaline and substance-P, that are released in the fetus brain during passing through the mother's birth canal, induced regular inspiratory bursts in the homozygous preparation. We suggest that the neuromodulators such as adrenaline and substance-P are important for the establishment and maturation of respiratory rhythm activity during perinatal period. The impairment of the inhibitory neural activity in the ventral

medulla and its transformation to excitatory activity in the homozygotes was due to the higher intracellular chloride ion concentration, resulting the respiratory defect in the homozygous mice when born through cesarean section.

Figures & Tables



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1) Biological Sciences
1-6) Pharmacology

Studies on a Role for PRIP in the Function of $GABA_A$ Receptor

Masato Hirata
Kyushu University, Faculty of Dental Scie
Hirata1@dent.kyushu-u.ac.jp

Introduction

PRIP (PLC-related inactive protein) was first identified as an inositol 1,4,5-trisphosphate binding protein. An attempt to explore interacting molecule(s) with PRIP revealed GABARAP (GABA receptor associated protein) and PP1 (protein phosphatsae-1), which prompted us to examine the possible involvement of PRIP in GABA(A) receptor signaling at several aspects.

Results

Biochemical and Behavioral Analysis of PRIP Double Knock-Out Mouse:

Our recent studies revealed that a novel $Ins(1,4,5)P_3$ binding protein, PRIP-1, regulates the assembly and/or function of $GABA_A$ receptors as assessed by analyzing the PRIP-1 knockout (KO) mice. There are two PRIP genes in mammals, thus the roles of PRIP-1 might partly be substituted by PRIP-2 in the PRIP-1 KO mice. In the present study, we produced the PRIP-1 and -2 double knockout (DKO) mice and examined the roles for PRIP molecules in regulating the assembly of $GABA_A$ receptors. The expression of α, β, γ and d subunits of $GABA_A$ receptors in the brain of DKO mice were slightly higher than those of wild type (WT) mice at both protein and gene levels, as analyzed by western blots and semi-quantitative RT-PCR. Ligand binding assays using [^{3}H]muscimol, a GABA agonist, and [^{3}H]Ro15-1788, a diazepam antagonist, showed that the cell surface expression levels of GABA binding site (α/β subunit interface) were increased in DKO, but the numbers of diazepam binding site (α/γ2 subunit interface) were reduced in the DKO mice, compared to WT mice. Diazepam sensitivity whose target is α/γ2 subunit interface, was reduced in DKO mice in electrophysiological and behavioral analysis. These findings indicate that PRIP are involved in trafficking of $GABA_A$ receptors to cell surface membrane.

Possible Involvement of PRIP in the Regulation of β-subunit Phosphorylation of GABA(A) Receptors:

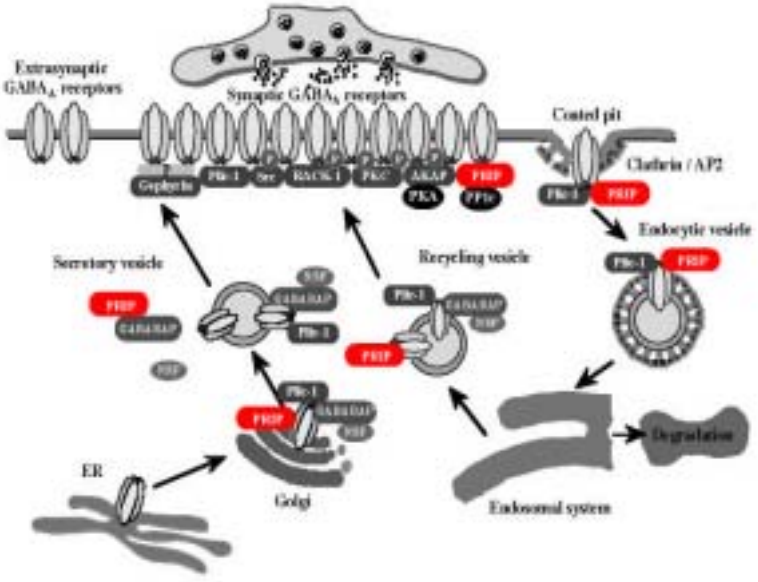
We have further examined the role for PRIP-1, which binds and inactivates protein phosphatase 1α (PP1α), in facilitating $GABA_A$ receptor phospho-dependent regulation, using PRIP-1 knock-out (*PRIP-1*$^{-/-}$) mice. In wild-type animals, robust phosphorylation and functional modulation of $GABA_A$

receptors containing the β3 subunits by cAMP-dependent protein kinase (PKA) was evident, which was greatly diminished in *PRIP-1*$^{-/-}$ mice. *PRIP-1*$^{-/-}$ mice exhibited equivalent levels of PKA activity compared to controls, and equivalent levels of PP1α at the protein level. However, the PP1α activity in *PRIP-1*$^{-/-}$ mice was enhanced compared to controls. Inhibiting phosphatase activity in *PRIP-1*$^{-/-}$ mice restored PKA mediated phosphorylation of $GABA_A$ receptor β3 subunit to levels seen in control animals. In addition, PRIP-1 was able to interact directly with $GABA_A$ receptor β subunits, and moreover, these proteins were found to be PP1a substrates. Finally, PRIP-1 was phosphorylated on threonine 94 and serine 96, and the phosphorylation of threonine 94 facilitated the dissociation of PP1α/PRIP-1 complexes, providing a local mechanism for the activation PP1a. Together, these results suggest an essential role for PRIP-1 in controlling $GABA_A$ receptor activity, *via* regulating subunit phosphorylation and thereby the efficacy of neuronal inhibition mediated by these receptors.

Discussion & Summary

All these results strongly indicate the involvement of PRIP in GABA(A) receptor signaling at many aspects including the transcription and expression of several subunit components of the receptors, the cell-surface expression of pentameric GABA(A) receptors, the phospho-dependent modulation of the receptors, and the internalization of the receptors once worked. Therefore, several impairments regarding GABA(A) receptor function were observed in PRIP-DKO mice compared to WT mice by electrophysiological and behavioral analysis. However, the molecular basis explaining these impairments are largely unknown, which is our future projects

Figures & Tables



1) Biological Sciences
1-7) Plant Biology

Molecular Genetic Studies on Maternal Inheritance of Chloroplasts Using a Model Plant System

Wataru Sakamoto
Research Institute for Bioresources, OkayamaUniversity
saka@rib.okayama-u.ac.jp

Introduction

Plastid and mitochondrion are the organelles that contain their own genomes. These "organelle genomes" are different from the nuclear genome in that they are 1) multiplicated in plant cells and 2) inherited from either parent. On fertilization, nuclear fusion takes place and one set of chromosomes from each parent is inherited in a zygote. However, it is not the case in chloroplasts and mitochondria: an organelle genome from either parent tends to be excluded, and the genome from the other parent is uniformally maintained. Recent survey implicated that about 20 % of flowering plants inherits plastid genome paternally (i.e. from pollen), while 80% inherits it maternally (i.e. through egg cell) (Zhang et al., 2003). This phenomenon, referred to as uniparental inheritance, has been revealed by genetic and cytological analyses, but little is understood on its molecular mechanism in higher plants. This study is to establish the experimental system that could allow us to study maternal inheritance at the molecular level.

Results

At the beginning of this research project, I proposed to conduct the following three experiments.

1) Transformation of the chloroplast genome by introducing a foreign gene (*aadA*) that confers resistance to an antibiotics spectinomycin (spec)
2) Spec resistance as a selection marker for investigating uniparental inheritance of plastid DNA
3) Isolation of a mutant in meternal inheritance in *Arabidopsis*

The first two projects concern the introduction of foreign genes into *Arabidopsis* chloroplasts in order to give chloroplast a genetically scorable marker. So far, transformation of chloroplast genomes has been reported only in limited plant species. We reasoned that the longer flanking region included in the transformation construct may improve the transformation efficiency in Arabidopsis. As an effort to design the DNA construct for chloroplast transformation, using the region between

rbcL and *atpE*, is still underway, we will test these construct for transformation in the future.

In the third project of screening mutants in maternal inheritance, we examined approximately 4,000 EMS-mutagenized M2 individual plants. We established a 'pollen-squash' method, by which we were able to fix pollens and simultaneously stain DNA by fluorescent dye DAPI (Sodmergen et al., 2002). In the wild-type pollens, one relatively large vegetative nucleus and two tightly packed sperm nuclei can be stained. In this method, however, DAPI stains corresponding to organelle DNAs are not detectable, suggesting that organelle DNAs diminish during pollen development.

In our screening, we were able to isolate putative mutants in which organelle DNAs could be detected by DAPI. So far, we isolated five mutants and classified into three categories. The first category included #1411 and #2214, and these mutants showed substantial intensities of fluorescent granules in the pollen cytoplasm (Figure 1). Although our initial genetic analysis (in progress) indicates that it is heritable, appearance of this phenotype is not 100% and variable in individuals. The mutant occasionally showed abnormal morphology in both vegetative and reproductive tissues.

The second category included #2084, in which the fluorescence is not as strong as #1411 but most of the pollens show this phenotype (Figure 1). The third category included #2213 and #2041, in which the DAPI fluorescence is similar to #1411, but the pollens looked immature and therefore we considered that pollen development is defective in this category. Because DAPI stains can be sometimes observed in the wild type at the younger stage of pollen development, it was assumed that pollen development itself might be deficient.

Given that the mutations obtained by EMS mutagenesis are likely recessive ones, our genetic screening suggests that a positive mechanism to eliminate organelle DNAs during pollen development exists. Further characterization of the granule bodies detected by DAPI stain may be necessary to confirm the containment of organelle DNAs in these mutants. Our genetic analysis is under progress to identify the genetic factors affecting maternal inheritance in higher plants.

In addition to the afore mentioned, we also isolated a mutant that has altered nucleoploidity. This mutant, #1415, segregated normal tri-nuclear pollen grains and abnormal di-nuclear pollen grains to 1:1. Our genetic analysis showed that the di-nuclear phenotype was transmitted only maternally, and the transmission rate was always 1:1. These results suggest that pollen mitosis II is impaired in #1415. Further characterization indicated that #1415 is an allele of *duo* mutant, which has been recently identified by Dr. Twell's group in U.K.

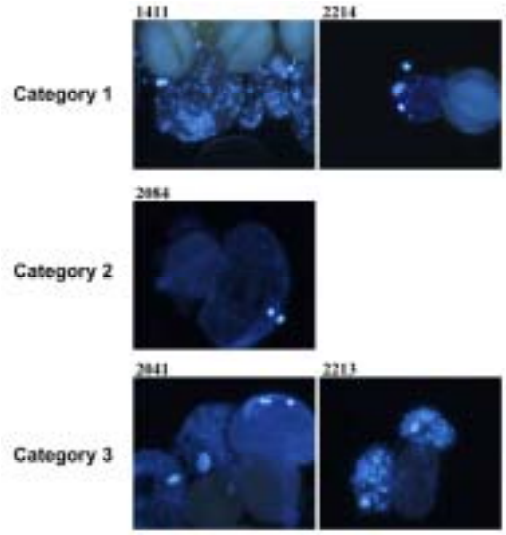


Figure1.
Arabidopsis mutants of altered organelle DNA containment in pollens. Shown are the squashed pollens simultaneously fixed and stained by DAPI (see Results).

Discussion & Summary

This work has been conducted in order to establish the experimental system that could allow us to study maternal inheritance at the molecular level. To this end, we utilize the model plant *Arabidopsis thaliana* to study and identify factors involved in maternal inheritance molecular genetically. Previous studies suggest that amount of organelle DNAs in pollens decreases during pollen development. Such diminish of organelle DNA could follow a regulatory mechanism that plays an important role in maternal inheritance in higher plants. We reasoned that some mutants can be obtained by EMS mutagenesis, based on these assumptions. Screening of the possible mutants by our pollen squash method indeed led us to isolate some mutants in which the organelle DNA containment was altered. Further characterization of these mutant, in terms of genetic analysis and subsequent map-based cloning, may enable to understand maternal inheritance in more detail.

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1) Biological Sciences
1-7) Plant Biology

Molecular genetic analysis of light signal transduction from the N-terminal domain of phytochrome B

Tomonao Matsushita
Department of Botany, Graduate School of Science, Kyoto University
matsushita@physiol.bot.kyoto-u.ac.jp

Introduction

Phytochrome is a major informational photoreceptor in plants. The phytochrome molecule has two domains: the N-terminal photosensory domain and the C-terminal domain which contains signaling motifs such as a kinase domain. The latter has been widely believed to transduce the signal to downstream components. Our recent study clearly demonstrated that the N-terminal domain, but not the C-terminal domain, of *Arabidopsis* phytochrome B (phyB) transduces the signal (1, 2), which forces us to reconsider our view on phytochrome. However, nothing has been known about the molecular mechanism of this phenomenon. In this study, we took a genetic screening strategy to address the following two points: 1) which amino acid residue within the N-terminal domain of phyB is directly involved in signal transfer? and 2) what is the downstream signaling component responsible for it?

Results & Discussion

In order to elucidate the molecular mechanism of the N-terminal domain signaling of phyB, we mutagenized transgenic *Arabidopsis* expressing the N-terminal domain of phyB and screened for long hypocotyl mutants in red light. We have so far screened at least 1,000,000 M2 seedlings which is derived from approximately 200,000 M1 plants. Then, we analyzed intragenic missense mutations and extragenic mutations to identify the amino acid residues critical for signaling within phyB N-terminal domain and novel downstream components mediating phyB signaling, respectively.

1) Analysis of intragenic missense mutations within the N-terminal domain of phyB

We identified 16 missense mutations within phyB N-terminal domain through the genetic screen described above. Although these mutations distributed throughout the N-terminal domain of phyB, some of them should affect spectral properties of phyB molecule instead of being directly involved in signaling, because it has been established that the N-terminal domain of phytochrome is required and sufficient for light perception. Therefore, we examined in detail the effects of these missense

mutations on the spectral properties of phyB N-terminal domain fragment. As a result, we identified 6 missense mutations that exhibited normal photochemical properties, which means these mutations abolished signaling competence of phyB without affecting its spectral nature. Interestingly, they fell within a relatively small region with significant homology to PAS domain, suggesting that this site is directly involved in phyB signal transduction. More experiments are needed to confirm this possibility, and they are currently in progress.

2) Analysis of extragenic mutations

We isolated 16 extragenic mutant lines to date and designated them as *pns* (*phyB N-terminal domain signaling*) mutants. We first examined their responses to various wavelengths of light, and the results of the photophysiological study indicated that *pns* mutants can be classified roughly into two categories: phyB pathway-specific ones that exhibit long hypocotyl only under red light, and phyB pathway-nonspecific ones with long hypocotyl also under other wavelengths of light than red light. Since our interest focuses on phyB signal transduction, we decided to further analyze only the former class of *pns* mutants. Then we performed rough mapping of three such *pns* mutants, and at least one of them was mapped in a region not known to contain any other loci involved in photomorphogenesis. We plan to continue rough mapping of more *pns* mutants and then to begin map-based cloning of the responsible genes for those *pns* mutants which are more likely to represent novel photomorphogenic loci.

We believe these two different types of analyses from inside and outside of the phyB molecule will give us a clue to unravel the long-standing mystery of phytochrome signal transduction.

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2) Medical Sciences

2-1) Immune System

Molecular Mechanisms of immune defenses against infection by IgM

Shibuya Akira

Department of Immunology, Institute of Basic Medical Sciences, University of Tsukuba

ashibuya@md.tsukuba.ac.jp

Introduction

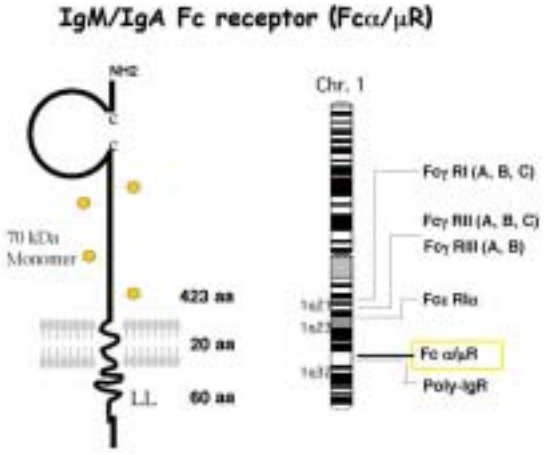
IgM is the first antibody to be produced in a humoral immune response and plays an important role in the primary stage of immunity. In addition, IgM is a major iostype of natural antibodies, which already exist in innate animal and are involved in the prevention of pathogen dissemination to vital organs. However, how this "innate type" of antibody are involved in immunity has not necessarily been elucidated.

Results

We previously identified an Fc receptor for IgA and IgM, designated, Fcα/μR which is a ~70 kDa monomeric protein containing a immunoglobulin-like domain in the extracellular region (**Fig 1**). No tyrosines are present in the cytoplasmic domain, but there is a di-leucine motif in the mouse Fcα/μR that may be involved in receptor internalization. These genes were mapped to syntenic regions of mouse chromosome 1 (1F) and human chromosome 1 (1q32.3), near several other Fc receptors, including Fcγ receptors I, II, and III, Fcε receptor, and the polymeric Ig receptor (**Fig 1**).

The Fcα/μR is constitutively expressed on the majority of B lymphocytes, including follicular, marginal and B1 B cells. The Fcα/μR mediates endocytosis *Staphylococcus aureus* /anti-S. aureus IgM antibody immune complexes by primary B-lymphocytes. We have recently found that the Fcα/μR is also expressed on follicular dendritic cells (FDC). To analyze in vivo role of the Fcα/μR, we have recently generated Fcα/μR -deficient and transgenic mice. These mice exhibited normal level of antibody subclasses, except of

Fig 1



IgA that is higher than that in control wild type mice.

