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In order to gain insights into regulations of various membrane structures in cell, Arfaptin, a protein involved in tubule formation at *trans*-Golgi network, and Atg16L, a protein involved in formation of autophagosomes, were subjected to structural studies.

BAR domain-containing protein Arfaptin is known to be associated with both tubule formation and newly identified tubule stabilization. Arfaptin-2, which was originally identified as binding partner of Rac1, induces membrane tubules in Golgi-membranes after being recruited onto the membrane by Arl1. Arfaptin-1, which is not a binding partner of Rac1, was recently shown to be associated with the regulation of the fission of secretory granules at the *trans*-Golgi network. The two isoforms share similar domain structure and amino acid sequence similarity of 68%, but recent reports suggest that they have specific functions and associated with different regulators. For instance, the membrane association of Arfaptin-2 is regulated by phosphorylation of Ser260 by Akt, where as Arf-association of Arfaptin-1 is regulated by phosphorylation of Ser132 by Protein Kinase D.

Arfaptin-2 was originally identified as a dual effector of the Rac and Arf, suggesting that the membrane-deformation induced by Arfaptin-2 is modulated by the two GTPases. The crystal structure of the Arfaptin-2 BAR domain in complex with Rac1 revealed that a Rac1 molecule binds to the concaved face of the Arfaptin-2 BAR homodimer, suggesting that the Rac1 binding interferes with membrane association of the BAR domain. It was also reported that Arf and Rac1 bind to the Arfaptin-2 BAR domain in a mutually exclusive manner. However, the molecular basis for the possible crosstalk between Arf and Rac1 GTPases mediated by Arfaptin-2 remains to be elucidated.

Arfaptins are known to localize in the Golgi region when exogenously expressed in cells. Man *et al.* have recently reported that endogenous Arfaptins are recruited onto *trans*-Golgi membranes through interaction with the Arf-like 1 (Arl1) GTPase rather than Arf GTPases. Arfaptin-2 is often found on dynamic vesicular and tubular structures emanating from the Golgi region where Arl1 colocalizes with, suggesting that Arl1 regulates Arfaptin-mediated membrane deformation at the *trans*-Golgi.

In this study, the crystal structures of Arfaptin-1 in free form and Arfaptin-1 or Arfaptin-2 in complex with Arl1 were determined. The complex structures showed that two molecules of Arl1 bind symmetrically on each side of the crescent-shaped homodimer of Arfaptin BAR, leaving the concave face open for membrane association, thus providing structural basis for recruitment of Arfaptins onto Golgi membranes by Arl1 and initiation of membrane remodeling. The inspection of the interface revealed that Arfaptin-2 associated with hydrophobic pocket in switch region of Arl1 through hydrophobic residues in α2 and α3 helices. The requirement of those residues for binding was confirmed by GST-pulldown assay. Surface Plasmon Resonance (SPR) experiments revealed that Arl1 and Rac1 bind to Arfaptin-2 with affinity in μM order. Structural comparison between Rac1-Arfaptin-2 and Arl1-Arfaptin-2 and SPR experiments indicated that Rac1 interferes with second molecule of

Arl1 on Arfaptin-2.

Two ubiquitin-like conjugation systems exist in Autophagy-related protein (Atg) family. One is the LC3 system, in which LC3, a mammalian homologue of yeast Atg8 is conjugated to phosphatidylethanolamine. The Atg16L complex is regarded as a novel type of the E3-like enzyme, which conjugate LC3 to a specific membrane component, phosphatidylethanolamine (PE). Ethanolamine-conjugated LC3 and its orthologue GATE-16, as well as yeast Atg8, promote tethering and hemi-fusion of membranes *in vitro*, and are thus suggested to be essential for elongation of isolation membrane in autophagy. Because the Atg16L complex dissociates from the membrane upon the completion of autophagosome, it's main role is suggested to be the regulation of this elongation step.

Yeast Atg proteins are more thoroughly studied compared to that of Mammal's. Multiple crystal structures of the components of yeast Atg5-Atg12/Atg16 have been reported. The model of the overall structure has been proposed; Atg5-Atg12 conjugate binds to N-terminal helix of Atg16, which dimerizes through its C-terminal coiled-coil domain to form complex that contains two molecules of the three Atg proteins. It has been shown that Atg5 is responsible for membrane-association of the complex, and that Atg5-Atg12/Atg16 complex alone is sufficient for homotypic tethering *in vitro*. It has been shown *in vivo* and *in vitro* that Atg16 is required for the efficient conjugation of LC3 and PE. More specifically, the dimerization of Atg16 at through its coiled-coil domain is reported to be essential for autophagy *in vivo*.

