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General Summary

Polyamines (putrescine, spermidine, and spermine) are ubiquitous biogenic amines that bind mainly to nucleic acids and are essential for cell proliferation. Ornithine decarboxylase (ODC) is a key enzyme of polyamine biosynthesis in mammalian cells. ODC converts ornithine to putrescine, which in turn leads to spermidine and spermine. ODC is degraded by interaction with antizyme (AZ). Three AZ isoforms (AZ1–3) are present in mammals. The AZs are expressed by translational frameshifting that is induced by polyamines and negatively regulate cellular polyamines. Cellular polyamine contents are maintained by the feedback mechanism involving AZ. The AZs are further regulated by proteins termed AZ inhibitors (Azins). Cancer cells generally contain elevated levels of polyamines. Our goal is to clarify the mechanism and biological significance of the elaborate regulatory system and to develop polyamine-related research or diagnostic tools.

Research Activities

Interaction between MYCN and AZ2 in neuroblastoma cells
We have found that AZ2 interacts with the cellular myelocytomatosis oncogene protein (c-MYC) in the nucleolus and nucleoli and accelerates c-MYC degradation in a ubiquitin-independent manner. This year we tested interaction between AZ2 and another member of the v-myc avian myelocytomatosis viral oncogene homolog (MYC) family, the MYC neuroblastoma derived homolog (MYCN), which is overexpressed in neuroblastoma cells. The high-level expression of AZ2 has been reported to correlate with increased survival in patients with neuroblastoma. Pull-down assay demonstrated that AZ2 specifically interacted with MYCN in a human neuroblastoma cell line, SH-SY5Y. In addition, binding of AZ2 accelerated MYCN degradation. This result suggests that AZ2 accelerates MYCN degradation in an ubiquitin-independent manner as it accelerates ODC or c-MYC degradation.

Analysis of interaction between AZ2 and ATP citrate lyase
Previous screening for AZ-binding proteins identified ATP citrate lyase (ACLY), a cytosolic enzyme that catalyzes the production of acetyl-CoA. We confirmed that both AZ1 and AZ2 bind to ACLY and that AZ colocalizes with ACLY to the cytoplasm. Unexpectedly, neither AZ1 nor AZ2 accelerated ACLY degradation. Instead, purified AZ, particularly AZ1, increased the activity of purified ACLY in a dose-dependent manner *in vitro*, suggesting that AZ activates ACLY through protein-protein interaction. Polyamines had no effect on ACLY activity *in vitro*. Knockdown of AZ1 or AZ2 or both in human cancer cells significantly decreased ACLY activity and the cellular levels of acetyl-CoA and cho-

lesterol, indicating that AZ regulates acetyl-CoA production in living cells.

Heterogeneity of hematopoietic stem cells in AZ1 *knockout mice*
We continued our research of *AZ1* knockout (*AZ1*$^{-/-}$) mice. The significant features of *AZ1*$^{-/-}$ mice are the increased putrescine level in tissues and the partial embryonic lethality with severe anemia. Last year, we showed the heterogeneity of hematopoietic stem cells (HSCs) from fetal liver, which have long-term bone marrow reconstruction ability. To study whether HSCs, which migrate from fetal liver to bone marrow in the late embryonic stage, have heterogeneity that continues for life, we collected *AZ1*$^{-/-}$ bone marrow cells from 6-month-old mice, transplanted the bone marrow cells including the HSCs into recipient mice, and assayed the repopulating potential of the HSCs. The *AZ1*$^{-/-}$ HSCs from the bone marrow indicated heterogeneity similar to that of HSCs from the fetal liver.

Analyses of physiological roles of Azin1
To address the regulatory roles of Azin1, we used spontaneously immortalized mouse embryonic fibroblasts (MEFs) from wild-type mice and mutant mice, in which expression of Azin1 is greatly decreased. We analyzed metabolite profiling of MEFs with capillary electrophoresis-mass spectrometry. Strong metabolic abnormalities in polyamines, folate, and nucleotide metabolism were noted in the mutant MEFs. Next, cell cycle progressions of MEFs were analyzed with flow cytometry using propidium iodide. The G2M ratio was found to be increased. The sub-G1 (hypodiploid) peak, which was regarded as specific for apoptosis, was noted in the mutant MEFs.

Analysis of AZ frameshifting mechanism with human in vitro *translation systems*
Using human *in vitro* translation systems, we are analyzing the molecular mechanism of ribosomal frameshifting of AZ expression. Polyamines induce both ribosomal frameshifting and read-through at stop codons. As a result, multiple translation products are detected in these systems, and the exact frameshift efficiency is difficult to analyze. To prevent this problem, the reporter for these systems was improved to synthesize single frameshift products and single read-through products. The stop codons in read-through frames were replaced with codons for alanine. In addition, the read-through product was translated as a green fluorescent protein fusion protein to be separated from the frameshift product on sodium dodecylsulfate-polyacrylamide gel electrophoresis. All synthesized peptides are released by the virus peptide release signal independent of the stop codon. This reporter expressed expected products in both the HeLa cell extract system and the human Protein synthesis Using Recombinant Elements (PURE) system. We are performing experiments with this reporter to examine the molecular mechanism of AZ frameshifting.

Interaction analysis of spermine-binding RNA aptamers with spermine
Aptamers of RNA are useful for exploring RNA-binding sequences and structures for target molecules. The spermine-binding aptamer SL_2 consists of 2 stems: the terminal-side stem and the loop-side stem separated by an internal loop. These structures were necessary for their spermine-binding activities. Replacement of an A-U base pair adjacent to

an internal loop in the terminal-side stem with a G-C base pair (SL_2 GC) reduced spermine-binding activity. Nuclear magnetic resonance signals of the terminal-side stem in SL_2 were observed to be broader than signals in SL_2 GC, indicating that the terminal-side stem of SL_2 takes a loose structure. We propose that the loose stem provides the plasticity to the RNA and is necessary for the spermine-RNA interaction.

Publications

Tajima A, Murai N, Murakami Y, Iwamoto T, Migita T (Jpn Fdn Cancer Res), Matsufuji S. Polyamine regulating protein antizyme binds to ATP citrate lyase to accelerate acetyl-CoA production in cancer cells. *Biochem Biophys Res Commun.* 2016; **471:** 646-51.