While immunization with T cell-dependent (TD) antigen NP-CG did not affect antibody production, enhanced production of IgM and IgG3 antibodies against T-independent (TID) antigen such as NP-Ficoll and NP-LPS was observed in Fcα/μR-/- mice. Moreover, we also observed that germinal center formation was augmented in response to TID, but not TD, antigen in Fcα/μR-/- mice. On the other hand, the Fcα/μR transgenic mice produced significantly lower amount of IgM and IgG3 than wild type mice in response to TID antigen.

Discussion & Summary

In the present study, we have demonstrated that Fcα/μR plays an important role for antibody production against TID antigen, suggesting that Fcα/μR is involved in innate immunity. However, it has remained undetermined how Fcα/μR mediates such an immune response. We have also shown an interesting result that FDC substantially express Fcα/μR. Because FDC plays an important role for antigen retention and presentation to B cells in germinal center and involved in germinal center formation and antibody production and maturation, Fcα/μR may regulate FDC function. Further studies should be required to clarify the in vivo rele of the Fcα/μR.

2) Medical Sciences

2-1) Immune System

Physiological roles of the cannabinoid receptors

Takayuki Sugiura

Faculty of Pharmaceutical Sciences, Teikyo University

sugiurat@pharm.teikyo-u.ac.jp

Introduction

2-Arachidonoylglycerol (2-AG) is an endogenous cannabinoid receptor ligand. Evidence is accumulating which shows that 2-AG is the true natural ligand for the cannabinoid receptors.

Two types of cannabinoid receptors have been identified to date. The CB1 receptor is abundantly expressed in the brain and assumed to be involved in the attenuation of neurotransmission. On the other hand, the physiological roles of the CB2 receptor, mainly expressed in several types of inflammatory cells and immunocompetent cells, have not yet been fully elucidated. In this study, we investigated possible pathophysiological roles of the CB2 receptor and 2-arachidonoylglycerol in acute inflammation in mouse ear.

Results

Precursors and the metabolic pathways for 2-AG.

In this study, we examined the effects of several metabolic inhibitors such a phosphatidylcholine-phospholipase C inhibitor, a diacylglycerol-lipase inhibitor, a phospholipase D inhibitor, a phosphatidic acid phosphohydrolase inhibitor, phosphatidylinositol- phospholipase C inhibitor on the level of 2-AG in inflamed mouse ear. We found that a phosphatidylcholine-phospholipase C inhibitor, a diacylglycerol-lipase inhibitor, a phospholipase D inhibitor and a phosphatidic acid phosphohydrolase inhibitor reduced both 2-AG formation and ear swelling. These results suggest that phosphatidylcholine is an important precursor molecule for 2-AG in inflamed tissues and that phosphatidylcholine-phospholipase C, diacylglycerol lipase, phospholipase D and phosphatidic acid phosphohydrolase are involved in the synthesis of 2-AG. We also found that the generation of various species of 2-monoacylglycerols such as 2-oleoylglycerol and 2-linoleoylglycerol in addition to 2-AG were generated in TPA-treated mouse ear. These observation support our hypothesis that 2-AG was generated from phosphatidylcholine. This is quite different from the case of the nervous system where 2-AG is generated mainly from inositol phospholipids and selective formation of 2-AG is observed.

Pathophysiological roles of 2-AG in inflammation.

We found that TPA-treatment of mouse ear induced the generation of 2-AG. The level of 2-AG was elevated by 4-fold. We also found that SR144528, a CB2 receptor antagonist, markedly reduced ear swelling induced by TPA. In contrast to SR144528, AM251, a CB1 receptor antagonist did not affect ear swelling. We also found that the level of LTB_4 in mouse ear was markedly elevated following the application of TPA. Notably, treatment of the ear with SR144528 blocked LTB_4 production triggered by TPA. Treatment of the ear with TPA also induced the infiltration of neutrophils. The TPA-induced infiltration of neutrophils was diminished by treatment of the ear with SR144528. Interestingly, the application of 2-AG or 2-AG ether, s stable analog of 2-AG, to mouse ear evoked ear swelling. On the other hand, anandamide, another endogenous cannabinoid receptor ligand did not induce ear swelling. These results strongly suggest that 2-AG rather than anandamide is crucially involved in acute inflammation induced by TPA. We also found that human eosinophils express high levels of the CB2 receptor. Notably, 2-AG induced the migration of human eosinophis. The migration evoked by 2-AG was abolished in the presence of SR144528, a CB2 receptor antagonist, or by pretreatment of the cells with pertussis toxin, suggesting that the CB2 receptor and Gi/o are involved in the 2-AG-induced migration. The migration of EoL-1 cells induced by 2-AG was suggested to be due to chemotaxis. In contrast to 2-AG, neither anandamide nor free arachidonic acid elicited the migration. These results suggest that the CB2 receptor and 2-AG are involved in allergic inflammation as well.

Effects of 2-AG on the vascular system.

We found that treatment of mouse ear with 2-AG induced the generation of nitric oxide. We also found that treatment of mouse ear with nitric oxide synthase inhibitors such as L-NMMA and 7-nitroindazole reduced ear swelling induced by 2-AG. L-NMMA and 7-nitroindazole also reduced TPA-induced ear swelling. These results strongly suggest that ear swelling induced by 2-AG or TPA is mainly due to the generation of nitric oxide. The mechanism by which 2-AG stimulated nitric oxide formation is not known. It is possible that p42/44 MAP kinase and p38 MAP kinase are involved in the production of nitric oxide in 2-AG- and TPA-treated mouse ear, because we found that U0126, an inhibitor of MEK that is upstream of p42/44 MAP kinase, and SB239063, a p38 MAP kinase inhibitor, markedly suppressed 2-AG-induced ear swelling (93 % inhibition and 87 % inhibition, respectively) and TPA-induced ear swelling (57 % inhibition and 81 % inhibition, respectively).

Discussion & Summary

The CB2 receptor is abundantly expressed in several types of inflammatory cells and immunocompetent cells and has been assumed to play an essential role in inflammatory reactions and immune responses. Nevertheless, its physiological function *in vivo* still remains an enigma. Based on our recent experimental results, we assume that the physiological roles of 2-AG in

inflammatory reactions are as follows: Various types of inflammatory cells and immunocompetent cells, such as macrophages, generate 2-AG when these cells are stimulated, and release 2-AG into the extracellular fluid. The released 2-AG then binds to the CB2 receptor expressed on other inflammatory cells or immunocompetent cells, induces the augmented production of chemokines such as IL-8 that induce the infiltration of neutrophils, and triggers direct migration of macrophages, eosinophils and natural killer cells, thereby stimulating inflammatory reactions. On the other hand, Δ^9-THC and SR144528, CB_2 receptor antagonists, interfere with the actions of the CB2 receptor and its physiological ligand 2-AG, thereby causing suppression of inflammatory reactions. Further detailed studies on the CB2 receptor and 2-AG are necessary for a thorough elucidation of the precise regulatory mechanisms of various inflammatory reactions.

Finally, 2-AG synthesis inhibitors and the CB2 receptor antagonists would be quite valuable as anti-inflammation drugs and immune suppressive agents.

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2) Medical Sciences

2-1) Immune System

Regulation of Inflammation and Obesity by MAP kinases

Masato OGATA

Mie university graduate school of medicine

ogata@doc.medic.mie-u.ac.jp

Introduction

It is well demonstrated that inflammation plays a critical role in the development of atherosclerosis. It is also reported that increased levels of CRP is a risk factor of Type 2 diabetes. These suggest the presence of common molecular mechanisms that link inflammation and metabolic syndrome. Mitogen-activated protein kinases (MAPKs) are critical components of signal transduction pathways. They are involved in the regulation of various cytokine productions and signal transductions of cytokine receptors. Cytokines and adipocytokines are regulators of inflammation and obesity. Thus, it is possible that MAPK is a critical regulator that is involved both in inflammation and obesity.

Results

There are three major subgroups of MAPK, namely, ERK, p38 and JNK. p38 can regulate its substrates, such as various transcription factors and kinases, through phosphorylation. p38 binds to substrates through the CD (common docking) site and the binding is very important for the phosphorylation and regulation of the substrates. We introduced mutations in the CD region of p38 and found that the sevenmaker (sem) type mutant of p38 ($p38^{sem}$) failed to associate with several substrates.

We have established $p38^{sem}$ knockin mice to study the physiological role of p38. It was reported that p38-deficient mice were embryonic lethal due to the defect in the placental development. In contrast, $p38^{sem}$ knockin mice are viable and fertile, demonstrating that the signaling pathway required for the placental development is not affected by the $p38^{sem}$ mutation. Since $p38^{sem}$ can still associate with limited number of substrates, activation of these substrates seems to be sufficient for the normal placental development. Though the $p38^{sem}$ knockin mice looked apparently normal, we found a profound defect in the production of TNF-a. When LPS was injected i.p. in the wild type mice, serum TNF-a level was increased transiently while little increase was observed in the $p38^{sem}$ knockin mice. Moreover, $p38^{sem}$ knockin mice were more resistant to LPS toxicity than wild type mice. These results clearly demonstrate that p38 is important for the LPS-induced TNF-a production in vivo. Furthermore, it is also shown that some signaling molecule(s) downstream of p38 is essential

for LPS-induced TNF-a production but not for the placental development.

Unexpectedly, the body weight of $p38^{sem}$ knockin mice was found to be lighter than that of wild type mice. p38 MAPK has been implicated in the differentiation of white adipose tissues and could be a modulator of the signaling cascade that promotes thermogenesis in brown adipose tissues. However, in vivo function of p38 remains to be determined since p38 knockout mice are embryonic lethal. We thus used $p38^{sem}$ knockin mice to delineate a potential role of p38 in adipose tissue metabolism and/or differentiation. As described above, $p38^{sem}$ knockin mice is a partial loss of function mutant and they are viable. Interestingly, low body weight and the reduction in white adipose tissue weight were observed in the $p38^{sem}$ knockin mice. In addition, the adipose tissue weight did not change very much with a high-fat diet. These results show that the p38 signaling pathway is involved in the regulation of adipose tissue weight probably through regulation of lipid metabolism and/or differentiation of adipocytes.

These observations demonstrate that p38 is involved in inflammation and obesity, suggesting p38 is a key molecule that links inflammation and metabolic syndrome.

Discussion & Summary

We have developed mutant mice of p38 MAPK and found that $p38^{sem}$ mutation results in the strong inhibition of proinflammatory cytokine productions as well as obesity. Thus, p38 could be involved in the regulation of inflammation and obesity as a key-signaling molecule connecting these conditions.

p38 is activated by LPS and cytokines such as IL-1, IL-6 and IL-10. p38 activation results in the production of proinflammatory cytokines such as TNF-a. Thus, the role of p38 in the regulation of inflammation is relatively well understood. In contrast, the mechanism that regulates obesity through p38 remains to be clarified. TNF-a is produced by not only inflammatory cells but also adipocytes and it induces insulin resistance. Thus, one possible mechanism could be regulation of soluble factors by p38. Our study suggests that inhibitors of p38 cascade could be candidate drugs to treat or prevent metabolic syndrome.

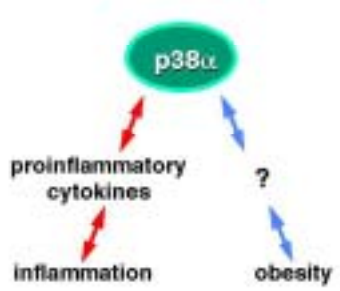

Fig. 1. Possible role of p38 MAPK in the linkage of inflammation and metabolic syndrome

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Takahiko Fujikawa, Yoshiko Mori, and Masato Ogata. Mutation of p38 MAPK in mice reduces adipose tissue weight and protects from high fat diet-induced obesity. (manuscript in preparation)

2) Medical Sciences
2-2) Central Nervous System

Analyses of molecular mechanisms of endoplasmic reticulum-localized Bcl-xL to ER stress induced cell death

Toshiyuki Nakagawa
Gifu University Graduate School of Medicine, Department of Neurobiology
tnakagaw@cc.gifu-u.ac.jp

Introduction

In response to functional disturbance, the ER sends distinct signals to the nucleus and cytosol resulting in the survival or demise of the cell. Such a response is indicative of ER stress, indicating the fundamental mechanisms of several disease, such as stroke, Alzheimer's disease, Diabetic Mellitus. Bcl-xL prevents apoptosis caused by proapoptotic Bcl-2 family proteins, such as Bax, Bak, and Bid. However, the function of antiapoptotic Bcl-2 family proteins at ER remains to be unknown. The objectives are to understand the molecular mechanisms and regulations of Bcl-xL against ER stress induced cell death and develop its therapeutic intervention.

Results

In C. elegans, the Apaf-1-like cell death activator CED-4 translocation from mitochondria to nuclear envelope may activate CED-3 procaspase to cause program cell death (Chen et al, 2000). However, it remains to be unknown the function at nuclear membrane and endoplasmic reticulum (ER) to activate caspases in mammal. To address this cascade, we examined the subcellular localization of caspases by western blotting using subcellular fractionation of PC12 cells. ER localizing proteins, such as caspase-12 (Nakagawa et al, 2000), p28Bap31, and grp78 (BiP), were detected membrane fractions. Cytosol localizing caspase, such as caspase-1, was detected in S100 fraction, but not in membranes fraction. Caspase-7 was detected in cytosolic fraction and membranes fraction. Next, We generated the stable PC12 cells constitutively expressed of mutant Bcl-xL. C-terminal transmembrane domain of membrane bound cytochrome b5 was substituted for that of Bcl-xL, which made Bcl-xL specifically translocate to ER (Figure). Immunostain indicated that ER-Bcl-xL specifically localized at ER, not mitochondria. To study the role of ER-Bcl-xL at ER during cell death, ER-Bcl-xL and control retroviruses were infected PC12 cells, which were treated by various drugs to induce cell death. The cell death of ER-Bcl-xL constitutively expressing PC12 cells were partially inhibited after non-ER stress inducible reagents: etoposide, staurosporine as well as ER stress treatment: thapsigargin, which was assayed by MTS. To examine the regulation of caspases

activation by ER-Bcl-xL, we carried out the western blotting of several caspases (caspase-12, -7, and –3) using ER-Bcl-xL expressing PC12 cells after thapsigargin treatment. 2 mM of thapsigargin was challenged into culture medium and incubated for six hours. After treatment, cells were washed twice with fresh medium and cultured in normal medium without reagent. Caspase-12 and -3 were activated even in control and ER-Bcl-xL expressing PC12 cells, whereas, interestingly, caspase-7 activation was specifically inhibited by the expression of ER-Bcl-xL. ER stress responses, such as, PERK phosphorylation (P-PERK), unfolded protein response (UPR) indicated by grp78 (BiP) induction, were similarly detected in both cells in time dependent manner. The splicing product of Xbp-1 mRNA, which was carried out by active Ire1a, was not discriminated in both PC12 cells by RT-PCR. ER stress stimulates JNK through Ire1a-TRAF2-ASK1. JNK activity was detected in ER-Bcl-xL expressing PC12 cells. These data indicated that ER-Bcl-xL did not have an effect on ER stress responses. Next, we investigated whether disease related mutation of proteins, such as a-synuclain (Parkinson's disease), SOD1 (Amyotrophic lateral schrelosis), and polyglutamine (polyQ78) (Huntington's disease), induce caspase-7. We transfected plasmids into 293T cells to examine caspases activation. Western blotting indicated that polyQ78 induced caspase-7 activation as well as caspase-8, but not caspase-3 in this experiment. Since polyQ induces ER stress through the dysfunction of ERAD-proteasome system, polyQ78 induced caspase-7 activation may be achieved at ER. To explore the mechanisms of caspase-7 activation, we examined the caspase-7 cleavage in several mutant MEF. Caspase-7 was cleaved in caspase-1 and caspase-12 knockout MEF cells after thapsigargin treatment like in wild type MEF cells. In contrast, caspase-7 activation was completely inhibited in bax;bak double knockout MEF cells. These data implied that caspase-7 activation might be required for Bax and Bak at ER during apoptosis.

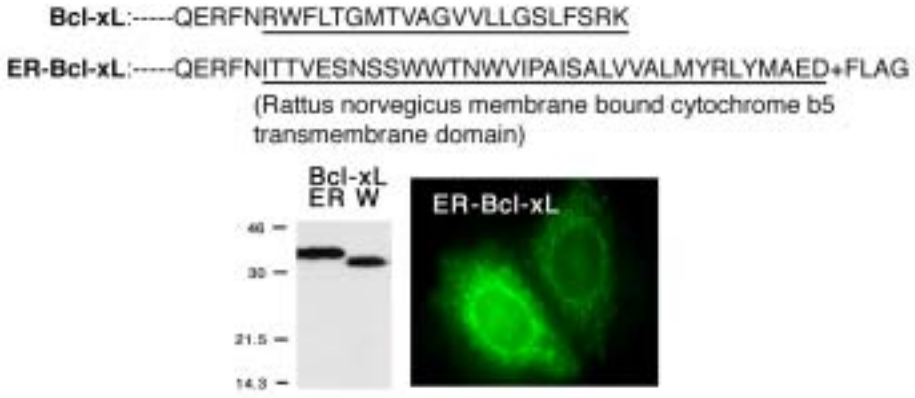

Figure. The mutant Bcl-xL (ER-Bcl-xL), C-terminal transmembrane domain of membrane bound cytochrome b5 was substituted for that of Bcl-xL, resulting in translocation of Bcl-xL specifically to ER. Western bloting showed the ER-Bcl-xL (ER) is slightly bigger than wild type Bcl-xL (W). Immunostain by anti-FLAG antibody showed that ER-Bcl-xL is specifically localized at ER.