Mammalian Atg5-Atg12/Atg16L complex too depends on the Atg5 for the membrane-association, dimerizes through the coiled-coil domain of Atg16L and has E3-ligase activity toward LC3. However, notable differences are present in Atg16L, which contains C-terminal WD40 domain and binds to small GTPase Rab33 at the region following the coiled-coil domain. A single nucleotide polymorphism in WD40 domain is associated with inflammatory Crohn's disease. As the defects in pathogenic clearance or regulation of immune response is the likely cause of this disease, the WD40 domain of Atg16L may play important function in the regulation of autophagy as part of immune response, a function absent in yeast.

It has been demonstrated that Rab33b, a Golgi-resident protein involved in the Golgi-to-endoplasmic reticulum (ER) retrograde membrane trafficking, directly interacts with Atg16L in a guanosine triphosphate (GTP)-dependent manner. Rab33b does not interact the coiled-coil region of Atg16L (residues 80-200), but does interact with slightly longer construct of Atg16L (residues 141-265) Therefore, Rab33b is likely to interact with Atg16L on the domain distinct from that required for oligomerization. Furthermore, GFP-Rab33b recruits not only Atg16L but also Atg12, which does not directly bind Rab33b. This indicates that Rab33b is able to recruit the Atg5–Atg12/Atg16L complex. Although the Rab33b-binding domain of Atg16L strongly inhibited autophagosome formation, depletion of Rab33b by siRNA has little effect on autophagosome formation. This suggests that recruitment of

Atg5-Atg12/Atg16L complex to the Golgi by Rab33b may be required for the types of autophagy other than macroautophagy.

In this study, the analytical size-exclusion chromatography was used to confirm the binding between Atg16L coiled-coil region and Rab33b. Small-Angle X-ray Scattering (SAXS) experiments were carried out to determine the conformation of the coiled-coil region tailed by Rab33-binding region. The crystal of Atg16L coiled-coil region revealed unexpected anti-parallel coiled-coil structure, a characteristic unobserved in its yeast homologue Atg16.

博士論文の審査結果の要旨

中村健介氏は、高エネルギー加速器科学研究科・物質構造科学専攻で、これまで5年間にわたって行ってきた研究成果に基づき、博士論文の発表を行った。

真核細胞内には生体膜によって囲まれた細胞小器官が存在し、それらの間で物質のやり取り（膜輸送）が行われることで、細胞の高度な生命活動が維持されている。この膜輸送の異常が原因となる病気も見つかっており、この細胞内膜輸送を理解することは、細胞生物学のみならず、医学の観点からも非常に重要である。同氏は、細胞内膜輸送に関わる二種類のタンパク質複合体について、それらの構造機能解析を行い、その博士論文も大きく２部からなっている。

一つは、ゴルジ体におけるチューブ状小胞の形成に関わる Arl1-Arfaptin 複合体についてである。ＢＡＲドメインは細胞膜を湾曲させる役割を果たすタンパク質群として知られているが、細胞内輸送で膜からのチューブ化に関わる Arfaptin タンパク質もこのＢＡＲドメインを保持し、共在する低分子量 GTPase である Arl1 と共に膜のチューブ化の制御に重要な役割を果たすと考えられていた。共同研究者らにより Arfaptin と Arl1 が試験管内で結合する知見から、中村氏はこれらタンパク質の発現精製を行い、Ｘ線結晶構造解析によりその複合体の立体構造を決定した。得られた構造から複合体形成に重要なアミノ酸を推定し、そこに変異を導入した変異タンパク質を作成して共同研究者と試験管内および細胞内での活性を調べ、結晶構造で見出した相互作用が実際に重要である事を示した。また、得られた複合体構造と表面プラズモン共鳴を用いての実験結果から、Arfaptin の co-factor である Arl1 と Rac1 は同時には Arfaptin に結合できないことを示し、Arfaptin による膜のチューブ化において Arl1 が重要である事を示した。

もう一つは、オートファゴソームの成長に関わる Rab33-Atg16L 複合体についてであり、この研究ではマウス Atg16L のコイルドコイル領域のＸ線結晶構造を解明した。得られた構造から、マウス Atg16L は逆平行のコイルドコイル構造を取ることを示した。これは、今までに報告された酵母 Atg16 が平行なコイルドコイル構造を取るのとは逆で、哺乳類では酵母とは Atg16L 複合体の全体構造が大きく異なることを示唆し、機能面でも大きな違いがある可能性を見出した。

本審査での発表は、予備審査の指摘事項を踏まえて大きく改善されていた。Atg16L の構造解析が不十分であった点については、十分に信頼できる結果を示し、発表の仕方についても導入部のわかりやすさ、研究目的と結果の位置づけ、そこからの考察など、非常に明快に説明がなされ、質疑応答にも的確に答え、研究内容について十分な理解と知識があることを示した。従って、審査員全員一致で、博士論文本審査会で合格と判定した。