研究説明資料

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・論文題目

Elucidation of RNA aptamer structure and interaction in living human cells through in-cell NMR spectroscopy (ヒト生細胞中における RNA アプタマーの構造と相互作用のインセル NMR 法による解明)

・現在までの研究の経過

The global health challenge posed by the human immunodeficiency virus type 1 (HIV-1) necessitates innovative approaches to combat its replication and pathogenicity. This Ph.D. thesis delves into the intricate realm of RNA aptamers, with a specific focus on the exceptional aptamer known as "TA". TA shows promise as both an anti-HIV therapeutic and a molecular tool functioning within living human cells.

RNA aptamers are oligonucleotides with a high affinity for binding to various target molecules, including metabolites, peptides, proteins, and even live cells. The Systematic Evolution of Ligands by Exponential Enrichment (SELEX) method is commonly employed to obtain RNA aptamers, which have found applications in nucleic acid drugs, drug delivery systems, biosensors, artificial riboswitches, and intracellular molecular imaging probes. Many of these RNA aptamers are functional within living cells, where they target endogenous molecules.

TA, designed using SELEX to specifically target the Tat protein in HIV-1, involved subjecting RNA libraries to multiple rounds of SELEX. This strategic process aimed at evolving RNA sequences with a high binding affinity for the Tat protein. As a result, TA exhibits a robust binding affinity, effectively inhibiting Tat's crucial role in HIV-1 replication. This characteristic makes TA a promising candidate for anti-HIV therapeutics, highlighting its potential significance in combating HIV-1 replication and suggesting its suitability for therapeutic applications.

Intracellular conditions differ significantly from *in vitro* conditions due to high molecular crowding. This crowding can lead to structural and interaction differences in RNA aptamers between in-cell and *in vitro* environments. Characterizing the atomic-level structures and interactions of RNA aptamers within the intracellular milieu is essential for the development of novel drugs and molecular tools for intracellular applications. This study not only emphasizes the importance of validating *in vitro* structural data within the cellular context but also underscores the significance of fully characterizing biological

macromolecules. This comprehensive understanding is crucial for elucidating their functions, relationships with molecular partners, and modes of interaction, as well as how changes in the molecular environment impact physiological processes. Thus, this study was meticulously designed to address these objectives.

In Chapter 1, we provided an introduction to RNA aptamers and their unique features, emphasizing the use SELEX method to identify these aptamers systematically, followed by a discussion on the diverse physiological and pathological roles of cellular environments. Transitioning seamlessly into the methodological aspect of how to explore this diversity, we summarize key techniques for achieving in-cell NMR. We introduce the methods for incorporation of the exogenous RNA molecules into living human cells. Simultaneously, the bioreactor system implements a flow technique during in-cell NMR measurements to prolong cell lifetime and uphold metabolic stability. These efforts culminate in the successful detection and investigation of RNA properties within living human cells using in-cell NMR, marking a pioneering achievement.

In Chapter 2, we adapted a previously reported tRNA-scaffold system in *E. coli* to cost-effectively obtain milligram quantities of isotopically labeled RNA for high-resolution heteronuclear 2D in-cell NMR experiments. These high-resolution heteronuclear 2D in-cell NMR spectra allow for the direct evaluation of the behavior of functional RNAs in living human cells, offering insights into their complex forms when interacting with target molecules, clearly distinguishing the signals of the introduced nucleic acids from those of the intracellular endogenous molecules and/or those of the molecules in the medium. They also improved the signal resolution of the introduced nucleic acids. The high-resolution 2D in-cell NMR spectra of RNA in the complex form with its target molecule enable direct evaluation of the behavior of functional RNAs in living human cells (Eladl et al., *Chem.Comm*., **2023).**

In Chapter 3, we successfully fully identify the RNA aptamer for HIV-1 Tat (TA). TA exhibits a binding affinity 100 times higher than that of the native trans-activation response element (TAR) in the HIV-1 genome RNA. Through in-cell NMR, we discover that TA forms two U-A*U base triples when binding to its target molecules, even within living human cells. This structural change widens the originally narrow major groove,

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facilitating the binding of two critical arginine residues of Tat. The interaction between Tat protein and TAR RNA plays a pivotal role in HIV-1 replication, positioning TA as a highly efficient selective antagonist and specific inhibitor of Tat. This chapter also extends in-cell NMR to the characterization of RNA-peptide interactions within living human cells (Eladl et al., *Int.J.Mol.Sci.,* **2023).**

In Chapter 4, we successfully detected RNA within living human Hela cells at a physiological temperature of 37°C. Our findings conclusively demonstrate the inherent instability of RNA in this cellular environment, with rapid degradation observed upon detection. This discovery sheds light on the nature of RNA within living cells and opens avenues for further exploration and understanding. (Eladl et al., *in preparation*., **2024)**.

In Chapter 5, a general conclusion is presented. Despite differences between in-cell and *in vitro* conditions, our results indicate that TA aptamer retains its conformation structure with a high specific binding affinity for RG peptide within living human Hela cells. This reaffirms TA's potential as a regulator of HIV-1 and a candidate drug target for cellular applications.

・将来の展望

This study demonstrates that in-cell NMR spectroscopy is a valuable tool for assessing the drug targeting of therapeutic RNA aptamers. It offers a promising future for RNA-drug screening and evaluating drug-target interaction efficiency under native intracellular environmental conditions with exceptional accuracy and resolution. While the elegance of in-cell NMR is evident, this knowledge can be leveraged in the development of more efficient and environmentally representative strategies for monitoring RNA-large protein interactions within human cells are undoubtedly necessary for future research.

・研究の位置づけ

The TA RNA aptamer, given its critical role in various biological processes and its implications in HIV-1 and cancer, warrants an in-depth understanding of its structure and interactions within living cells. This study, through *in vitro*, in lysate, and in-cell NMR spectroscopy, provides comprehensive insights into TA's structure and interaction mechanisms with Tat peptide. It demonstrates that TA undergoes a conformational

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change upon binding to Tat, forming a unique U-A*U base triple. This structural alteration, widening the originally narrow major groove, leads to a 100-fold increase in aptamer binding affinity compared to the native TAR, positioning our aptamer as an efficient selective antagonist and specific inhibitor of Tat in the HIV-1 replication cycle.

By successfully addressing these objectives, this study significantly advances our understanding of RNA aptamer functionality within living cells and their potential for novel therapeutic applications. It not only enhances our comprehension of the interaction between TA and HIV-1 Tat but also establishes in-cell NMR as a valuable tool for RNA studies within the cellular environment.

In conclusion, this Ph.D. thesis represents a crucial step toward unlocking the potential of RNA aptamers as versatile tools for both anti-HIV therapeutics and molecular investigations within living human cells.