Discussion & Summary

The Bcl-2 protein plays an important role in apoptosis by antagonizing Bax and Bak function at mitochondria and regulating initiator caspase activation independently of the apoptosome (cytochrome c/Apaf-1/caspase-9). In this study, we showed that ER targeting BH3 proteins, polyglutamine (pQ), and ER stress caused caspase-7 activation, which was specifically inhibited by Bcl-xL at ER. These data indicated that the Bcl-2/Bcl-xL proteins function to regulate the release of proapoptotic molecules from mitochondria as well as the caspase-7 activation at ER by competing with BH3 proapoptotic proteins.

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2) Medical Sciences
2-2) Central Nervous System

Molecular mechanisms of inflamed pain hypersensitivity in spinal cord neurons

Tatsuro Kohno
Division of Anesthesiology, Niigata University Graduate School of Medical and Dental Science
kohno@med.niigata-u.ac.jp

Introduction

Bradykinin has long been established to be a key inflammatory mediator playing an integral role in the pathophysiological processes that produce acute and chronic inflammatory pain. For decades, most studies on the algesic (pain-promoting) effects of bradykinin have been focused principally on the periphery. Besides its peripheral effects, bradykinin may also modulate pain processing in the spinal cord. The major plan here is to decipher the role of bradykinin and its receptors in pain transmission in the spinal cord.

Results

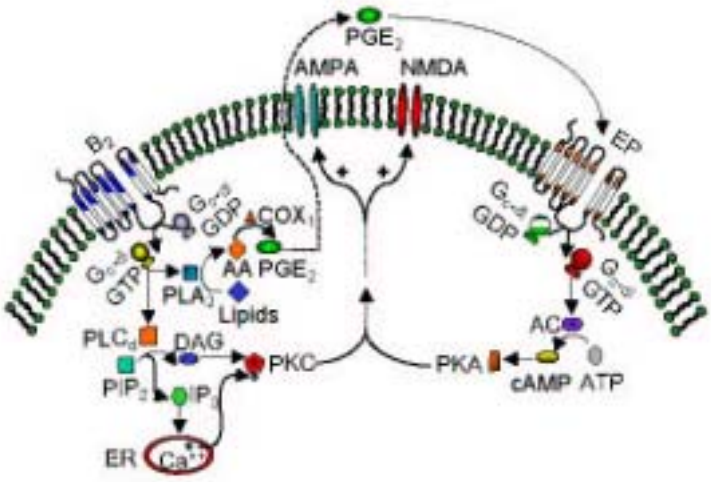
We show that central sensitization, an activity-dependent increase in synaptic efficacy in the spinal cord, requires bradykinin B_2 receptor activation. B_2 receptors are expressed in dorsal horn as well as dorsal root ganglion neurons and B_2 agonists substantially augment AMPA and NMDA receptor-mediated currents in lamina II neurons of spinal dorsal horn, recorded in adult rat slices, acting pre- and post-synaptically. Augmentation of these currents by bradykinin requires co-activation of protein kinase C (PKC) and A (PKA), the latter downstream of cyclooxygenase-1 (COX1) and extracellular signal-regulated kinase (ERK). Administration of bradykinin to the spinal cord *in vivo* induces an NMDA- and ERK-dependent hyperalgesia, while both an intrathecal B_2-selective antagonist suppress behavioral manifestations of central sensitization. Bradykinin is a synaptic neuromodulator that potentiates glutamatergic synaptic transmission in the spinal cord by activating multiple kinases, producing pain hypersensitivity.

Discussion & Summary

The major form of therapy targeted at acute central sensitization has been NMDA receptor antagonists. These antagonists are effective for reducing central sensitization-mediated pain in laboratory animals but their use in patients is severely limited by their psychotomimetic side effects. A better target, based on our findings, may well be bradykinin as an initiator both of NMDA and

AMPA use-dependent synaptic potentiation. The action of bradykinin upstream of multiple signaling pathways that modify neuronal excitability in the peripheral nervous system to produce peripheral sensitization, and in the central sensitization, indicate that preventing its production (using kallikrein inhibitors) or action (with B_2 receptor antagonists) may provide useful means for controlling acute pain hypersensitivity.

Fig.



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

The role of IL-6/gp130 signaling and its negative regulator SOCS3 in viral myocarditis

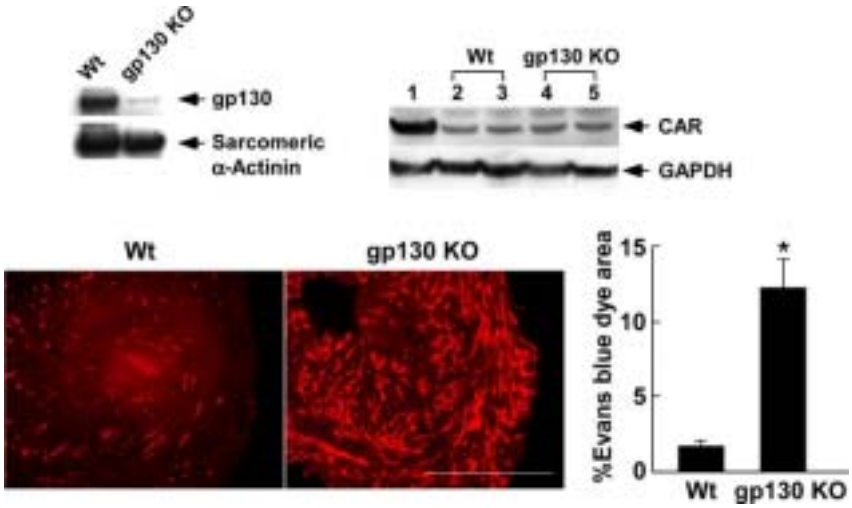
Hideo Yasukawa
Cardiovascular Research Institute, Kurume University, Fukuoka Japan
yahideo@med.kurume-u.ac.jp

Introduction

Enterovirus infection can cause acute myocarditis, a debilitating and potentially lethal disease in adults and children, and has been implicated in the development of chronic dilated cardiomyopathy, one of the main indications for cardiac transplantation. Interleukin-6 (IL-6) family cytokines including leukemia inhibitory factor (LIF) and cardiotrophin1 (CT-1) activate JAK/STAT pathway. IL-6 transduces signals through gp130, the common receptor component of IL-6 family cytokines. IL-6 family cytokines have multi-functions in cells, however, little is known regarding the role of IL-6 family cytokines in anti-viral defense in viral myocarditis.

Results

The purpose of this research project is to investigate the role of IL-6/gp130 signal and its negative regulator SOCS3 in viral myocarditis. We generated cardiac-specific SOCS3 transgenic mice. These mice and cardiac specific gp130 knockout mice were backcrossed into a susceptible, Balb/c, strain and infected with coxsackievirus B3 (CVB3). JAK-STAT signaling and antiviral effects were evaluated in isolated cardiac myocytes. Cardiac-specific SOCS3 expression resulted in a marked increase in the percent of infected myocytes, virus titer (nearly 100 fold) and cardiac cell damage from Hematoxylin-Eosin staining. There was also a loss of cardiac function by echocardiogram and early mortality (80% vs. 5%) compared to wild-type, inbred littermates. In isolated adult cardiac myocytes, SOCS3 expression had no effect on IFN-mediated JAK-STAT signaling or anti-viral effects. However, gp130 signaling measured by cardiotrophin-1-stimulated STAT3 phosphorylation was completely inhibited. Furthermore, CVB3 infection of cardiac-specific gp130 knockout mice increased the percent of infected myocytes and cardiac cell damage (Figure). Interestingly, gp130 stimulation with cardiotrophin-1 has no effect on the viral replication in isolated myocytes, but increases cardiac cell survival even with CVB3 infection. Adenoviral gene transfer of dominant-negative STAT3 but not PI3-kinase inhibitor nor MEK1 inhibitor treatment abolished the cytoprotective effect of gp130 cytokines on CVB3 infected myocytes



Figurelegend

In vivo significance of cardiac gp130 signaling. Knockout efficiency of gp130 in the adult ventricular myocytes from the knockout mice was confirmed by immunoblotting with anti-gp130 antibody. Evans blue dye uptake in the cardiac cells was significantly increased in gp130 KO on the fifth day after the virus inoculation.

Discussion & Summary

1) There are important susceptibility factors within the cardiac myocyte that can be inhibited by SOCS3 expression. 2) These factors are independent of IFN signaling. And 3) gp130 signaling could account for a portion of the protective effect provided by JAK-STAT signaling. 4) STAT3-dependent signaling pathway is essential for the antiviral effect of gp130 cytokines during CVB3 infection. These data demonstrate that a cardiac myocytic innate immune response has a powerful effect on viral infection in the cardiac myocyte, and gp130 signaling plays important roles in this innate immune response during viral myocarditis.

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2) Medical Sciences
2-7) Infection

Role of lipid rafts on the infection of intracellular bacteria into macrophages

Masahisa Watarai
Obihiro University of Agriculture and Veterinary Medicine
watarai@obihiro.ac.jp

Introduction

Brucella species are facultative intracellular pathogens that survive in a variety of cells, including macrophages, and their virulence and chronic infections are thought to be due to their ability to avoid the killing mechanisms within macrophages (1, 2). The molecular mechanisms of their virulence and chronic infections are incompletely understood. *Brucella* internalizes into macrophages by swimming on the cell surface and then the bacteria are enclosed by macropinosomes. Lipid raft-associated molecules are selectively incorporated into macropinosomes containing *B. abortus* (3-6). In this study, we investigated receptor for the bacteria on macrophage surface and roles of lipid rafts on signal transduction through the receptor.

Results

1)SR-A promotes internalization and intracellular replication of *Brucella.*

To investigate the role of class A scavenger receptor (SR-A) in *Brucella* infection, several phenotypes of *B. abortus* virulence were tested by using bone marrow-derived macrophages from SR-A deficient mice. Macrophages from parent mice supported internalization of the wild type *B. abortus* strain, but macrophages from SR-A-deficient mice inhibited the internalization. Macrophages from SR-A-deficient mice also showed inhibition of internalization of the *virB4* mutant, suggesting that the type IV secretion system does not contribute to bacterial internalization dependent on SR-A. The wild type *B. abortus* strain replicated slightly in macrophages from SR-A-deficient mice between 24 h and 48 h after infection, but the intracellular growth rate was still lower than those macrophages from parent mice.

2)Roles of LPS in the SR-A dependent internalization of *Brucella.*

LPS from smooth and rough *B. abortus* inhibited internalization of *B. abortus*, but not LPS from *E. coli*. Interestingly, LPS from *Salmonella enterica* serovar Typhimutium also inhibited internalization of *B. abortus* into macrophages, suggesting that soluble LPS from *B. abortus* and *S.*

enterica serovar Typhimurium was competing with LPS on the surface of *B. abortus* for receptors on the macrophage plasma membrane. In addition to LPS, internalization of *B. abortus* was inhibited by another SR-A ligand, polyinosinic acid, whereas a control reagent, polycytidylic acid that does not block the SR-A receptor had no effect. Furthermore, 2F8, a monoclonal antibody that specifically recognizes mouse SR-A and that blocks phagocytosis of apoptotic thymocytes mediated by SR-A, inhibited internalization of *B. abortus*. These inhibitors did not affect internalization of opsonized bacteria.

3)Decreased proliferation of *Brucella* in SR-A deficient mice.

Many bacteria were recovered from the spleen of parent mice infected with the wild type *B. abortus* strain at 10 and 20 days after infection, but markedly fewer bacteria were recovered from SR-A-deficient mice, judged from the number of CFU in each spleen. These results indicated that bacterial internalization mediated by SR-A contributed to *B. abortus* proliferation in mice.

4)Association between SR-A and lipid rafts during bacterial internalization

Our previous studies showed that lipid raft-associated molecules were selectively incorporated into macropinosomes containing *B. abortus* to induce signal transduction into macrophages and to form replicative phagosomes (3-6). If SR-A has an important role in the signal transduction into host cells, it may be contained in lipid rafts on the macrophage plasma membrane infected with *B. abortus*. To ensure that SR-A localizes in lipid rafts, we prepared detergent-resistant membranes (DRMs) fraction from macrophages infected with wild type *B. abortus* strain or *virB4* mutant. Lipid rafts can be isolated as DRMs from cells. To isolate DRMs from infected macrophages, we extracted them in cold 1 % Triton X-100 lysis buffer, and analyzed them on sucrose gradients for DRMs that float in light membrane fractions away from bulk cellular proteins. SR-A was contained in detergent soluble fractions isolated from the uninfected control or macrophages infected with the *virB4* mutant and it was not detected in detergent insoluble fractions, which were fractions 1-3 of the gradient (15-30% sucrose). In macrophages infected with the wild type strain, in contrast, most SR-A was contained in detergent insoluble fractions. These results suggest that SR-A shifted into detergent insoluble fractions, lipid rafts, from detergent soluble fractions by bacterial internalization dependent on the type IV secretion system and that SR-A may participate in the signaling pathway in *B. abortus* infection into macrophages (7).

Discussion & Summary

Brucella abortus is a facultative intracellular bacterium that can survive inside macrophages (1). Intracellular replication of *B. abortus* requires the VirB complex, which is highly similar to the

conjugative DNA transfer system (2). In this study, we showed that a class A scavenger receptor (SR-A) of macrophages is required to internalize *B. abortus* and contributes to the establishment of bacterial infection in mice. Macrophages from SR-A-deficient mice inhibited internalization and intracellular replication of both wild type strain and the *virB4* mutant, and that bacterial proliferation was inhibited in SR-A-deficient mice. Adding lipopolysaccharide from *B. abortus* and *Salmonella enterica* serovar Typhimurium, but not from *Escherichia coli*, to macrophages inhibited bacterial internalization. VirB-dependent bacterial internalization induced localization of SR-A into detergent-resistant membrane lipid rafts. These results indicate that *B. abortus* internalizes into macrophages by using SR-A as a receptor and that the VirB type IV secretion system of *B. abortus* regulates signal transduction dependent on SR-A to form replicative phagosomes, and which is mediated by lipid rafts (7).

Figures & Tables

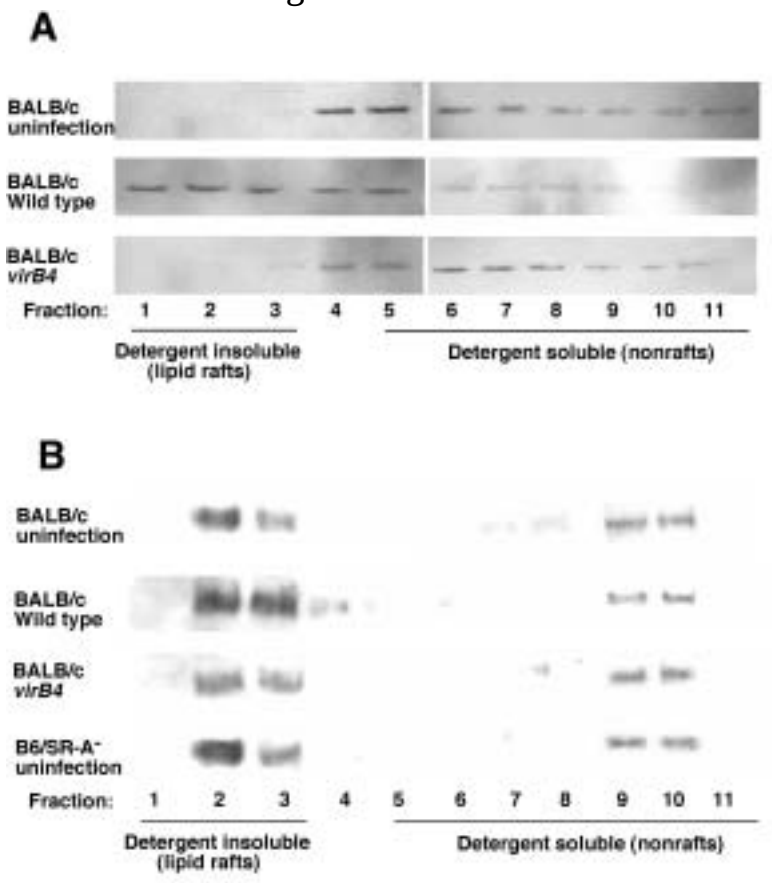

Fig. 1. Association between SR-A and lipid rafts in *B. abortus* internalization. Macrophages infected with or without the wild type or *virB4* mutant were lysed in 1% Triton X-100 lysis buffer, and the whole lysates were fractionated by sucrose density gradient ultracentrifugation. Individual fractions, indicated at the bottom, were analyzed for the presence of SR-A (A) or raft-associated GPI-anchored protein CD48 (B) by immunoblotting.

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2) Medical Sciences
2-7) Infection

Identification of new disease genes and development of new gene therapy protocol for primary immunodeficiency

Yoshiyuki Minegishi
Tokyo Medical and Dental University
yminegishi.mbch@tmd.ac.jp

Introduction

Patients with genetic defects in B-cell development suffer from severe recurrent infections including pneumonia and sepsis, resulting in poor prognosis in the absence of appropriate diagnosis and treatment. All of the known genetic defects that result in a failure of B-cell development involve signaling through the pre-B-cell receptor. Most of the male patients having X-linked inheritance possess mutations in the X chromosome-encoded cytoplasmic tyrosine kinase Btk. Some sporadic patients having autosomal recessive inheritance possess defects in immunoglobulin m heavy chain, Igα, λ5 or B-cell linker protein gene. These findings demonstrated that the components of pre-BCR and its downstream molecule are critical for normal human B cell development.

Results

Although we have identified 90% of genetic origins of human B cell deficiency, genetic basis of 10 % of the patients are still unknown. To determine the genetic origin of these patients, we decided to screen genomic DNA samples from the patients without mutations in Btk, μ heavy chain, Igα, λ5, or BLNK using SSCP analysis. To determine if mutations in Bam32 or LAB could give rise to human B cell immunodeficiency, we designed to amplify individual exons of these two genes by PCR for analysis by single-strand conformation polymorphism (SSCP). Genomic DNA samples were analyzed from 25 patients with a Btk-deficient phenotype, in whom we had not identified mutations in Btk, μ heavy chain, Igα, Igβ, BLNK or the surrogate light chain.

