学位論文の要約

論文題目 Studies on protein liquid-liquid phase separation and its functional roles

(タンパク質の液液相分離とその機能に関する研究)

広島大学大学院 統合生命科学研究科 数理生命科学プログラム

学生番号 D191511　　氏 名 劉 蘇

In recent years, protein liquid-liquid phase separation (LLPS) has been widely reported to play important roles in biological systems as well as in virus replication. LLPS is microscopically observed as a droplet-like condensation (granulation) *in vitro* and shown as "membraneless" organelles (MLOs) *in vivo*. LLPS is commonly driven by weak, multivalent interactions between the residues from the intrinsically disordered proteins (IDPs) and proteins harboring intrinsically disordered regions (IDRs) that typically enriched in specific repetitive residues with low complexity. The basic driving forces of LLPS include electrostatic interactions, hydrophobic contacts, π-π stacking and cation-π interactions.

In this thesis, we first discussed about the glycosaminoglycans (GAGs) from a traditional Chinese medicine named colla corii asini (CCA). GAGs were reported to affect some proteins that can undergo LLPS. This raised our interest in these granulation-prone proteins. Then, we used two kinds of granulation-prone proteins for study: one is the C-terminal part (residues 404-551) of cleavage and polyadenylation specificity factor (CPSF6), an important component of nuclear speckles (NSs) which is a kind of MLO concentrating many factors for genetic transcription, pre-mRNA processing and export; the other one is fused in sarcoma (FUS), a structure well-characterized protein associated to amyotrophic lateral sclerosis (ALS).

In Chapter 1, we explored the structural characteristics of three types of GAGs named chondroitin sulfate (CS), dermatan sulfate (DS) and hyaluronic acid (HA) in CCA. We found that CCA contains a heterogeneous mixture of CS/DS and HA, in which CS/DS possesses a heterogeneous structure with different sulfation patterns and densities in its constituent chain, while HA structure might vary in its chain length. This was the first time the structure characteristics of CS/DS and HA from CCA were explored. The structural characterization of CS/DS from CCA may provide possibilities for further elucidating the medicinal properties of

CCA. GAGs were reported to change the LLPS properties of Tau protein which plays important roles in Alzheimer's disease. Many granulation-prone proteins have important function in biological activities, disease development and virus replication. These findings raised our interest in the study about proteins that can undergo LLPS and form droplets. Next, we explored protein LLPS and their functional roles through *in vitro* experiments.

In Chapter 2, we explored the droplet behavior of LLPS-prone protein named CPSF6 and the role of CPSF6 droplets in HBV replication. We found CPSF6 formed droplets in a polyethylene glycol (PEG)-dependent way. And these droplets were more stable in higher ionic strength of the buffer, which was different from other IDPs, such as Tau protein. It suggests that CPSF6 LLPS is mainly driven by cation-π between the Arg guanidinium and other residues (such as Tyr) in its Arg-rich domain, instead of electrostatic interactions that is critical for Tau LLPS. Through optimizing the parameters for CPSF6 droplets formation, the recommended protocols were confirmed and performed in other following experiments. Secondly, we investigated how the hepatitis B virus (HBV) core protein (HBc) works with CPSF6 liquid droplets. We extend the work from Prof. Kazuaki Chayama (Graduate School of Biomedical and Health of Sciences at Hiroshima University) whose research group found that CPSF6 was associated with HBV covalently closed circular DNA (cccDNA) and CPSF6 (residues 404-551) was co-immunoprecipitated with HBc. It is known that HBc capsids consist of 90 or 120 HBc dimers. We found that HBc capsid flocked in CPSF6 droplets while HBc dimers distributed in the droplets uniformly. This was the first time that the interaction of CPSF6 droplet and HBc were observed. From our observations and the related literatures, we raise the hypothesis that CPSF6 droplets may provide the condensate "compartment" for HBc assembly in HBV replication steps (**Fig. 1**): CPSF6 interacts with HBc dimers distributed in the nucleolus and transports the dimers to nuclear speckle; after HBc interacts with the genomes (polymerase and pgRNA) and the concentration reaches to a local "threshold" in CPSF6 granule, the capsid assembly would be triggered followed by pgRNA-contained capsid export.

