Department of Molecular Biology

Senya Matsufuji, *Professor*
Noriyuki Murai, *Assistant Professor*
Akihiro Oguro, *Assistant Professor*

General Summary

Polyamines (putrescine, spermidine, and spermine) are ubiquitous biogenic amines that bind to nucleic acids and play essential roles in DNA replication and gene expression. They are also involved in various cellular processes, such as apoptosis, autophagy, and control of ion channels. Cellular polyamine contents are maintained by a feedback mechanism involving the key regulatory proteins antizymes (AZs). AZs are expressed by translational frameshifting in a polyamine-dependent manner and negatively regulate cellular polyamines. AZ is widely conserved in eukaryotes. In mammals there are 3 AZ isoforms (AZ1–3). AZs are further regulated by proteins termed antizyme inhibitors (Azins). Our goal is to clarify the mechanism and biological significance of the elaborate regulatory system and to develop polyamine-related research tools.

Research Activities

Protective effect of AZ1 against over-intake of polyamines
Because polyamines are essential for normal cell function and because the polyamine content in tissues declines with age, polyamines have been evaluated as possible antiaging agents. On the other hand, excess polyamines are known to be harmful. We have shown that elevated levels of putrescine in AZ1-knockout mice disturb ontogenesis and hematopoietic cellular differentiation. To investigate the potential protective role of AZ1 against over-intake of polyamines, we performed a long-term feeding experiment. Adult AZ1-deficient mice and control mice were fed composite meals containing a high dose of polyamines (25 times that of normal meal) for 1 year. At the end of the experiment, animal tissues were subjected to biochemical or morphological examinations. Although no difference was found in body weight or life span between the groups, multiple tumors developed in the livers of only AZ1-deficient mice. The result suggests that AZ1 plays a protective role against tumorigenesis induced by over-intake of polyamines.

Mechanism and significance of c-Myc degradation mediated by AZ2
AZ binds to ornithine decarboxylase (ODC), a key enzyme for polyamine biosynthesis, triggering degradation of the enzyme by proteasomes. It has also been reported that AZ1 binds to and destabilizes various proteins other than ODC. Last year we found that AZ2 binds to c-Myc and accelerates its degradation in cultured cells. We further investigated the mechanism and biological significance of AZ2-mediated c-Myc degradation. Antiproliferative signals are known to accelerate proteasomal degradation of c-Myc via a mechanism that requires phosphorylation at 2 amino acid residues, threonine

58 and serine 62, and subsequent polyubiquitination of c-Myc by a ubiquitin ligase termed F-box and WD repeat domain-containing 7 (FBW7). To characterize the AZ2-mediated c-Myc degradation, we used a mutant c-Myc in which the 2 residues are substituted with alanine (T58A/S62A). In cultured cells, the T58A/S62A mutant was destabilized by coexpressed AZ2, but not by FBW7, suggesting that AZ2-mediated c-Myc degradation is independent of polyubiquitination. The significance of AZ2-mediated c-Myc degradation was investigated in UV-irradiated cells, because UV-induced c-Myc degradation is mediated, at least in part, by an ubiquitin-independent pathway. We observed that c-Myc degradation was accelerated by UV irradiation and that the acceleration was suppressed by knockdown of AZ2 through RNA interference. Thus, AZ2 likely mediates c-Myc degradation with certain stresses, such as UV irradiation.

Fluorescent visualization of cancer cells by monitoring intracellular polyamines

Cancer cells generally contain elevated levels of polyamines. We are developing a novel method to visualize cancer cells by combining the polyamine-dependent frameshift mechanism of AZ, an endogenous cellular polyamine sensor, and the fluorescent protein techniques. The frameshift signal sequence of AZ1 messenger (m) RNA, either single or in tandem, was inserted between 2 fluorescent proteins, enhanced cyan fluorescent protein (ECFP) and Keima-Red, so that the ECFP-Keima-Red fusion protein was synthesized only under high-polyamine conditions. The constructs were introduced into mammalian cultured cells and analyzed with fluorescent microscopy. We observed fluorescence of both ECFP and Keima-Red in the transfected cells, but the intensity of Keima-Red fluorescence did not correspond to the polyamine concentration. The system should be improved by utilizing more appropriate AZ1 frameshift signals to gain sensitivity to polyamines.

Expression analysis of Azin1

Azin1 is a homolog of ODC that positively regulates cellular polyamines by binding to and inhibiting AZs. Cellular levels of Azin1 change with growth stimuli or tumorigenesis and are regulated by polyamines. To clarify the mechanism of transcriptional regulation of Azin1, we analyzed various transcripts caused by alternative initiation and splicing in both wild-type mice and homozygous *Azin1* gene trap mice. The gene trap mice have a partially lethal condition with decreased tissue levels of ODC and putrescine. However, Northern blotting detected a certain level of *Azin1* mRNA remaining in the homozygous gene trap mice, and Western blotting analysis revealed that embryonic fibroblasts from the homozygous gene trap mice contained Azin1 protein at a level 20% to 30% of that in wild-type controls. These results raised the possibility that alternative forms of *Azin1* mRNA are transcribed to skip the trapping insertion. Therefore, we determined transcriptional start sites (TSSs) of *Azin1* gene using the oligo-capping method and the reverse transcriptase-polymerase chain reaction with primers designed after the TSS database. The analyses revealed that the *Azin1* gene trap mice contain a small amount of the full-length transcript and a large amount of a transcript lacking exon 2, both starting from the authentic TSS. In addition, the gene trap mice express alternative transcripts with exon 3, which are minimally utilized in wild-type mice. The expression of the tran-

scripts with exon 3 showed a tissue-specific pattern. Furthermore, other forms of alternative spliced transcripts were detected in both wild-type mice and gene trap mice. Some of the transcripts did not contain translational regulatory elements of polyamines or were expected to encode a form of *Azin1* lacking the AZ-binding domain. These results, together with previous findings, demonstrate the complicated regulation of *Azin1* expression.

Development of a cancer diagnostic system with RNA aptamer for polyamines
RNA aptamers are functional RNAs that bind to their target biomolecules with high affinity and can distinguish similar chemical structures. We aimed to develop a simple diagnostic system for cancer using RNA aptamers for polyamines, which are promising biomarkers for cancer. Last year we isolated an RNA aptamer with high affinity for spermine (spermine aptamer). To determine the binding specificity of the spermine aptamer, we compared the affinity of the aptamer to spermine and related amines with the systematic evolution of the ligand exponential enrichment (SELEX) method. The spermine aptamer was shown to have specificity to linear tetra-amines that contain primary amines at both ends and 3 or 4 carbon atoms between amine moieties. Next, we made an affinity column with immobilized spermine aptamer and performed a semiquantitative analysis of spermine in the solution. It detected spermine with a range of 0.01 to 3 mM. In addition, the spermine aptamer was labeled with fluorescein and used to visualize spermine in the solution under UV light.

Publications

Murai N, Murakami Y, Matsufuji S. Protocols for studying antizyme expression and function. *Methods Mol Biol* 2011; **720:** 237–67.

Reviews and Books

Ivanov IP (Univ Col Cork), Matsufuji S. Autoregulatory frameshifting in antizyme gene expression governs polyamine levels from yeast to mammals. In: Atkins JF, Gesteland RF, editors. Recoding: Nucleic acids and molecular biology 24. New York: Springer; 2010. p. 281–300.