The representative results of SSCP were shown in Figure 1. Left panel showed the SSCP result of exon 2 of LAB gene. Patient 9 demonstrated altered migration pattern in this analysis, indicating the presence of heterozygous sequence alteration in this patient (C: control). Right panel showed the normal pattern of SSCP, indicating all the patients and a control revealed identical migration pattern, indicating no sequence alteration in all the individuals evaluated. We also identified homozygous sequence alteration on exon 6 of Bam32. The sequence analysis revealed that the patients had homozygous sequence alteration at the –3 splice acceptor site. We also sequence entire Bam32

cDNA in eight patients and found the insertion of111 bp aberrant intronic sequences between exon 7 and exon 8, which resulted in truncation of the PH domain of Bam32. The third alteration was a ATC to ATG substitution, occurred at the third base-pair position in codon 39 of LAB gene, which resulted in missense mutation from isoleucine to methionine. These three alterations were located at the site, which is important for normal function of the genes and were not present in 100 normal individuals.

In order to evaluate the functional consequences of the expression of this mutant, we expressed I39M mutant of LAB protein using pMX-IRES-GFP retroviral vector into B cell line WEHI-231 (Figure 2). WEHI-231 can induce apoptosis by B cell receptor cross-linking. Under our experimental condition, approximately 20 % of WEHI-231 cells expressed GFP. By staining with apoptosis marker annexin V and 7AAD, we evaluated the apoptosis of WEHI-231 in GFP negative control population and GFP positive population separately. Anti-μ treatment increased the apoptotic cells from 0.6% to 6.4 % in GFP negative cells. In GFP positive fraction the increase is more drastic, from 0.4% to 13%, suggesting the expression of the mutant protein might have negative effect for the survival of B cells in the patient. These studies suggested the presence of new genetic origin of human B cell deficiency and should help to diagnose early in childhood for prompt treatment of the patients.

Figure 1

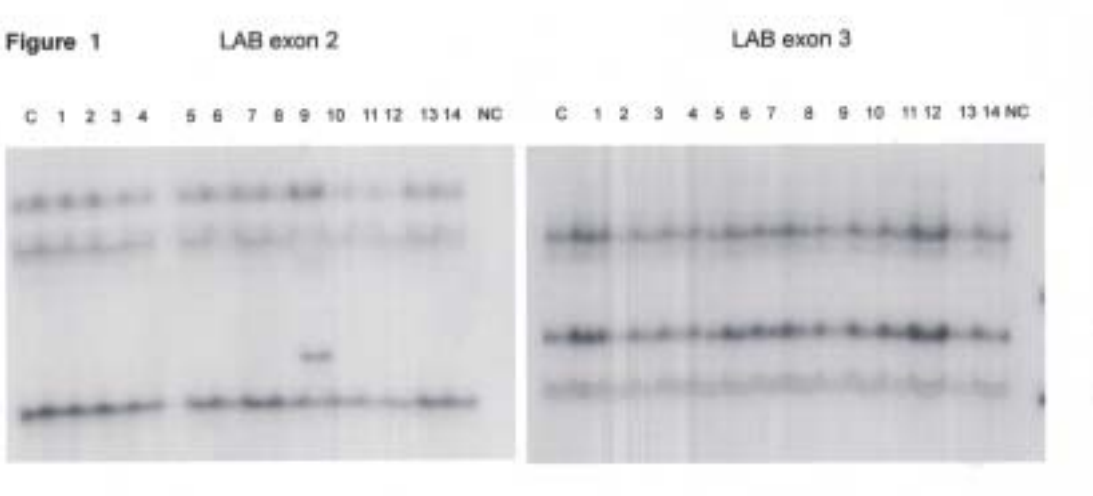
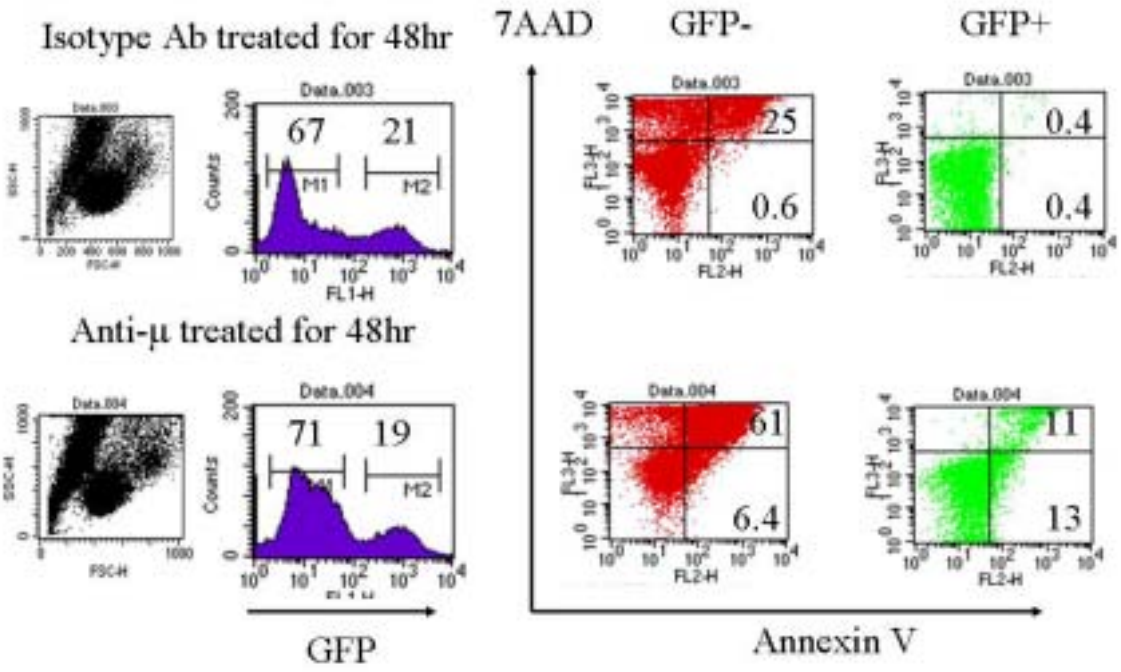


Figure 2



Discussion & Summary

The identification of genes that cause human agammaglobulinemia has both clinical and biologic implications. Early diagnosis and therapy is essential to prevent morbidity due to the recurrent pulmonary infections in childhood. Further, as improved therapies for immunodeficiencies becomes available, it should be critical to know the exact nature of the genetic defect. Mutations in the specific genes provide new intellectual framework to the normal development of the human B-cell lineage. The findings in our patients support the hypothesis that signaling from pre-BCR and BCR is essential for the progression of B-cell differentiation beyond the pre-B-cell stage.

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2) Medical Sciences
2-8) Oncology

Molecular cloning of the gene for mitotic spindle checkpoint deficiency

Shinya MATSUURA
Research Institute for Radiation Biology and Medicine, Hiroshima University
shinya@hiroshima-u.ac.jp

Introduction

Cancer-prone syndrome of premature chromatid separation with mosaic variegated aneuploidy (PCS syndrome) is a rare autosomal recessive disorder characterized by growth retardation, microcephaly, childhood cancer, premature chromatid separation of all chromosomes and mosaicism for various trisomies and monosomies. Biallelic BUB1B mutations were recently reported in five of eight families with MVA syndrome (which we assume is identical with PCS syndrome). We carried out molecular and functional analysis of BUB1B (encoding BubR1) in seven Japanese families with PCS syndrome.

Results

BUB1B mutations were found in all seven families studied: a single base deletion (1833delT) in four families; and a splice site mutation (IVS10-5A>G), a nonsense mutation (670C>T), and a missense mutation (107G>A) in one family each. Transcripts derived from the patients with the 1833delT and IVS10-5A>G mutations were significantly reduced, possibly due to nonsense-mediated mRNA decay, and hence these alleles were effectively null. No mutation was found in the second allele in each of the five patients studied, including the full coding regions, intro-exon boundaries, the promoter region 5-kb upstream of the translation initiation site, and the 3'-untranslated region. cDNA sequencing indicated that the second allele expressed intact mRNA transcripts.

We analyzed the seven families for microsatellite marker 26020GT situated 54-kb upstream of the gene; SNP 1046G/A at exon 8 of BUB1B; and microsatellite marker D15S994, 69 kb downstream of the gene. The 26020GT-1046G/A-D15S994 haplotype of 2A7 was linked to the 1833delT mutation in three of the four families analyzed, a finding that suggested a common origin of the mutation. The haplotype in the second allele in which no mutation was detected was 6G3 in five of the seven families.

Western blotting analysis of BubR1 expression was performed on immortalized fibroblast cell

lines using anti-BubR1 polyclonal antibody. Consistent with the mutation analysis, BubR1 bands of normal size but reduced intensities were detected in these cell lines. We also found that the expression of BubR1 from the 6G3 haplotype was moderately reduced.

We examined the effect of colcemid treatment on the mitotic index of immortalized fibroblast cells. Control cells showed an increase of mitotic index with a peak at 24 hr, whereas the cells from the patients showed abnormal response with no clear peak in mitotic index with an increasing accumulation of micronucleated cells. Introduction through microcell-mediated chromosome transfer of human chromosome 15 containing the BUB1B locus into the cells restored the accumulation of mitotic cells and abolished the accumulation of micronucleated cells.

Immortalized fibroblast cells were analyzed for mitotic checkpoint proteins, with HeLa cells serving as a normal control. In the patients' cells, normal levels of Mad2, Bub1, and Bub3 proteins were detected with western blotting analysis, and Mad2, Bub1, Bub3, and CENP-B, -C, -E, and –F proteins localized to the kinetochores efficiently.

Western blot analysis of the patients' cells demonstrated reduced expression of BubR1 but normal levels of p55cdc protein (data not shown). Kinetochore signals of BubR1 were faint, and kinetochore p55cdc signals were markedly reduced. Transfer of chromosome 15 (containing BUB1B) into the patients' cells restored kinetochore association of both BubR1 and p55cdc. These results suggest that in these patients reduced expression of BubR1 caused abolished kinetochore localization of 55cdc and abnormal checkpoint signaling.

Discussion & Summary

BUB1B mutations were found in all seven families studied: a single base deletion (1833delT) in four families; and a splice site mutation, a nonsense mutation, and a missense mutation in one family each. Transcripts derived from the patients with the 1833delT mutation and the splice site mutation were significantly reduced due to nonsense-mediated mRNA decay. No mutation was found in the second alleles in the seven families studied, but RT-PCR, western blot, and haplotype analysis of BubR1 indicated a modest decrease of their transcripts. BubR1 showed both reduced protein expression and diminished kinetochore localization in cells from two patients. The expression level of p55cdc, a specific activator of anaphase-promoting complex, was normal but its kinetochore association was abolished. Microcell-mediated transfer of chromosome 15 (containing BUB1B) into the cells restored normal BubR1 levels, kinetochore localization of p55cdc, and the normal responses to colcemid treatment. These findings emphasize the importance of BubR1 in p55cdc-mediated mitotic checkpoint signaling, and indicate that PCS syndrome results from >50% decrease in expression (or activity) of BubR1.

Figures & Tables

Table 1. Clinical features in patients with PCS syndrome

Patient	Age at death (y.)	IUGR	Micro-cephaly	DWC[a]	Cataracts	Cancer
1	3	+	+	+	+	Wilms tumor
2	0.4	+	+	+	-	Wilms tumor
3	0.3 (alive)	+	-	-	-	-
4	8 (alive)	+	+	+	+	Wilms tumor
5	0.8	+	+	+	+	Wilms tumor; ERMS[b]
6	1.5	+	+	+	+	Wilms tumor
7A	1.1	+	+	+	?	Wilms tumor; ERMS[b]
7B	0.6	+	+	+	?	Wilms tumor

[a] DWC = Dandy-Walker complex

[b] ERMS = embryonic rhabdomyosarcoma

Table 2. Mutations in *BUB1B* identified in patients with PCS syndrome

Patient	Exon	Mutation	Type	Effect on Protein	Inheritance
1	15	1833delT	Frameshift	F611fs625X	Maternal
2	11	IVS10-5A>G	Splice site	Q468fs480X	Paternal
3	6	670C>T	Nonsense	R224X	Maternal
4	2	107G>A	Missense	R36Q	Maternal
5	15	1833delT	Frameshift	F611fs625X	?
6	15	1833delT[a]	Frameshift	F611fs625X	Paternal
7A, B	15	1833delT[a]	Frameshift	F611fs625X	Maternal

[a] Deduced from the analysis of family members

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2) Medical Sciences
2-8) Oncology

Biochemical and molecular mechanisms of drug resistance of gastrointestinal stromal tumors against Imanitib.

Toshirou Nishida
Osaka University Graduate School of Medicine
toshin@surg1.med.osaka-u.ac.jp

Introduction

Gastrointestinal Stromal Tumor (GIST) has been demonstrated to be caused by gain-of-function mutations in the KIT or PDGFRalpha gene. Mutations in the KIT or PDGFRalpha gene are found in regulatory domains (Exon 9 and 11 for KIT and Exon 12 for PDGFRalpha) and kinase domains (Exon 13 and 17 for KIT and Exon 18 for PDGFRalpha). Downstream kinases including RAS-GAP-MAP kinases, PI3K- AKT kinases also play an important role in progression and neoplastic proliferation of GIST. Imatinib mesylate, a selective inhibitor for KIT and PDGFRalpha, are active for advanced GISTs. However, primary or secondary resistance is going to appear after long time-use of imatinib. The purpose of this project is to elucidate molecular mechanisms of secondary resistance to imatinib. In this project, we have evaluated the target gene mutations and activation of its downstream kinases in resistance clones.

Results

In GISTs, we and others have shown that mutations in the kinase domains of the KIT or PDGFRalpha gene are involved in the primary resistance for imatinib. GIST without any mutations in the KIT and PDGFRalpha genes also shows primary resistance to the drug. In CML, The mechanisms of acquired drug resistance of GIST are shown to be newly generated target gene mutation in the activation loop. Similar mechanisms are postulated to be in secondary resistance of GIST. In the present study, we have examined newly developed KIT and PDGFRalpha mutations, over-expression of KIT and PDGFRalpha, and mutations in downstream kinases in 9 secondary resistant GISTs.

All resistant GISTs are viable, express strongly KIT protein in the immunohistochemical examinations, and also show activation of downstream kinases including RAS-GAP-MAP kinases and PI3K- AKT kinases. Full sequencing of the KIT and PDGFRalpha genes revealed that three resistance GISTs have newly generated mutations in the kinase domains of the KIT gene. The new mutations are occurred in the same allele to the primary mutation. Kinetic analysis indicated that

these newly generated KIT mutations showed resistance to imatinib. Three GISTs with primary KIT gene mutations have no additional mutation in the KIT and PDGFRalpha genes.

We have also investigated primary resistance mechanisms of GISTs without any mutations in the KIT and PDGFRalpha genes for imatinib. At present , we observed that these GISTs also express phosphorylated KIT protein, that downstream kinases of the KIT protein are activated, and that the KIT protein in these GISTs are smaller in the molecular weight than the wild type and mutated KIT protein. Now, we are investigating activation mechanisms of these KIT proteins using proteome analysis and differential display methods.

Discussion & Summary

In summary, we have examined resistance mechanisms of GISTs which showed secondary resistance to imatinib. Although we have now analyzed the limited number of patients with resistant GISTs, almost half of GISTs show newly generated c-kit gene mutations in the kinase domain. Although the other GISTs show strong expression of activated KIT protein and its downstream kinases, precise mechanisms of KIT protein activation under imatinib are still investigated.

Figures & Tables

Table 1. Analyzed cases

	Primary lesion	Imatinib	Best response		primary	resistance
Case 1	stomach	600	PR	Alive	c-kit exon11 (del codon557-558)	Unknown at present
Case 2	intestine	600	PR	Alive	c-kit exon11 (Lys558Asn)	same to the primary
Case 3	duodenum	400	PR	Alive	c-kit exon11(Del:559-574)	same to the primary
Case 4	stomach	600	PR	Alive	c-kit exon11(Del:557-558 TrpLys)	c-kit exon17 (c-kit Trp 823 Asp)
Case 5	intestine	400	SD	Alive	c-kit exon11(Del:557-558 TrpLys)	c-kit exon17 (c-kit Asp 816 His)
Case 6	duodenum	600	PR	Alive	c-kit exon11(Del:560-572)	c-kit exon17 (c-kit Ala829-Pro)
Case 7	stomach	400	SD	Death	wild type KIT	wild type KIT
Case 8	intestine	400	PR	Alive	c-kit exon11 (Val560Asp)	same to the primary
Case 9	stomach	600	PR	Alive	Unknown at present	Unknown at present

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2) Medical Sciences
2-12) Hematology

A role for transcription factors in hematopoiesis and leukemogenesis

MINEO KUROKAWA
Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo
kurokawa-tky@umin.ac.jp

Introduction

Embryonic development of multilineage hematopoiesis requires the precisely regulated expression of lineage-specific transcription factors including AML1/Runx1 (also CBFA2 or PEBP2αB). In vitro studies and findings in human diseases including leukemias, myelodysplastic syndromes (MDS) and familial platelet disorder with predisposition to acute myelogenous leukemia also suggest it plays a pivotal role in adult hematopoiesis. However, this role in vivo has not been fully disclosed due to the embryonic lethality of AML1-targeted mice.

