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General Summary

Polyamines (putrescine, spermidine, spermine) are ubiquitous biomolecules necessary for cell growth. Growth stimuli induce marked increases in the polyamine content of cells, whereas a feedback mechanism prevents over-accumulation of polyamines. A protein called antizyme (AZ) plays a key role in the feedback system. AZ is induced by polyamines and blocks the cellular polyamine supply by accelerating degradation of ornithine decarboxylase, a rate-limiting enzyme for polyamine biosynthesis, and by inhibiting cellular polyamine uptake. AZ is conserved from yeasts to humans, and in mammals there are 3 AZ paralogs (AZ1-3). These AZs are further regulated by another set of regulatory proteins, antizyme inhibitors (AZINs) 1 and 2. Our goal is to clarify the significance of the complex regulatory system mediated by so many proteins and the differential roles of the regulatory proteins, as well as to develop related research tools.

Research Activities

Study of the physiological roles of AZ1 in knockout mice

In homozygous AZ1-knockout mice, tissue levels of polyamines are markedly increased. We analyzed urinary polyamines in AZ1-deficient mice and found an increase in acetylated polyamines, which suggests up-regulation of tissue polyamine catabolism via acetylated derivatives. In fact, spermidine/spermine *N*¹-acetyl transferase (SSAT) activity was elevated in several tissues, including the liver and spleen, but not in the brain or liver. In addition, we found that the SSAT activity was negatively correlated with the AZ2 level in the tissues. Maximal inducible levels of AZ2 may differ among organs, and when AZ1 is absent, the lower levels of AZ2 may lead to the higher influx of polyamine into cells, resulting in the induction of SSAT.

To study a possible protective role of AZ1 against over-intake of polyamines, we administered excess spermidine (10 times standard intake) to AZ1-deficient mice and wild-type controls daily for 1 week. This treatment caused a greater weight loss in AZ1-deficient mice. After 1 week of treatment, the polyamine contents of the whole blood and liver, respectively, of AZ1-deficient mice were 3 times and 1.5 times as high as those in wild-type controls. These results suggest that the excess spermidine intake affects polyamine kinetics throughout the body, particularly in AZ1-deficient mice.

Study of the physiological roles of AZIN1 in knockout mice

The physiological role of AZIN1 was studied in knockout mice. We confirmed in 2 co-isogenic lines of AZIN1-knockout mice back-crossed to C57BL/6J and BALB/c that a major phenotype of AZIN1-deficient mice is partial embryonic death, although we had previously reported that AZIN1-deficiency in a mixed genetic background was

completely lethal. Biochemical analysis revealed decreases in the tissue levels of putrescine and spermidine and in urinary polyamine excretion, confirming that total polyamine synthesis is suppressed in AZIN1-knockout mice.

Analysis of AZ2-interacting proteins

Previously we found a specific interaction between AZ2 and cerebellar degeneration-related protein 2 (CDR2), which binds to the proto-oncoprotein c-Myc. Pull-down assays showed that AZ2 interacts directly with c-Myc. This interaction was confirmed by the expression of fluorescent protein-tagged AZ2 and c-Myc in COS-7 cells. AZ2 was distributed in both the cytoplasm and nucleus, and c-Myc was localized in the nucleus when expressed alone, but the 2 proteins were co-localized in the nucleus when co-expressed. In addition, we found that AZ2 accelerated degradation of c-Myc, just as it does for ornithine decarboxylase, in a transient expression system in 293-F cells.

We have identified 2 AZ2-interacting protein from a mouse complementary DNA library using 2-hybrid analysis, pull-down assays, and analysis of the cellular localization of fluorescent-tagged proteins. One of these protein, zinc finger HIT domain-containing protein 1 (Znhit1), interacts with the tumor suppressor p53. Using a pull-down assay with epitope-tagged proteins, we demonstrated that AZ2 and p53 competitively bind to Znhit1.

Improvement of a bacterial system to select RNA-binding peptides targeting AZ pseudoknot RNA

The induction of AZ expression by polyamines is mediated by a unique translational frameshift mechanism. A signal for the translational frameshifting is a pseudoknot structure on AZ messenger RNA. To understand the mechanism of pseudoknot action, we used a bacterial selection system to screen for an artificial peptide that binds to the pseudoknot structure, but we failed to find such a peptide. The selection system is based on a reporter system in which expression of the reporter gene depends on bacteriophage lambda anti-termination activity mediated by N protein that binds to an RNA sequence termed boxB. Substitution of the combination of N protein-boxB interaction with library peptide-target RNAs allows us to screen artificial RNA-binding peptides. A possible reason screening failed is the limitation in the size of heterologous RNAs that can be accommodated in the antitermination system. The effects of the lengthening of the boxB stem were therefore examined. We found that an extension of the boxB stem led to a loss of activity, which was partially reversed by extending the RNA spacer located between boxB and boxA, another element of the antitermination system. The findings will be useful for improving the screening system for novel AZ pseudoknot-binding peptides.

Isolation and characterization of RNA aptamers against polyamines

Rapidly growing tissues actively synthesize polyamines and contain large amounts of polyamines. Urinary polyamines have been utilized as diagnostic and prognostic indicators of malignant disorders. An enzyme immunoassay system for urinary polyamines has already been developed and commercialized. However, currently

available anti-polyamine antibodies cannot discriminate polyamine species with similar chemical structures. RNA aptamer is a functional RNA selected from a random RNA pool with an *in vitro* amplification system, called SELEX (systematic evolution of ligands by exponential enrichment), and specifically binds to the target. Aptamers are usually superior to antibodies for structural discrimination and, therefore, are potential tools for detecting biomolecules. We attempted to isolate RNA aptamers against each polyamine and develop a diagnostic system for polyamines. As a first step, SELEX was performed using spermine as a target, and 2 RNA aptamers were selected. These aptamers are specific for spermine; they do not bind to putrescine or *N*¹-acetylspermine and bind to spermidine only weakly. The 2 aptamers share 2 secondary structure regions, which may be important for their binding activities. Selection of RNA aptamers for other polyamines is underway.

Publications

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