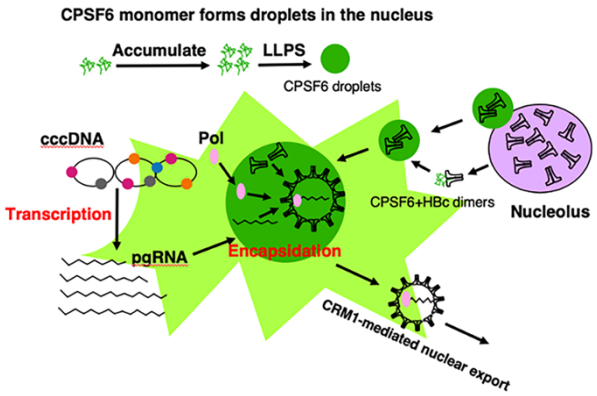

Fig. 1 Hypothesis: the role of CPSF6 droplets in HBV replication.

In Chapter 3, we intended to explore the interaction mechanism between CPSF6 and HBc. Few HBc specific binding to CPSF6 was found in pull-down and size exclusion chromatography experiments, suggesting that CPSF6-HBc interaction was very weak. We prepared eleven site-directed mutagenesis and seven deletion mutants of CPSF6: in the site-directed mutagenesis proteins, the charged residues Lys and Glu were replaced by Ala; in the deletion mutants (D1-D7), the proteins were truncated with 20 or more residues by sequence. All the CPSF6 mutants did not defect the droplet formation. We found HBc can still localize with all the CPSF6 mutants, suggesting that CPSF6 and HBc have multi-binding sites. Compared with CPSF6 free proteins and HBc dimers, CPSF6 droplets and HBc capsids showed tighter interactions. This may be caused by the higher density of protein: the local concentration of both CPSF6 droplets and HBc capsids are higher and provide more, concentrated binding sites than their monomers and oligomers. HBc recruiting to CPSF6 droplets in a granule size-dependent way and less dependent on the sequence of CPSF6. Overall, our results showed that the interaction between CPSF6 droplets and HBc was mediated by weak multivalent interactions, instead of by the adoption of specific protein-protein interactions. These findings provide a better understanding about the interaction between the viral folded proteins and the host LLPS-prone proteins.

In chapter 4, we explored how different enantiomers (L-, D-) of amino acids Tyr and Arg work on fused in sarcoma (FUS) granulation. We found both Tyr and Arg show enantio-dependent modulation of FUS granulation. D-Tyr had higher affinity for FUS granular compared with L-Tyr, and L-Arg had a higher affinity for FUS granular surface compared with D-Arg. We speculate that FUS granular surface is chiral and L-amino acid exclusively assemble there to enhance enantio-selective interactions (**Fig. 2**), which shares similar properties with the chiral stationary phase (CSP) used in chiral liquid chromatography for enantiomer separation. The impact of L- and D-amino acids on FUS granules assembly indicate that besides the side chain of the residues, the backbone chirality of amino acids also affects the interaction between residues

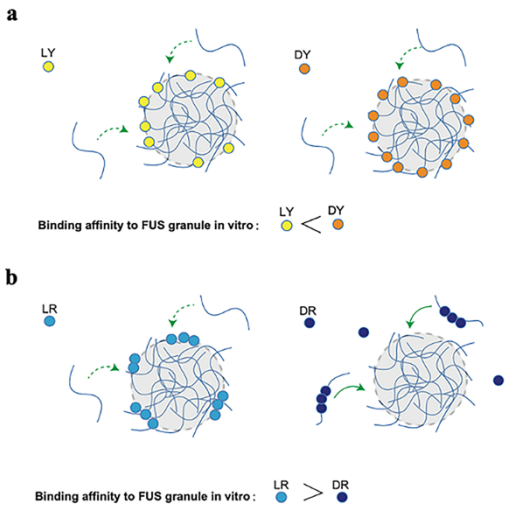

Fig. 2 Schematic mechanism for retarding FUS granulation in vitro by L-/D-Tyr and L-/D-Arg amino acids.

in FUS granulation. Our results show the interactions of small molecules with the granules are enantio-dependent, and these findings will give additional insights into designing small molecules that disturb LLPS with higher efficacy.

In summary, we explored the structural characteristics of GAGs from CCA, and we investigated two granulation-prone proteins here. We demonstrated the disaccharide compositions of CS/DS in CCA. This structural analysis may contribute to understanding the biological and medicinal properties attributed to CCA. We observed the viral protein HBc localized in host protein CPSF6 droplets, and the interaction was mediated by weak multivalent binding, instead of specific protein-protein interactions. We raised the hypothesis that CPSF6 droplets may provide these condensations for HBc assembly in the nucleus for HBV replication. Defecting CPSF6 droplet formation and CPSF6-HBc interaction would be the further study and shed new light on drug target for treating HBV infection. In addition, we found FUS granulation can be modulated in an enantio-dependent way by small molecules, such as Tyr and Arg. These results show us a new way to modulate protein LLPS by small molecules.