Results

We assessed the requirement of AML1 in adult hematopoiesis by an inducible gene targeting method. Using the Cre-loxP sequence-specific recombination system, we generated mutant mice in which exon 5 of the AML1 gene can be selectively deleted by the expression of Cre recombinase. We bred the mutant mice with Mx-cre transgenic mice to generate mice, in which deletion of AML1 allele could be evoked in hematopoietic progenitors by inducible expression of Cre reconbinase by the injection of polyinosinic-polycytidylic acid (pIpC). Injection of pIpC did not cause significant differences in neutrophil counts or hemoglobin levels between AML1-deficient and control mice. However, platelet counts for AML1-deficient mice declined to one third to one sixth of those for the control mice, immediately after pIpC administration. Lymphocyte counts were slightly depressed after more than four weeks in AML1-deficient mice. These results suggest that AML1 is required for the maintenance of platelets and lymphocytes, but not for the sustained production of erythrocytes and neutrophils. AML1-deficient bone marrow revealed the absence of normal megakaryocytes with abundant cytoplasm and lobulated nuclei. Instead, it contained a small number of immature megakaryocyte-like cells with occasionally separated round nuclei resembling abnormal “micromegakaryocytes” observed in human MDS. Given the prominent immaturity of megakaryocytes in AML1-deficient mice, we examined the ultrastructure of the megakaryocytes. In addition to the smaller size, AML1-deficient megakaryocytes showed poorly developed demarcation membranes compared to those of the control. During the maturation process,

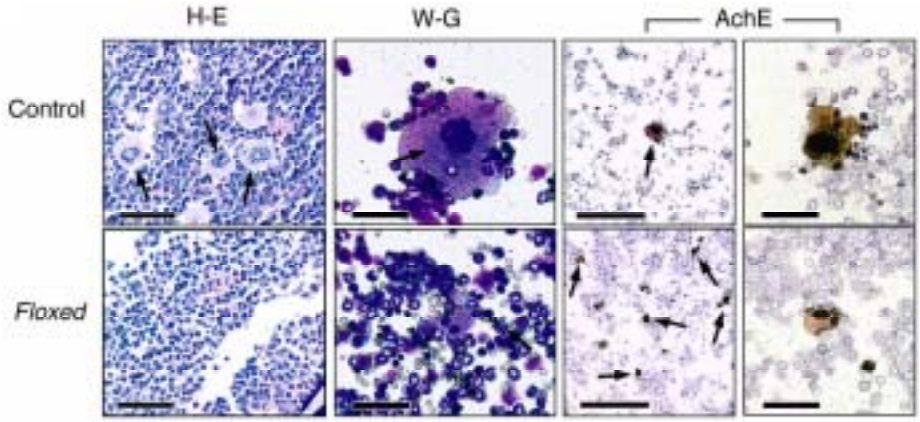
megakaryocytes acquire high DNA ploidy (>4 n) through a cell cycle process known as endomitosis. As expected from the smaller size of nuclei, $CD41^{+}$ megakaryocytes from AML1-deficient mice showed a remarkably lower polyploidy level (≤8 n) than those from the control mice (modal ploidy, 16 n) as revealed by the flow cytometric measurement of DNA content. This demonstrates that AML-1 deletion also causes defective polyploidization in megakaryocytes. Subsequently, we performed in vitro colony forming assays to evaluate the frequency of hematopoietic progenitors. AML1-deficient bone marrow cells formed more megakaryocytic colonies (CFU-Meg) than control bone marrow cells in semisolid media. The numbers of myeloid and mixed (myeloid and erythroid) colonies were also elevated in AML1-deficient bone marrow. By single colony PCR genotyping, $AML1^{-/-}$ genotype was detected in 149 of 150 myeloid colonies, 48 of 50 erythroid colonies, 35 of 36 mixed colonies and 14 of 14 megakaryocytic colonies from pIpC-treated AML1-deficient bone marrow cells, thus excluding the possibility that those hematopoietic cells might be predominantly derived from progenitor cells still expressing intact AML1. Using flow cytometry, we observed an increased cell number in the Lin^{-} c-Kit^{hi} $CD41^{hi}$ fraction, which was presumed to contain CFU-Meg, in AML1-deficient bone marrow. Similarly, The number of cells defined by $CD34^{-}$ $Lin^{-/lo}$ c-Kit^{hi} Sca-1^{hi}, which represent the most immature hematopoietic progenitor cell population was elevated in AML1-deficient bone marrow. Since immature hematopoietic progenitor cell fractions were elevated in AML1-deficient bone marrow, we assessed their ability to reconstitute adult hematopoiesis using a competitive repopulation assay. We employed isotypes of the pan-hematopoietic marker CD45 (Ly5) to distinguish the origins of the repopulating cells. Sublethally irradiated Ly5.1 recipient mice ($Ly5.1^{+}Ly5.2^{-}$) were intravenously injected with a mixture of bone marrow cells from Ly5.1–Ly5.2 competitor mice ($Ly5.1^{+}Ly5.2^{+}$) and AML1-deficient or control mice ($Ly5.1^{-}Ly5.2^{+}$) previously injected with pIpC. Subsequently, we analyzed Ly5 isotypes on the repopulating cells by flow cytometry. Notably, the floxed bone marrow cells reconstituted neither peripheral T (Thy-1.2^{+}) nor B ($B220^{+}$) cells, while there were no significant differences in the mature neutrophil (Gr-1^{hi} Mac-1^{+}) and monocyte populations (Gr-1^{lo} Mac-1^{+}).

Discussion & Summary

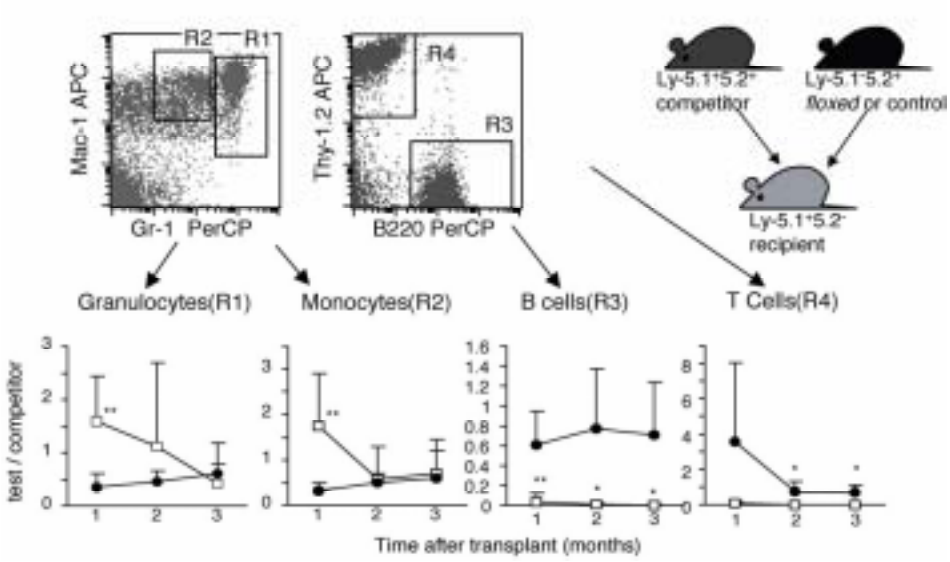
In the absence of AML1, hematopoietic progenitors are fully maintained with normal myeloid cell development. However, AML1 deficient bone marrow showed megakaryocytic maturation block, increased hematopoietic progenitor cells and defective T and B lymphocyte development. AML1 is thus required for the maturation of megakaryocytes and differentiation of T and B cells, but not for hematopoietic stem cell maintenance in adult hematopoiesis. Our present data also demonstrate that AML-1 is essential for the terminal differentiation of hematopoietic progenitors of megakaryocytic and lymphocytic lineages, which establishes AML-1 as a multi-role regulator in the

maintenance of lineage-committed cells in adult hematopoiesis. However, our results also suggest that the maintenance of hematopoietic stem cells and their commitment to more mature progenitors in adult hematopoiesis does not always require a transcription factor essential for hematopoietic ontogeny during embryogenesis, and can be induced by other hematopoietic genes.

Figures & Tables



Megakaryocytic maturation arrest of AML1-deficient mice. In contrast to control mice, small and dysplastic megakaryocytes were increased in AML1-deficient mice (Floxed). Bone marrow (BM) sections stained with hematoxyllin and eosin (H-E), cytocentrifuged specimens of the BM cells stained with Wright-Giemsa (W-G), and cytochemically stained for acetylcholinesterase (AchE). BM was collected from mice 2 months after pIpC injection. Arrows : megakaryocytes, bars : 50 μm (H-E, W-G, and AchE-right) ; 250 μm (AchE-left).



AML1-deficient BM cells show maturation block in T and B cells. BM cells from AML1-deficient (floxed) or control ("test") mice (Ly-5.1-5.2+) 1 – 3 months after pIpC injection were injected simultaneously with "competitor" Ly-$5.1^{+}5.2^{+}$ BM cells, and the contribution to peripheral blood cells was analysed by FACS. AMl1-dificient BM cells contribute to granulocytes and monocytes, but not to B cell or T cells. Data are shown as mean (AML1-deficient : squares, control : closed circles) + S.D. (error bars) of Ly-5.1^{-}Ly-5.2^{+} objective cell number / Ly-5.1^{+}Ly-5.2^{+} competitive cell number ratios of each point. * : $P < 0.05$, ** : $P < 0.01$ (Wilcoxon rank sums test).

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3) Chemical Sciences

3-1) Organic Chemistry

Development of Stereoselective Synthetic method of Procyanidin Oligomers and Their Activities

Noriyuki Nakajima

Biotechnology Research Center, Toyama Prefectural University

nori@pu-toyama.ac.jp

Introduction

Proanthocyanidins are known as condensed tannins and/or oligomeric flavonoids. The investigation of proanthocyanidins is now increasingly important because of their various strong bioactivities. However, the structure-activity relationship of proanthocyanidin oligomers has not been proved yet, because a large number of isomers in the plants make it very difficult to purify and supply each pure compound for assay. So we have undertook a stereoselective synthesis of procyanidin oligomers, and further investigated their bioactivities.

Results

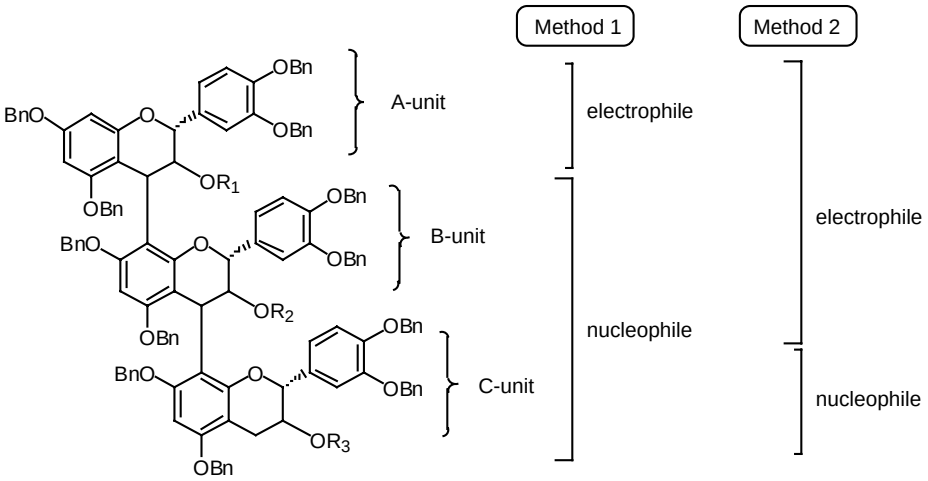
We applied the intermolecular TMSOTf-catalyzed condensation to the synthesis of procyanidin trimers and galloyl-substituted procyanidin dimers. A stereoselective condensation of (2*R*,3*R*,4*S*)-5,7,3'4'-tetra-*O*-benzyl-4-(2''-ethoxyethyloxy)flavan (epicatechin electrophile) with four dimeric nucleophiles in CH_2Cl_2 at –40°C to –20°C gave the corresponding four trimers in excellent yields (**Table 1**, entries 1-4). On the other hand, when (2*R*,3*S*,4*S*)-5,7,3'4'-tetra-*O*-benzyl-4-(2''-ethoxyethyloxy)flavan (catechin electrophile) was condensed with four dimeric nucleophiles in CH_2Cl_2 at –78°C, only procyanidin C2 (catechin trimer) was obtained in excellent yield (entry 5). The condensation yields of other two trimers were only in 47% and 44 % yields yield (entries 7 and 8) and catechin-catechin-epicatechin trimer was not obtained yield (entry 9). The structure of condensed product was confirmed by comparing the ^{1}H-NMR spectra of products, that was synthesized by two different condensation approaches (entries 5 and 6). Finally, deprotection of (+)-catechin and (-)-epicatechin trimers derivatives gave seven natural procyanidin trimers in good yields.[1] Twelve galloyl-substituted procyanidin B1, B2,[2] B3[3] and B4[4] 3-*O*-gallate, 3"-*O*-gallate and 3,3"-di-*O*-gallate, were also synthesized under our condensation conditions using TMSOTf as a catalyst.

Their bioactivities, antioxidant activities on UV-induced lipid peroxide formation using the TBA method, DPPH radical scavenging activity and inhibitory activity of DNA polymerases, of all

synthesized compounds were investigated (**Fig 1**).

Figures & Tables

Table 1 Synthetic studies of procyanidin trimers



Entry	A-unit	B-unit	C-unit	Method	Temp, °C	Yield, %
1	Epicatechin	Catechin -	Catechin	1	-20	70
2	Epicatechin	Catechin -	Epicatechin	1	-20	97
3	Epicatechin	Epicatechin -	Catechin	1	-40	100
4	Epicatechin	Epicatechin -	Epicatechin	1	-40	91
5	Catechin	Catechin -	Catechin	1	-78	100
6	Catechin	-Catechin	Catechin	2	-78	44
7	Catechin	Epicatechin -	Catechin	1	-78	47
8	Catechin	Epicatechin -	Epicatechin	1	-78	44
9	Catechin	Catechin -	Epicatechin	1	-78	0

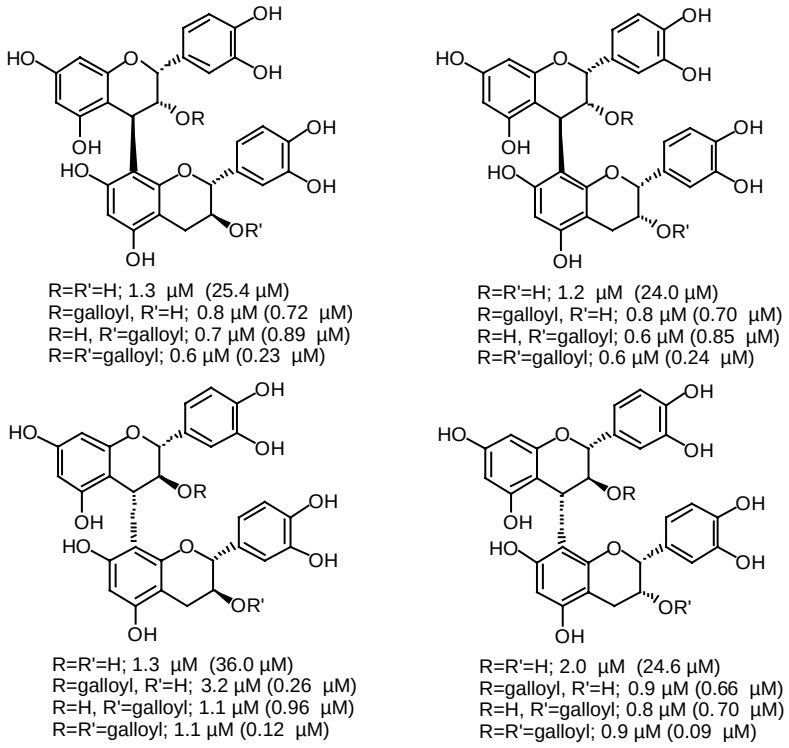


Fig. 1 IC_{50} Values of DPPH radical scavenging activity and enzyme inhibition against mammalian DNA polymerase α in parenthesis for procyanidin dimers.

Discussion & Summary

The TMSOTf-catalyzed intermolecular condensation method was effective for the synthesis of 3,4-*trans* procyanidin trimers. Seven procyanidin trimers and twelve galloyl-substituted procyanidin dimers were systematically synthesized. From the results of the activity tests, the galloyl group of is very important for activities. 3,3"-Di-*O*-gallate dimers acted as strong inhibitor against DNA polymerase α and β, whereas the desgalloyl and monogalloyl compounds did not exhibit any appreciable inhibitory activity against the DNA polymerase β.

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3) Chemical Sciences
3-1) Organic Chemistry

Synthetic studies on insect anifeedants

Shigefumi Kuwahara
Graduate School of Agricultural Science, Tohoku University
skuwahar@biochem.tohoku.ac.jp

Introduction

The use of antifeedants as eco-friendly agrochemicals to reduce crop damage by insect pests has recently been attracting increasing attention among academic circles as well as manufacturers of agrochemicals. Among antifeedants so far reported, which are produced by plants and prevent insect pests from eating the plants, azadirachtin (**1**), and clavigerins A and B (**2** and **3**, respectively) are known for their extremely potent antifeedant activity. In this report, our studies directed toward the total synthesis of these substances are described.

