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General Summary

We have been studying the biological role and the regulatory mechanism of polyamines by antizyme (AZ). Polyamines, such as putrescine, spermidine, and spermine, are biogenic polycathions that are present in cells of all types. Polyamines are essential for cellular growth and regulate the function of various biomolecules. Polyamines also induce a regulatory protein called AZ. AZ accelerates the degradation of ornithine decarboxylase (ODC), the key enzyme of polyamine biosynthesis, and inhibits the cellular uptake of polyamines. The induction of AZ by polyamines involves a unique mechanism, translational frameshifting. AZ is evolutionally conserved in a wide range of eukaryotes and, in mammals, 3 paralogues (AZ1, AZ2, and AZ3) have diverged.

Research Activities

Study of the physiological roles of AZ1 in knockout mice

AZ1 is a major form of AZ expressed systemically. The phenotype of homozygous AZ1 knockout mice is partial embryonic death. Embryos lacking AZ1 show decreased numbers of hematopoietic cells in the liver. With the colony forming assay, burst-forming unit-erythroid (BFU-e) was decreased in each secondary hematopoietic organ, namely the aorta-gonado-mesonephros (AGM) region, the fetal liver, and the adult bone marrow. Maternal administration of an ODC inhibitor, α-difluoromethylornithine (DFMO), prevented the hematopoietic disturbance in embryos lacking AZ1. Further analysis demonstrated that maternal administration of DFMO during the AGM period prevented the decrease in BFU-e in the fetal liver and the adult bone marrow. In addition, pretreatment with putrescine of floating cells from the AGM region of wild-type mice resulted in a decrease of BFU-e, but the addition of putrescine to soft agar medium during the colony-forming assay did not have this effect. These results suggest that exposure of early hematopoietic cells in the AGM region to a high concentration of putrescine is a major cause of the hematopoietic disturbances in AZ1 knockout mice.

Previous studies have shown that AZ1 knockout mice that survive until adulthood have body weights lower than those of their wild-type littermates. We examined the mechanism of this lean phenotype. AZ1 knockout mice born after maternal administration of DFMO showed body weights equal to those of their wild-type littermates at birth, but after 9 to 12 weeks they weighed less than their wild-type littermates. Blood hemoglobin levels were identical in AZ1 knockout mice and wild-type controls both at birth and at 9 to 12 weeks. Body fat mass measured with X-ray computed tomography (CT) was significantly lower in AZ1 knockout mice than in wild-type controls 12 to 14 weeks after birth, and the difference increased thereafter. Polyamine synthesis of adult

AZ1 knockout mice was significantly elevated, and spermidine/spermine N^1 acetyltransferase (SSAT) activity was 50% as high as in wild-type controls. Transgenic mice overproducing SSAT reportedly have a lean phenotype due to overconsumption of acetyl-CoA. A similar mechanism may account in part for the low body fat mass in AZ1 knockout mice. However, because the increase in SSAT activity in AZ1 knockout mice was much smaller than that in SSAT transgenic mice, other mechanisms for the body fat loss are likely involved.

Analysis of AZ2-interacting proteins

AZ2, the second form of AZ, is expressed throughout the body, as is AZ1, but at much lower levels. To search for AZ2-specific functions, we screened AZ2-interacting proteins from mouse brain and liver cDNA libraries with a yeast two-hybrid system and discovered cerebellar degeneration-related protein 2 (CDR2) as an AZ2-binding protein. CDR2 binds to AZ2 but not to AZ1 or AZ3. Although the function of CDR2 is unknown, it reportedly binds to c-Myc through its leucine zipper domain. This year we examined the interactions among AZ2, CDR2, and c-Myc with pull-down assays in human-derived 293F cells. The interaction between AZ2 and CDR2 was decreased with c-Myc expression, whereas the interaction between c-Myc and CDR2 was decreased with AZ2 expression; this result suggests competitive binding of AZ2 and c-Myc to CDR2. Next, the effect of AZ2 on the stability of CDR2 was analyzed in transfected 293 cells to assess the biological significance of the interaction. We postulated that AZ2 could destabilize CDR2, as does its main target, ODC. However, CDR2 was stabilized with co-transfection of AZ2. Finally, the subcellular localization of AZ2 and CDR2 in Neuro2a cells was examined using fusion proteins with enhanced cyan or yellow fluorescent proteins (ECFP, EYFP). When separately expressed, AZ2 was mainly localized in the nucleus, and CDR2 was localized in the cytoplasm. Co-expression of the 2 molecules changed the localization of AZ2, which became co-localized with CDR2 in cytoplasm, indicating their interaction in the cells.

In a related project, we screened AZ2-interacting proteins from mouse kidney cDNA libraries with a yeast two-hybrid system and obtained 75 candidate clones. We re-screened these candidates under stricter conditions and obtained 8 molecules. Pull-down assay was performed to confirm the specificity of interaction between AZ2 and these molecules and demonstrated that 3 of the 8 proteins interact only with AZ2 and that 2 proteins interact with both AZ1 and AZ2. Next, these proteins were tagged with EYFP and expressed in cells either alone or with ECFP-tagged AZ2. Observation with fluorescent microscopy revealed that AZ2 had a markedly different subcellular localization and was now co-localized with candidate proteins when co-expressed with 2 of the candidate proteins.

Study of RNA-binding proteins that bind to the pseudoknot structure of AZ mRNA

The mRNA of AZ has a pseudoknot structure as a signal for polyamine-dependent translational frameshifting, but the precise mechanism of this regulation has not been clear. We hypothesized that a protein that binds to the pseudoknot structure is involved in the translational frameshifting; to test this hypothesis we surveyed such

RNA-binding proteins with the UV-crosslinking method. A protein of 34 kDa that specifically bound to a mutant version of AZ1 mRNA pseudoknot was detected in human-derived 293F cells and was successfully purified using the pseudoknot RNA as an affinity ligand. Analysis with the peptide mass fingerprint method identified 2 candidates, namely heterogeneous ribonucleoprotein (hnRNP) A1 and a related protein, hnRNP A1 like (hnRNP A1L). The cDNAs of these proteins were introduced into 293F cells with the dual luciferase reporter gene system to measure their effects on AZ1 translational frameshifting. Dual luciferase assay revealed that hnRNP A1L doubled the frequency of frameshifting, whereas hnRNP A1 had no effect. Stimulatory effects of hnRNP A1L were observed, however, both on the reporter genes with the wild-type pseudoknot and with the mutant pseudoknot. We are now confirming the binding of these proteins to the pseudoknot structure and their effects on frameshifting in vivo by preparing recombinant hnRNP A1 and hnRNP A1L proteins from *Escherichia coli*.

Publications

Isome M*[1]*, Lortie MJ*[1]*, Murakami Y (Musashino Univ), Parisi E*[1]*, Matsufuji S, Satriano J*[1] *([1]Univ California San Diego). The antiproliferative effects of agmatine correlate with the rate of cellular proliferation. *Am J Physiol Cell Physiol* 2007; **293:** C705-11.