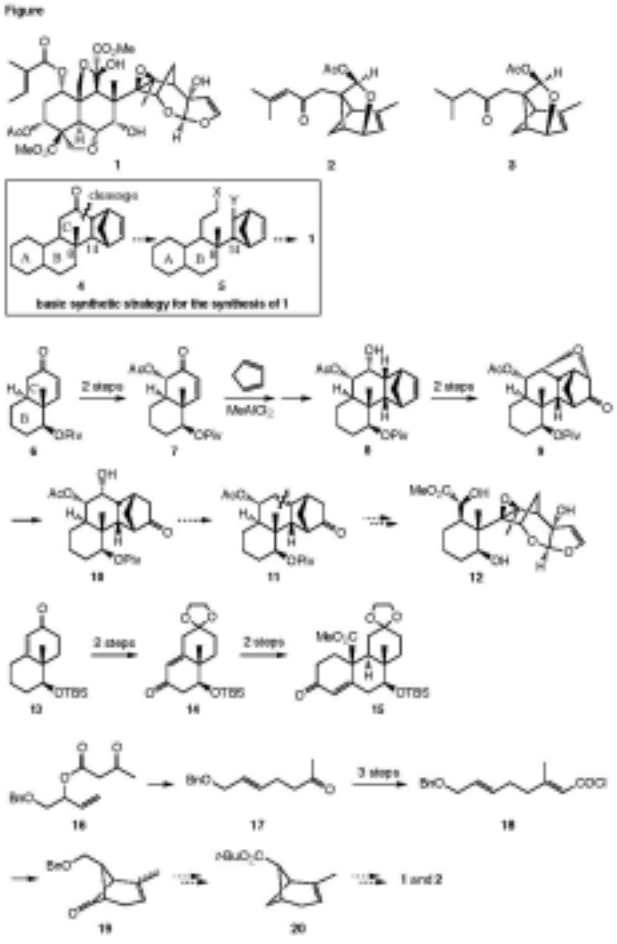
Results

As a basic synthetic strategy, we envisaged installing the highly sterically congested C8–C14 bond of **1** by dissecting the C ring of pseudo-steroidal compound **4**. Along this basic plan, enone **6**, obtained readily from the Wieland-Miescher ketone, was converted into **7** through oxidation and acetylation. The enone (**7**) was subjected to Diels-Alder reaction conditions with cyclopentadiene in the presence of methyl aluminum dichloride, affording quantitatively the corresponding adduct, which was then reduced to give **8**. Epoxidation of **8** with mCPBA brought about cyclization of the resulting epoxy alcohol to furnish an pentacyclic alcohol, which was then oxidized to give **9**. Reductive cleavage of the a-C–O bond of **9** gave **10**, which could be convertible to an azadirachtin model compound (**12**), although lacking the A-ring of azadirachtin, via oxidative cleavage of **11**.

For the synthesis of clavigerins A and B, compound **16**, obtained from benzyloxyacetaldehyde in 2 steps, was exposed to the Carroll rearrangement conditions to give **17**, which was converted into acid chloride **18** by a three-step sequence including a Wittig reaction. Heating of **18** in xylene in the presence of tributylamine afforded the desired bicyclo[3.1.1]heptane derivative (**19**) via a [2 + 2] cycloadditon reaction of a ketene intermediate. This bicyclic compound is considered to be convertible to **1** and **2** via alkylation of the ester with an equivalent of the side chain.

Discussion & Summary

Based on a proposed biosynthetic pathway for azadirachtin, a new strategy for its total synthesis, featuring the construction of its C8–C14 moiety by cleaving the C-ring of a fused polycyclic intermediate, was examined. Starting from the Wieland-Mischer ketone, a substrate for the cleavage reaction was prepared. Since the cleavage proceeded smoothly with some analogous compounds, application of the conditions to intermediate **11** would produce the corresponding C-ring cleaved intermediate, which would probably lead to our initial goal, an azadirachtin model compound lacking only its A-ring portion.



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For a recent synthetic study of azadirachtin, see: K. C. Nicolaou et al., Angew. Chem. Int. Ed., 44, 3447 (2005).

For structural determination of clavigerins, see: N. B. Perry et al., Tetrahedron Lett., 44, 1651 (2005).

3) Chemical Sciences

3-1) Organic Chemistry

Global identification of Akt substrates by a novel phosphoproteome analysis.

Seisuke Hattori, Dsc.

Institute of Medical Scinece, The University of Tokyo

hattoris@ims.u-tokyo.ac.jp

Introduction

Akt is a serine/threonine kinase the abnormal regulation of which is implicated in the carcinogenesis of several types of cancer. Akt is also a major signal transducer for insulin signaling pathways. Therefore, it is important to globally identify Akt substrates and characterize their functions. We addressed this issue by a novel phosphoproteome strategy.

Results

We established conditions to purify phosphoproteins by immobilized metal affinity chromatography (IMAC) by optimizing pH and ionic strength. Under the established conditions, the purity of phosphoryated Akt substrates increased about ten-folds by the chromatography.

Next, we analyzed the phosphoprotein profiles from Akt-activated (NIH3T3 cells treated with PDGF) and Akt-suppressed cells (NIH3T3 cells treated with PDGF in the presence of wortmannin, an inhibitor for PI3-kinase, an upstream activator for Akt). The proteins were labeled with Cy5- (Akt-activated cells) and Cy3-derivatives (Akt-suppressed cells), and the combined sample was analyzed on 2-D differential fluorescence gel electrophoresis (Fig.1). Proteins specific to Akt-activated cells give red spots, while proteins specific to Akt-suppressed cells give green spots. Proteins with similar amounts between two samples give yellow spots (as an overlap of red and green). As shown in the figure, multiple red or orange spots were observed, among the many spots being yellow. Since, phosphoprotein-enriched fraction was used as a sample for this analysis, the result suggests that these spots are Akt substrates. Currently, we are trying to identify the proteins in these spots and characterized the function of these proteins.

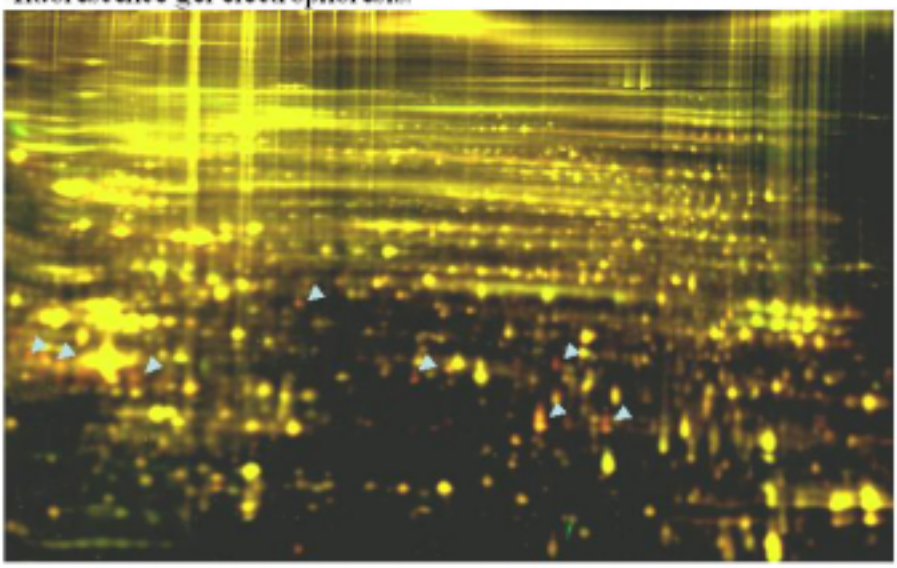
Discussion & Summary

This system could be applied to other kinases if the activation conditions would be established and if the specific inhibitor would be available. Therefore, the system may be very useful to analyze the signaling pathways of a given kinase.

Summary:

We established the conditions to purify phosphoproteins from total cell lysate by using immobilized metal affinity chromatography. The phosphoproteins were analyzed by 2-D fluorescence differential gel electrophoresis where multiple spots unique to Akt-activated cells were identified.

Figure 1. Identification of Akt substrates by 2-D differential fluorescence gel electrophoresis.



Phosphoprotein fractions from Akt-activated (red) and Akt-suppreseed (green) cells were run on a same gel. Proein profiles were obtained by scanning the gel at Cy5 (red) and Cy3 (green) wavelengths, and illustrated as overlapping figure. Proteins unique to Akt-activated cells give red or orange spots (arrowheads).

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3) Chemical Sciences

3-1) Organic Chemistry

Development and Application of Chiral Ligand based on N-Ar Chiral Mimetic

Kazuhiro Kondo

Graduate School of Pharmaceutical Sciences, Nagoya City University

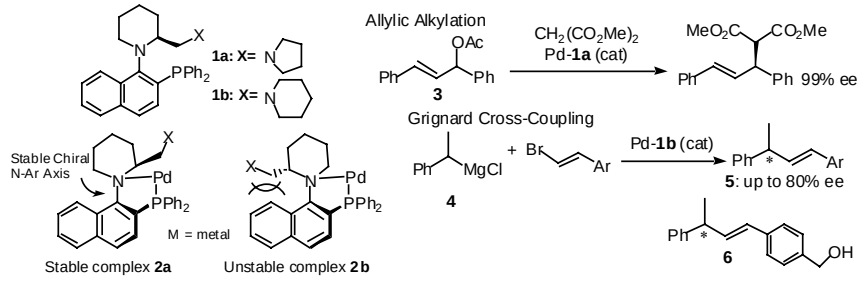
kazuk@phar.nagoya-cu.ac.jp

Introduction

We designed the ligand **1** on the following concept: the ligand **1** may have the two diasteromers due to the N-Ar axis and the chiral carbon in solution. But, if the N-Ar axis in **1** is configurationally flexible, and the complexation of **1** and a metal is largely reflected by the asymmetric center in **1**, one of the two diastereomer complexes due to the N-Ar axis, is expected to be selectively formed (**2a**), leading to the creation of favorable asymmetric environment.

Results

We synthesized the ligand **1a**,**b**. The diastereomers of **1a**,**b** due to the N-Ar axis and the chiral carbon, were not observed. With the ligands **1a**, we first concentrated on a palladium-catalyzed asymmetric allylic alkylation, using the allylic acetate **3** as the substrate and dimethyl malonate as the nucleophile. The ligand **1a** was found to be effective (98% yield and 99% ee). We carried out NMR studies on the complex **2a**. The ^{1}H-and ^{31}P-NMR spectra of the complex **2a** showed only one isomer complex. Since it is important to develop versatile chiral ligands, which afford good enantioselectivity in a variety of reactions, we next were interested in the asymmetric Grignard cross-coupling reaction of 1-phenylethylmagnesium chloride with alkenyl halides. One type of substrate with which it has been more difficult to achieve high selectivity is *E*-bromostyrene derivatives. We performed in the asymmetric cross-coupling reaction with the ligand **1b**, and 1-phenylethylmagnesium chloride (**4**), affording enantioselectivity of up to 80%. The product **5** was transformed into lipoxygenase inhibitor **6**.



Discussion & Summary

We have shown that an N-Ar axially chiral mimetic-type ligand **1a**,**b** are efficient in the standard asymmetric allylic alkylation and the asymmetric Grignsrd cross-coupling reaction of 1-phenylethylmagnesium chloride with *E*-bromostyrene derivatives. Our ligand **1a** can be considered to be one of the best ligands for the standard asymmetric allylic alkylation described in the literature to date, and our ligand **1b** are, to the best of our knowledge, the third best enantioselectivity in the reported literature for the asymmetric Grignsrd cross-coupling reaction of 1-phenylethylmagnesium chloride with *E*-bromostyrene derivatives. Further application to other catalytic asymmetric reactions is now in progress.

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3) Chemical Sciences

3-1) Organic Chemistry

Synthetic Studies on Marine Cytotoxic Halogen-Containing Oxasqualenoids

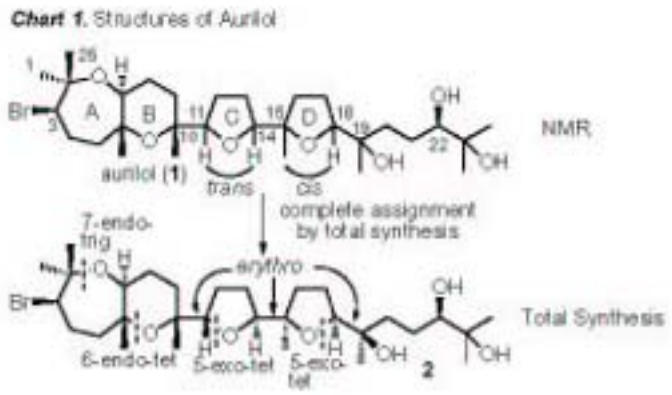
Yoshiki Morimoto

Graduate School of Science, Osaka City University

morimoto@sci.osaka-cu.ac.jp

Introduction

The plane structure and partial stereochemistry of a bromotriterpene polyether (+)-aurilol (**1**), a member of marine cytotoxic halogen-containing oxasqualenoids, were mainly elucidated by NMR methods; however, determination of the entire stereochemistry has not been reached.[1] Although there have also been many other types of oxasqualenoids, it is often difficult to determine their stereostructures even by the current highly advanced spectroscopic methods, especially in acyclic systems including quaternary carbon centers such as C10–C11, C14–C15, and C18–C19 in **1**. The purpose of this research is the total assignment of the incomplete stereostructure of **1** by chemical synthesis (Chart 1).



Results

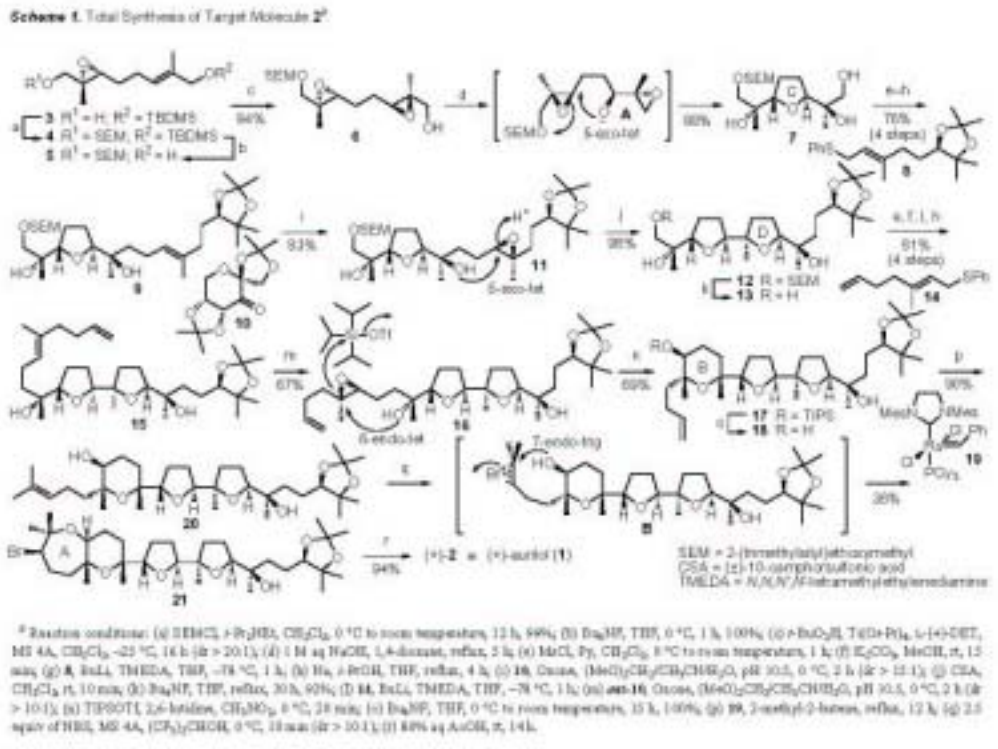
In the course of our structural studies on oxasqualenoids, which could biogenetically be derived by epoxide-opening cyclizations of squalene polyepoxides, we predicted the unknown stereochemistry to be *erythro* configuration as shown in **2**.

The synthesis of target molecule **2** began with SEM protection of the hydroxy group in the known optically active epoxy alcohol **3** (98% ee) (Scheme 1). Selective deprotection of the TBDMS ether in **4** and Sharpless' epoxidation of the allylic alcohol **5** using L-(+)-DET afforded diepoxide **6**. The C

ring formation from **6** under the alkaline condition stereoselectively proceeded via a regioselective 5-exo-tet epoxide opening of the intermediate **A** to provide triol **7** in 88% yield. The following sequence, (1) mesylation; (2) epoxidation; (3) chain extension with the C_{10} unit **8**; and (4) desulfurization, uneventfully yielded diol **9** from **7**. Shi's epoxidation of **9** catalyzed by chiral ketone **10** diastereoselectively gave labile bishomoepoxy alcohol **11**. Treatment of **11** with a protic acid underwent a regioselective 5-exo-tet cyclization to produce diol **12** in 98% yield.

Deprotection of the SEM ether in **12** afforded triol **13**, from which diene **15** was derived by the same sequence as that of **7** to **9** except for employing sulfide **14**. Shi's epoxidation of the diene **15** with ***ent*-10** proceeded in a regioselective manner to provide monoepoxide **16** with the terminal alkene intact. The construction of the desired B ring was regioselectively carried out by treating the bishomoepoxy alcohol **16** with TIPSOTf and 2,6-lutidine in CH_3NO_2 at 0 °C for 20 min.

After removal of the TIPS group in **17**, cross metathesis of olefin **18** with 2-methyl-2-butene using Grubbs' catalyst **19** produced alkene **20** in 90% yield. The regio- and stereoselective 7-endo-trig bromoetherification of **20** was brought about under the optimal condition to give the A,B-ring system **21**, wherein removal of the acetonide, finally furnished the target molecule **2**. The spectral characteristics (^{1}H and ^{13}C NMR) of the synthetic **2** were identical to those reported for the natural product. Thus, it has been found that the hitherto unknown relative configuration of (+)-aurilol (**1**) is *erythro* as indicated in **2**.



Discussion & Summary

The unprecedented 6-endo-tet cyclization promoted by a silyl triflate against Baldwin's rule would be very stimulating because Brønsted acid-catalyzed cyclizations for these types of bishomoepoxy alcohols, such as **11**, generally afford 5-exo-tet regioselectivity, regardless of the relative configurations of the tertiary alcohol-epoxide substrates. It has been found that $(CF_3)_2CHOH$ with high polarity and non-nucleophilicity is the solvent of choice to successfully form the A ring.

In conclusion, we have accomplished the first asymmetric total synthesis of a cytotoxic bromotriterpene polyether (+)-aurilol (4.74% overall yield in 21 steps from **3**) featuring the highly regio- and stereocontrolled biogenetic-like A–D ether ring formations. The total synthesis has realized the total assignment of the incomplete stereostructure of aurilol (**1**), which is difficult to determine the stereochemistry otherwise.[2]

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3) Chemical Sciences

3-1) Organic Chemistry

Studies on the structure and the chemical synthesis of marine toxin Azaspiracid

Masato Oikawa

Graduate School of Life Sciences, Tohoku University

mao@bios.tohoku.ac.jp

Introduction

Azaspiracids are family of polyether toxins isolated first from Irish mussels as causative organic compounds for Azaspiracid poisoning prevailed in Europe [1]. The novel structure as well as its unique biological activity, the mode of which is supposed to be much different from those for other known marine polyether toxins, prompted us to develop studies on the chemical structure and the synthesis.

Results

Though we have already successfully achieved the chemical synthesis of the FGHI-ring domain of Azaspiracid [2], the domain can not be used for further homologation of the E-ring moiety toward the total synthesis because of the lack of a suitable functional group. In addition, we have noticed in the previous work that the Boc group used for protection of C_{40}-amino group was not robust enough under even mild acidic conditions used for construction of the FGHI-ring domain. To deal with these issues we have studied the possibility for Alloc and Ns protecting groups for the amino group.

The precursors **4** and **5** were independently synthesized by the modified manner used before [2], and submitted to cyclization reaction using $Yb(OTf)_3$ in CH_2Cl_2. It was found that natural-type and unnatural-type aminal diastereomers **6** and **7** were selectively obtained from **4** and **5**, respectively in reasonable yields. In both cases undesirable decomposition of these protecting groups was not observed.

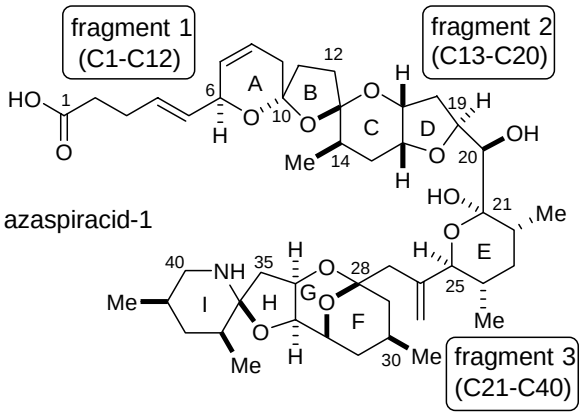
The synthesis of the E-ring moiety was also studied. In the present study, we have developed a new strategy using intramolecular cyclization starting from α-halosulfide **9** by the successive treatment with NCS and then with AgOTf. By this reaction, the thiopyranoside **10** was obtained in low yield but with high stereoselectivity.

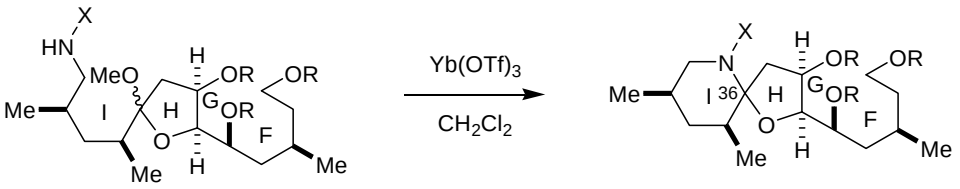
Discussion & Summary

The development of high-yield and stereoselective synthesis of the FGHI domain is essential for the total synthesis of Azaspiracid-1. In the present study, we have explored amenable protecting groups for C_{40}-amino functionality stable under conditions for the construction of the FG-ring moiety. In the event, we have found Ns and Alloc protecting groups were suitable for this purpose and, quite interestingly, provided unnatural and natural diastereomers, respectively, stereoselectively in good yield. The efficient method for construction of the E-ring moiety was also developed and the conditions are now being under optimization to improve the yield.

Our efforts are continued to synthesize the EFGHI-ring moiety toward the total synthesis of Azaspiracid on the basis of the knowledge found in these studies.

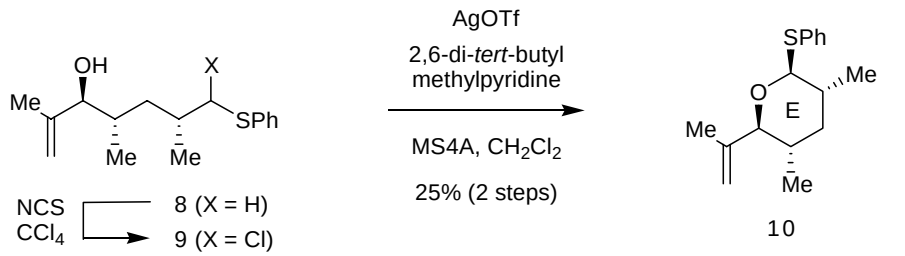
Figures & Tables





4 (X = Alloc, R = Bn)
5 (X = Ns, R = Ac)

(36*S*)-6: 45% desired (from 4)
(36*R*)-7: 89% undesired (from 5)



10

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[2] M. Sasaki, Y. Iwamuro, J. Nemoto and M. Oikawa, *Tetrahedron Lett.*, **44**, 6199-6201 (2003).

3) Chemical Sciences
3-1) Organic Chemistry

Synthesis of optically active a-aryl carboxylic acids by catalytic asymmetric protonation

Akira YANAGISAWA
Chiba University
ayanagi@scichem.s.chiba-u.ac.jp

Introduction

Asymmetric protonation of enolates is one of the most effective methods for preparing optically active α-substituted carbonyl compounds. Main objective of this research is to develop a truly practical catalytic asymmetric protonation of prochiral metal enolates or silyl enolates. Especially, we focus on enolates derived from α-aryl carboxylic acids, some of which are known to have biological activity, and aim to develop chiral proton sources or chiral catalysts, which can provide both enantiomers of α-aryl carboxylic acids with high optical purity.

Results

BINAP·AgF is an efficient chiral catalyst for asymmetric aldol reaction of silyl enolates[1] and also for asymmetric allylation of aldehydes with allylsilane.[2] Because the activation of a trimethoxysilyl group by the fluoride ion is remarkable, we envisaged that the silver fluoride complex would behave as a chiral catalyst also for asymmetric protonation of silyl enolates with an appropriate achiral Brønsted acid such as methanol. We first studied the protonation of 2-methyl-1-tetralone–derived trimethylsilyl enolate **1** (shown in entry 1 of Table 1) with methanol to find the optimum reaction conditions. We attempted the reaction using various ratios of a mixture of BINAP and AgF at –20 ˚C and found that a 0.6:1 mixture was more effective in obtaining a nonracemic product with higher enantioselectivity than a 1:1 mixture.

We then examined solvent effects on the chemical yield and enantioselectivity. Among the solvents tested, THF or chlorinated hydrocarbons indicated better ee than did methanol, and dichloromethane was the solvent of choice. In fact, when the protonation was carried out in a 20:1 mixture of dichloromethane and methanol, (*S*)-enriched 2-methyl-1-tetralone was obtained with 56% ee. Further improvement in the enantiomeric ratio was attained when a twofold volume of methanol and dichloromethane was employed. Additionally, with other achiral alcohols, we investigated the enantioselectivity of this catalytic protonation and as a consequence, methanol was found to be the most effective.

This asymmetric protonation was applied to diverse trimethylsilyl enolates and the results are summarized in Table 1. Not only the 5-MeO derivative, but also the 2-ethyl derivative afforded good optical purities similar to that of 2-methyl-1-tetralone (entries 1-3). Although simple ketones possessing no aromatic group at the α-carbon are known to give unsatisfactory results in the catalytic asymmetric protonation under acidic conditions, employment of 2,2,6-trimethylcyclohexanone-derived silyl enolate in the present protonation resulted in high enantioselectivity of more than 80% ee (entry 4). A very high enantiomeric ratio was obtained with the silyl enolate of 2-phenylcyclohexanone (entry 5). 2-Arylcycloalkanones are the best substrates for the present asymmetric protonation and, for example, trimethylsilyl enolate of 2-phenylcycloheptanone also showed high enantioselectivity and reactivity (entry 7). As for *p*-methoxyphenyl, *p*-tolyl, and 2-naphthyl derivatives, almost perfect asymmetric induction was observed and (*R*)-enriched products were formed nearly quantitatively in every case (entries 8-10).[3]

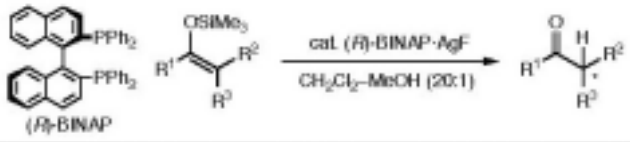
On the basis of these results, we further attempted the catalytic asymmetric protonation of α-aryl carboxylic acid enolates under the optimized reaction conditions. When ketene bis(trimethylsilyl) acetal of Naproxen **2** was treated with a catalytic amount of (*R*)-BINAP·AgF complex in a 20:1 mixture of dichloromethane and methanol, (*S*)-enriched Naproxen (**3**) was obtained in 93% yield with 8% ee (eq 1). Although we tried several other α-aryl carboxylic acids, satisfactory results were not obtained at all. Further studies on the reaction conditions including reaction temperature and solvent are necessary to improve the enantioselectivity.

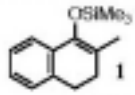
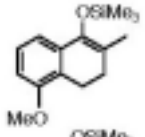
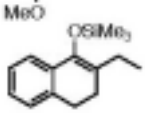
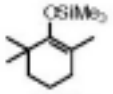
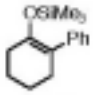
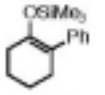
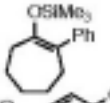
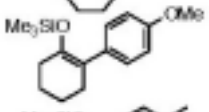
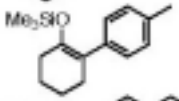
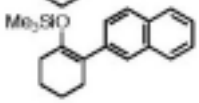
Discussion & Summary

The reaction mechanism was not fully elucidated; however, two mechanisms can be considered for the catalytic asymmetric protonation. As a probable catalytic mechanism, a BINAP·AgF complex is regenerated from an initial transition-state structure accompanied by the formation of the protonated product and methoxytrimethylsilane. In contrast, another mechanism generates BINAP·AgOMe and fluorotrimethylsilane from the transition-state structure via a transmetallation step. In the second cycle and thereafter, BINAP·AgOMe is recycled and acts as a chiral catalyst in the second transition-state structure.

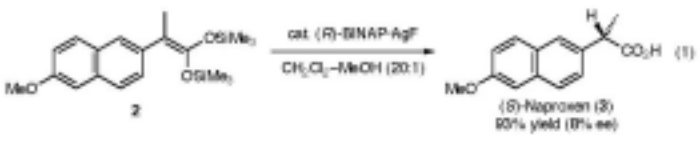
In summary, we have developed a novel catalytic asymmetric protonation system. By using a BINAP·AgF complex as a chiral catalyst and MeOH as an achiral proton source, various nonracemic ketones were prepared with enantioselectivity up to 99% ee. Further studies on the protonation of silyl enolates of α-aryl carboxylic acids and other carbonyl compounds are currently underway.

Table 1. (*R*)-BINAP·AgF-Catalyzed Asymmetric Protonation of Various Trimethylsilyl Enolates[a]



Entry	Trimethylsilyl Enolate	Conditions	Yield, %[b]	% ee (confign)[c]
1	 1	-20 °C, 36 h	72	62 (*S*)
2		-20 °C, 48 h	82	67 (*S*)
3		-20 °C, 48 h	75	64 (*S*)
4		0 °C, 48 h	82	87[d] (*S*)
5		-30 °C, 24 h	96	98 (*R*)
6[e]		-30 °C, 24 h	75	99 (*R*)
7		-30 °C, 48 h	95	97 (*R*)
8		-40 °C, 48 h	93	>99 (*R*)
9		-40 °C, 48 h	96	>99 (*R*)
10		-40 °C, 48 h	89	>99 (*R*)

[a] Unless otherwise noted, the reaction was carried out using (*R*)-BINAP (0.06 mmol), AgF (0.1 mmol), trimethylsilyl enolate (1 mmol), and MeOH (1 mL) in dry CH_2Cl_2 (20 mL). [b] Isolated yield. [c] The enantioselectivity was determined by HPLC analysis using a chiral column (OD-H). [d] The enantioselectivity was determined by GLC analysis using a chiral column (γ-TA). [e] (*R*)-*p*-Tol-BINAP was used.



(1)

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3) Chemical Sciences
3-1) Organic Chemistry

Synthetic Study on Glycopeptide Using Glycosyl Amino Acid Having Natural Oligosaccharide

Toshiyuki INAZU
Department of Applied Chemistry, School of Engineering, Tokai University
inz@keyaki.cc.u-tokai.ac.jp

Introduction

Recently we have reported the coupling efficiency of Fmoc-Asn(GlcNAc)-OH during solid phase synthesis of glycopeptide. In order to confirm the coupling efficiency of glycosyl amino acid having natural oligosaccharide for solid phase synthesis of glycopeptide, we prepared the Fmoc derivatives of amino acids having natural oligosaccharides from natural glycoproteins, such as ovalbumin, ovomucin, and so on.

Additionally we found the method for recovery of glycosylated amino acid derivatives, which were usually expensive, and valuable derivatives.

Results

1) Preparation of glycosylated amino acids

First we prepared ovalbumin from hen eggs. We are provided fresh eggs by the collaborated chicken yard. These eggs were laid at the same day by the same aged hens and were available within 2 days.

Ovalbumin was prepared by the combined method using saturation of ammonium sulfate and isoelectric point of the corresponding protein. We got ovalbumin in good yield under the conditions of a half saturation of ammonium sulfate at pH 4.6 from egg white. The purity of ovalbumin was detected by gel electrophoresis. We have prepared pure ovalbumin by reprecipitation method, which step was repeated 3 times.

Next the limited hydrolysis of ovalbumin using a-chymotrypsin was carried out. After several purification steps, such as ion-exchange chromatography and gel chromatography, we obtained glycosylated peptides.

Hydrolysis of glycosylated peptides by pronase, which step was repeated twice times, gave the mixture of glycosylated asparagines having several kinds of natural N-linked oligosaccharides. Finally the reaction of these glycosylated asparagines and Fmoc-OSu (*N*-9-fluorenylmethyloxycarbonyloxy-succiniimide) gave the corresponding Fmoc-Asn(Sugar)-OH,

which were isolated by HPLC.

In this study we also attempted to prepare ovomucin to obtain mucin-type (O-linked) glycosylated amino acids, such as Fmoc-Ser(Sugar)-OH and Fmoc-Thr(Sugar)-OH. It was found ovomucin was prepared easily from egg white in good yield by the direct centrifugation. Pure ovomucin was stored as lyophilized powder. The preparation of glycosylated amino acid having O-linked natural oligosaccharide is now in progress.

2) Solid phase synthesis of glycpeptides

The solid phase synthesis of glycopeptides by dimethylphosphinothic mixed anhydride (Mpt-MA) method has been studied. No protective group for hydroxyl function of sugar moiety was necessary during the coupling reaction using Mot-MA method.

Usually solid phase synthesis of glycopeptide was carried out using a large excess of glycosylated amino acid derivatives. The recovery of glycosylated amino acid derivative has been expected. Mpt-MAs were expected to decompose to Fmoc-amino acid and dimethylphosphinothic acid by only addition of water because they are classified into acid anhydride. Coupling reaction of Mpt-MA proceeded in the presence of tertiary amine, such as triethylamine, *N, N*-diidopropylethylamine (DIEA), and so on. When the reaction mixture after coupling reaction by Mpt-MA method was poured into water, glycosylated amino acid would be regenerated. We tried the recovery of Fmoc-Thr(Man)-OH during the solid phase synthesis of mannosyl peptide by Mpt-MA method.

After the solid phase coupling reaction of Fmoc-Thr(Man)-OMpt, which was derived from Fmoc-Thr(Man)-OH and Mpt-Cl, in the presence of DIEA in *N, N*-dimethylformamide (DMF), all of DMF filtrate and washings were poured into water. The decomposition of Mpt-MA was detected by TLC. A large excess of water was added and the aqueous solution was charged on a polymeric adsorbent, such as Dia-ion HP-20 and Amberlite XAD-2 resin. The resin was washed sufficiently with water and eluted with methanol. The methanol solution was corrected and evaporated under reduced pressure. We obtained Fmoc-Thr(Man)-OH in 98 % recovery yield. Recovered glycosylated amino acid was able to use for the synthesis of the corresponding Mpt-MA. This simple recovery system was applicable for only Mpt-MA method because there are Mpt-MA, tertiary amine, and DMF in the reaction medium by Mpt-MA. Because of this simplicity, this recovery step would be applied for the automated synthesizer.

3) The coupling efficiency of glycosylated amino acid derivative

The solid phase syntheses of several kinds of H-AA-Asn(Sugar)-Ala-Leu-Ala-resin and the evaluation of their coupling efficiency are now in progress.

Discussion & Summary

We prepared Fmoc-Asn(Sugar)-OH having natural N-linked oligosaccharide from ovalbumin. Similary we attempted to prepare ovomucin in order to obtain Fmoc-Ser(Sugar)-OH and Fmoc-Thr(Sugar)-OH having natural O-linked oligosaccharide. It was found the preparation of ovomucin was easily by only centrifugation of egg white.

Syntheses and evaluation of glycopeptide using these glycosylated amino acid derivatives are now in progress.

Usually a large excess of glycosylated amino acid was used for the synthesis of glycopeptide. We tried the recovery of glycosylated amino acid and found a simple method for Mpt-MA method. This recovery method consisted 2 steps, 1) decomposition of mixed anhydride to acid; 2) recovery of the Fmoc glycosylated amino acid by polymeric adsorbent. We are now studying the optimum conditions for this recovery system.

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4) Others

Identification of new peptide agonists specific for circulatory orphan receptors using internalization assay

Kenji Kuwasako
First Department of Internal Medicine, Miyazaki Medical College, University of Miyazaki
kuwasako@fc.miyazaki-med.ac.jp

Introduction

It is well known that almost G protein-coupled receptor molecules are rapidly internalized after agonist exposure to inhibit over signaling *in vivo*. Nothing is reported about new peptide isolation using internalization assays of orphan receptors. In this study, we will establish stable transfectants for orphan receptors that abundantly expressed in circulatory systems and identify their specific peptides from extracts of human pheochromocytoma or normal porcine adrenal medulla using receptor internalization assay.

Results

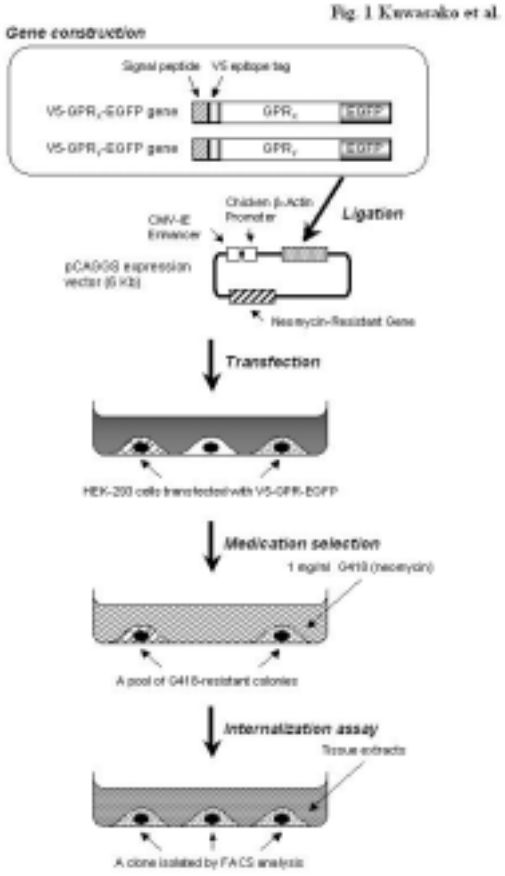
We initially selected orphan receptors that would need peptide agonists by computer research and limited the two ones (GPR_X and CGR_Y) that fully expressed in the circulatory organs and up-regulated in cardio-vascular diseases. We next amplified the two targeted orphan receptor genes by PCR methods using appropriate primers sets. To quantify the receptor internalization using flow cytometry, double V5 epitope tags were used in-frame to the 5' end of cDNAs encoding V5-GPR_X and V5-GPR_Y, and the native signal sequences were removed and replaced with MKTILALSTYIFCLVFA (Guon et a. *J Biol Chem*. 1992; 267: 21995-21998). In addition, EGFP genes were ligated in-frame to the 3' end of the V5 epitope-tagged gene constructs to directly observe receptor internalization using fluorescence microscopy. Both sided, tagged receptor genes (V5-GPR_X-EGFP and V5-GPR_Y-EGFP) were then cloned into the mammalian expression vector pCAGGS/Neo using the 5' *Xho* I and 3' *Not* I sites (1), termed pCAGGS/Neo-V5-GPR_X-EGFP and -V5-GPR_Y-EGFP. We selected human embryonic kidney (HEK)-293 cells for transfections because they possess adequate components required for receptor internalization, such as G protein-coupled receptor kinases (GRKs). Cells grown to 70% confluence were transfected with pCAGGS/Neo-V5-GPR_X-EGFP and -V5-GPR_Y-EGFP using LipofectAMINE transfection reagent. Two days after transfection, cells were plated in selective medium containing 1 mg/ml G418. A pool of G418-resistant colonies was trypsinized followed by limited dilution and single cell plating in to 96-well plates. Three weeks after transfection, EGFP-expressing clones were all screened for

expression of V5 epitope tags by flow cytometry. We selected the most suitable clones for internalization assay. These two transfectants were maintained in the same buffer containing 0.25 mg/ml G418. On the other hand, bovine and porcine adrenal medullae and porcine atria, ventricles and lungs were extracted by our previous methods by Kitamura, K., et al (2).

Discussion & Summary

We have succeeded to establish the transfectant cell lines stably expressing V5-GPR_X-EGFP and V5-GPR_Y-EGFP and to fully obtain the aforementioned tissue extracts. The following experimental procedures will be performed from now on: Unknown peptides are purified using gel filtration, ion-exchange chromatography, various reversed-phase HPLC, etc. Following 30-min sample treatment at 37°C, cells are stained by rhodamine-labeled antibody at 4°C and the loss of cell surface receptor expression are quantified using flow cytometry. The fractions that induced full internalization are collected and used for the next step of peptide purification. The final purifications are analyzed by protein sequencer. The tissue distribution and functional properties of the unknown peptides are further examined.

Figures & Tables



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Part II

Reports from the Recipients of Garants for International Meetings

(Fiscal Year 2003)

11th International Conference on the Cell and Molecular Biology of Chlamydomonas (Chlamy2004)

1. Representative
   Yoshihiro Matsuda, the chairman of organizing committee

2. Opening period and Place
   May 11-15, 2004, International Conference Center Kobe, Kobe, Japan

3. Number of Participants
   167 persons (89 persons from 10 foreign countries)

4. The total costs
   Eleven million yen

5. Main use of the subsidy
   Costs for setting of poster panels and for equipment rent at the opening place

6. Result and Impression
   The *Chlamydomonas* conference has been held every 2 years since1982 – seven times in USA, twice in Europe, and once in Canada. The present conference was held in Japan for the first time. Three organizers (Y. Matsuda, R. Kamiya and H. Fukuzawa) hoped to have participants from many countries who use *Chlamydomonas* and related organisms, especially to have Japanese phycologists who have been doing excellent works on the fields of molecular biology and biotechnology, in order to enhance scientific exchange. For this purpose, Chlamy2004 was supported by 6 foundations and backed by 6 Societies in Japan, related to plant and algal researches. We are pleased to report that these financial aids were able to support a part of travel expenses of 55 young scientists and 18 invited persons.
   This conference aimed at providing a platform on which world-wide researchers using *Chlamydomonas* and related organisms can present their new findings, and thereby promoting development of the cell and molecular biology of this organism. Since the value of *Chlamydomonas* as an excellent model organism has recently been further raised, owing to the development of new biotechnology as well as completion of its genome project, this meeting was particularly timely for constructing a strong international network of researchers in the forthcoming post-genome era.
   During 5 days of this meeting, we had 72 oral presentations and 85 poster presentations, dividing into 11 sessions: (1) Photo- and Chemosensory Processes, (2) Structure and Function of the Photosynthetic Apparatus, (3) Genomics and Proteomics, (4) Basal Body and Flagellar Assembly, (5) Acclimation to Environmental Stress, (6) Genomic Techniques, (7) Flagellar Motility: Regulation and Mechanism, (8) Organelle Biogenesis, (9) Mating and Cell Division, (10) Little

Green Cells and Major Evolutionary Questions, and (11) Discussion on Genome Annotation. All sessions were held at the same one big room from 9 am to 9 pm everyday so that all participants could listen to all sessions of different research fields. Furthermore, we reserved a room at the Portopia Hotel for further discussion after the night sessions. Especially, the participation of many Japanese scientists with different research careers and interests other than *Chlamydomonas* to this meeting provided a proper jolt to their biological knowledge of *Chlamydomonas* biologists.

7. Additional description

At the business meeting, it was decided that the meeting report on the Chlamy2004, Kobe, will be published soon in Protist and that the next *Chlamydomonas* conference will be held at Oregon (USA) in 2006.

Report of 21st International Lectin Meeting (Interlec 21)

1. Representative

Kenichi Kasai, Chairman of the Organizing Committee of 21st International Lectin Meeting

2. Opening Period and Place

May 23 - 28, 2004.

Shonan Village Center and Hotel Okuyumoto in Hakone

3. Number of Participants

182 persons (52 persons from foreign countries)

4. Total Cost

18,190 thousand yen

5. Main use of the subsidy

Support for travel expenses of invited speakers

6. Result and Impression

Importance of research on lectins, natural molecules having the role of deciphering glycocodes, has been growing day by day, in concordance with the significant development of glycobiology. Glycomics should be the most important field of life science after the era of genomics and proteomics, and lectinology will certainly take an essential part in it. Interlec meeting, only one series of international scientific forum focused on lectin research, have been held every two years around the world, and made a great contribution to the progress of life science, especially glycobiology. It was extremely important to organize the Interlec meeting for the first time in Japan at such a significant moment.

Thanks to many scientists who have submitted excellent abstracts, we were able to make a splendid program. A number of papers on the latest results in a variety of aspects of lectins were presented by both established and young researchers. Principal topics were as follows: 1) Structure, function and specificity of lectins. 2) Regulation of cells by lectins. 3) Mechanism of sugar recognition. 4) Characterization of newly identified lectins. 5) Lectins as tools for life science. 6) Modification of lectin function by genetic engineering. 7) Medical application of lectins. 8) Application of lectins to agricultural and technological sciences. 9) Molecular evolution of lectin. 10) New methodology for lectin research.

During the meeting, 61 papers were presented orally and 70 papers were presented as posters.

Participants could gain a comprehensive view on current state of lectin research and enjoy the scientific program. For all oral presentations, there were active questions, comments and discussions between the presenters and audience. Although the poster sessions were assigned after dinner, discussions by enthusiastic participants did not end until late hours in the evening.

The meeting seems to be extremely fruitful especially for young researchers because it was a valuable experience for them to have been able to meet for the first time many established and famous lectin researchers whom they knew only by name, listen to their lectures, and make personal contact. Such an experience should certainly have greatly stimulated and encouraged these young researchers. It seems also very fruitful for them to get aquainted with many other young lectin researchers because they will be able to collaborate each other and contribute to further development of lectin research in the future.

During the course of the meeting, many items were planned to entertain participants both scientifically and socially. We especially wished to offer participants from foreign countries opportunities to experience Japan's unique culture, so different from that of the west, and gain a new perspective on human activities, which may help to produce new ideas for scientific research. We have already received letters from many participants which are kindly enough to estimate that the meeting was the most exciting and fruitful one ever held. They also acknowledge all efforts made by the organizing committee. Therefore, we now feel very happy to be albe to finish our task successfully, and would like to express our sincere gratitude for the support from your foundation.

7. Additional description

Nationality of participants from foreign countries: USA, Canada, Argentin, Brazil, Urguai, Australia, Taiwan,

China, Thai, India, Israel, Germany, The Netherland, Switzerland, Belgium, France, United Kingdom, Sweden,

Denmark.

International Narcotics Research Conference 2004/Kyoto

1. Representative

Masamichi Satoh

2. Opening period and Place

July 18- July 23, 2004

3. Number of participants

291 persons (including 140 persons from 18 foreign countries and areas)

4. The total cost

¥29,663,521-

5. Main use of the subsidy

Support for travel expenses of an invited speaker and 4 young scientists.

6. Result and impression

The International Narcotics Research Conference (INRC) is held annually at the middle of July, where a lot of scientists from diverse places on earth and various study fields discuss the novel findings regarding Opioids. The first international conference was held in a classroom of the University of Basel (Switzerland) as a satellite session of narcotics of International Union of Pharmacology (IUPHAR) on July 19, 1969. The meeting was organized by the Late Drs. Hans Kosterlitz and Harry Collier. After that, the conference has been held every year. In 1981, the 12th meeting of INRC was held in Kyoto, Japan, organized by Dr. Hiroshi Takagi, as a satellite meeting of IUPHAR held in Tokyo. And 23 years later, we had this present conference again in Japan and it was the 35th meeting of INRC, held in Kyoto from July 18-23, 2004, and named "INRC2004/Kyoto".

INRC2004/Kyoto was jointly sponsored by the Japan Foundation for Neuroscience and Mental Health, the Japanese Narcotics Research Conference and the National Institute on Drug Abuse (NIDA), and supported by 5 societies and associations in Japan (including the Japanese Pharmacological Society, the Pharmaceutical Society of Japan, Japan Neuroscience Society, Japanese Association for the Study of Pain, and Japan Society of Pain Clinicians). There were financial aids, the subsidies by NIDA and 6 foundations, including yours, and the donation from the Osaka Pharmaceutical Manufacturers Association, the Pharmaceutical Manufacturers' Association of Tokyo, and 20 enterprises.

In this conference, two plenary lectures were presented as follow; “Psychological Basis of Compulsive Drug-Seeking: Implications for Treatment” by Dr. Barry J Everitt (University of Cambridge, UK), and “Regulatory Mechanisms of Synaptic Transmission in the Neural Circuit” by Dr. Shigetada Nakanishi (Kyoto University, Japan). A Founders’ Lecture, “Acupuncture, Endogenous Opioids and Anti-opioids” was presented by Dr. Ji-Sheng Han (Peking University, China). It showed us the surprising effectiveness of acupuncture for the pain control. In 10 symposia, (I) Intractable Pain and Analgesia, (II) New Molecules in Receptor Trafficking/ Signaling-I, (III) Gene Regulation and Human Genetics, (IV) Neurobiology and Opioids, (V) New Compounds/Molecules in Opioid World, (VI) New Trends in Opioid-related Peptide Research, (VII) New Molecules in Receptor Trafficking/ Signaling-II, (VIII) Molecular Psychiatry, (IX) CB1-Opioid-Glutamate Cross-talk in Reward and Addiction, and (X) New Frontier in Addiction Research, 29 symposiasts and 21 regular speakers selected from regular paper gave us lectures. In the poster session, 129 posters remained on display throughout the conference, and were excitingly discussed for three hours on Monday 19 and Thursday 22. In addition, two luncheon lectures were presented as follow; “Clinical Utility of Oxycodone in the Management of Pain” by Dr. Barry A. Eagel (Purdue Pharma L.P.), and “Locus-specific Rescue of Opioid and Anti-opioid genes into Knock-out Mice for the Study of Opioid Tolerance and Dependence” by Dr. Hiroshi Ueda (Nagasaki University, Japan). Total 184 papers were fully presented for 5 days. The sessions started at every 8:30 am, and there was no break except for lunchtime. We all had a solid time for discussion throughout the conference. In addition, because all oral presentations were held in the same room, the participants were able to fully listen to all lectures.

Although opioids have been widely used in the clinical management of pain including chronic cancer pain, their liability to develop tolerance and dependence often restricts the range of application. The mechanisms of the development of opioid tolerance and dependence have been vigorously investigated for a long time, and many recent researchers have tried to elucidate them at the molecular level. In this conference, there were a lot of advanced findings about the molecular mechanisms from various points of view. These studies proposed novel points of view and new methods in succession, indicating the high activities of opioid researchers. Furthermore, it was reported that opioidergic system plays an important role in the dependence on other drugs of abuse such as cannabinoids and nicotine. Subsequently more researchers in the field of other substance dependence are thought to take part in the field of opioid research. Opioids and their receptors are the issue of discussion for not only their side effects such as tolerance and dependence, but also their possible application for depression, anxiety, learning and memory, itch, and immune suppression. There is a body of evidence about the relationship between opioids and glial cells, reported in some symposia, “Neurobiology and Opioids” and so on. All together, the field of opioid research is still deriving a large amount of interest from many scientists in the world.

It was decided that the next 36th meeting of INRC were going to be held at Annapolis, Maryland in the U.S.A. from July 10-15, 2005.

The 4th Tateshina Conference on Organic Chemistry

1. Representative

Eiichi Nakamura, the chairman of organizing committee

Keisuke Suzuki, the general secretary of organizing committee

Takuzo Aida, Makoto Fujita, Terunori Fujita, Takehiko Iida, and Keiji Maruoka, the members of organizing committee

2. Opening period and place

November 12-14, 2004 at Lodge Mitsui-no-Mori and Tateshina Forum in the city of Chino, Nagano prefecture, Japan

3. Number of participants

73 persons (16 persons from foreign countries)

4. The total cost

2.5 million yen

5. Main use of the subsidy

Support for travel expenses of invited foreign speakers

6. Results and impression

The Conference was hold at Tateshina Mitsui-no-Mori Lodge, located at an altitude 1,100 m in the city of Chino, Nagano prefecture between November 12 (Fraiday) and November 14 (Sunday). We had seven scientific sessions, including six lectures and one poster session. Six invited speakers presented general description of their subject area as well as the most recent discovery from the speaker's laboratories. The session included 50-min talk and 30-min discussion so that participants could fully appreciate the present status and the future development of the field where you are active.

The organizing committee gave one-paragraph explanation of the Conference for the information. For sometime, relatively young generations of Japanese organic chemists and myself have been pondering on a possibility of having a conference for Asian chemists. The organizing committee therefore consulted some eminent chemists in Japan, China, Korea, Taiwan, and Hong Kong about a new style conference to be held in our country. Professors Mukaiyama and Noyori were among those who strongly supported this idea. We therefore gave a trial meeting in November 2000 with industrial and academic people from Japan, Korea, and Thailand including topics of synthetic,

supramolecular, natural product chemistry and molecular biology. There, all participants agreed to put forward the idea of East Asian conference. Professor Eiichi Nakamura was elected to become Chairman of the 4th Conference.

One of the most attractive features of the Conference was high-standard speakers, Professor C.-H. Wong of The Scripps Research Institute, Professor K. Nakanishi of Columbia Univ., Dr. Y. Hsiao of Merck, Mr. T. Arimoto of MEXT, Professor R. F. Ismagilov of The Univ. of Chicago as well as Professor A. Fujishima of KAST. The participants are as important as speakers, and about sixty-five of them included people of all area related to organic chemistry, from industry and academia, and from various parts of East Asia.

The organizing committee and Asian liaison members are confident that the Conference is unique in its style, and effective in generating a friendly, cooperative and creative atmosphere among participants, providing Asian chemists with a forum for the advancement of organic chemistry in this part of the world.