Structural Study on the Extracellular Signaling Molecule Reelin Involved in the Cortical Layer Formation

Reelin, a gigantic extracellular glycoprotein produced by Cajal-Retzius neurons and other neurons in the cortex, plays a central role in cortical layer formation during mammalian brain development. Reelin was originally identified as a gene product absent in *reeler* mice exhibiting malformations of the cerebral cortex. Reelin acts on migrating neuronal precursors, and regulates correct cell positioning in the cortex and other brain structures. It is now accepted that reelin binds to the lipoprotein receptors ApoER2 or VLDLR on neurons and initiates a signaling cascade involving phosphorylation of the adaptor molecule Dab-1 (Fig. 1(a)). The biochemical basis for the reelin-receptor interaction, however, has remained poorly understood owing to the lack of structural information.

Reelin is a modular protein composed of a signal sequence, an F-spondin-like region, nine sequentially concatenated repeat units of ~380 amino acids, and a C-terminal basic tail region (Fig. 1(b)). The N-terminal repeat is incomplete and shows a markedly lower similarity to other repeats. Each of eight complete reelin repeats contains a central epidermal growth factor (EGF) module flanked by two homologous subrepeats of 150-190 amino acids. The EGF-like module is ubiquitous among extracellular proteins, but the two subrepeats are unique to reelin and do not show any sequence similarities to other protein families. Therefore, structure determination was indispensable for elucidation of the molecular architecture of reelin.

In this study, we first attempted to obtain structural data on a single reelin repeat. We designed and tested a series of deletion constructs of reelin to find a fragment suitable for structural study. As a result, the third repeat, R3, showed the highest expression level, and was subjected to crystallization. The R3 fragment was produced in the mammalian expression system, and the structure has been solved by the Se-SAD method at 2.05 Å resolution [1]. X-ray diffraction data for phasing and refinement were collected at beamline **BL44XU**. As shown in Fig. 2(a), the resulting structure has a horseshoe-like globular structure, in which the two subrepeats separated by the EGF-like module make close contact. Subrepeats A and B have a common 11-stranded β-jelly roll fold, which shows a structural similarity to carbohydrate-binding proteins. In addition, the subrepeats share a common feature with carbohydrate-binding proteins in metal binding. Many carbohydrate-binding proteins contain a calcium ion, and we also identified a metal ion, presumably a calcium ion, at the corresponding site.

We carried out functional analysis of reelin-receptor interaction, and narrowed down the receptor-binding unit to the R5-6 fragment. Interestingly, neither the R5 nor R6 fragment is capable of binding to the receptor. In addition, we designed an artificial fragment R6-5, an inverted version of R5-6 in which the positions for R5 and R6 were swapped, but it does not bind with the receptor, neither. These observations indicate that the correct positioning of R5 and R6 is critical for receptor binding. Therefore, we next determined the structure of R5-6.

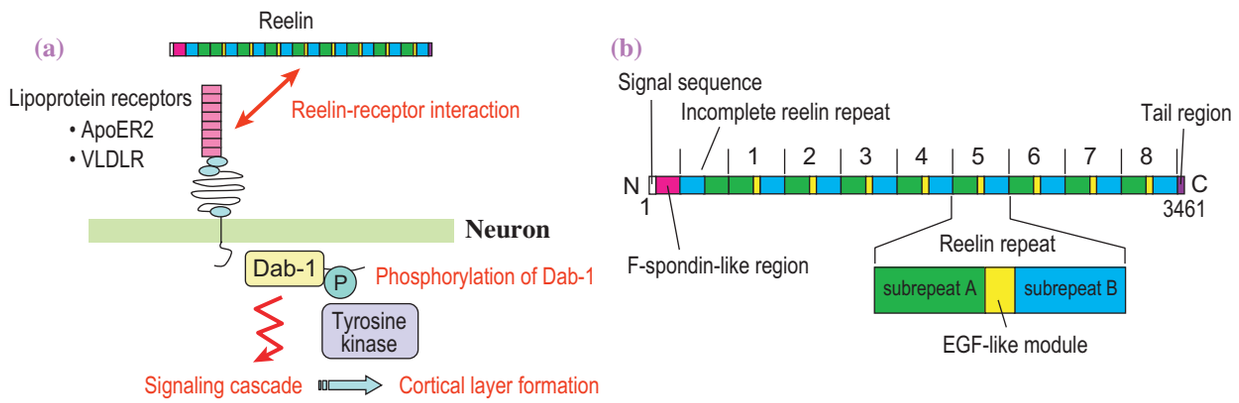

Fig. 1. **(a)** Diagram of reelin signaling on neuron. **(b)** Modular structure of reelin molecule.

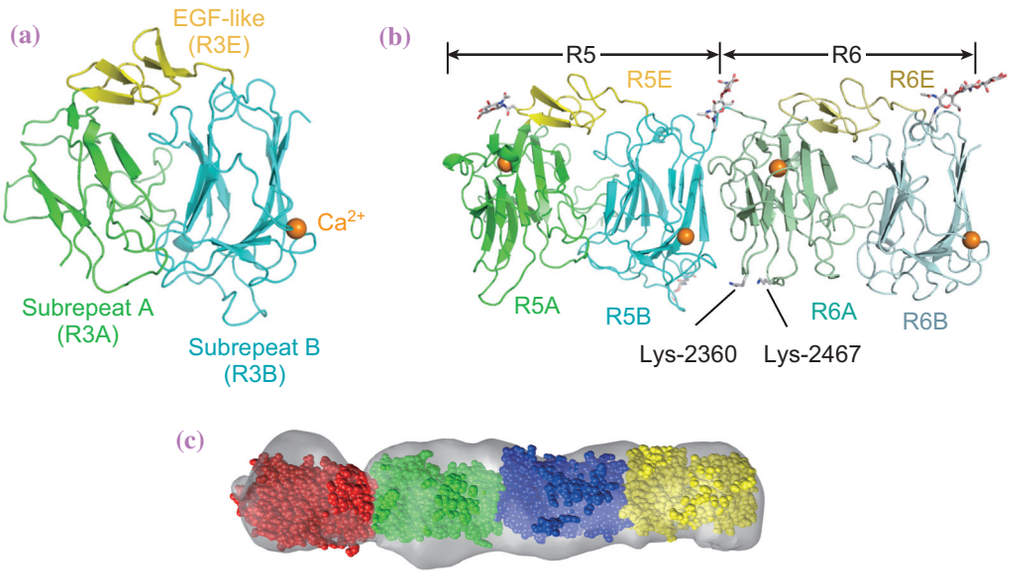

Fig. 2. **(a)** Crystal structure of single reelin repeat R3. The reelin repeat is composed of N-terminal subrepeat A (R3A, green), the central EGF-like module (R3E, yellow), and C-terminal subrepeat B (R3B, cyan). A metal ion presumed to be calcium ion was found in subrepeat B. **(b)** Crystal structure of two-repeat fragment R5-6. Two repeat units are arranged side by side and related by an almost perfect translation. The two lysine residues critical for receptor binding (Lys-2360 and -2467) are highlighted in the stick model. Calcium ions and N-glycans are shown in sphere and stick-model, respectively. **(c)**Averaged electron tomogram of four-repeat fragment R3-6. Space-filling models of four reelin repeats are fitted onto the envelope calculated from electron microscopy.

The recombinant R5-6 fragment was also produced in mammalian cell. For structure determination, we collected diffraction data at beamline **BL41XU**. We solved the structure by the molecular replacement method at 2.0 Å resolution [2] (Fig. 2(b)). The structural features of the R5 and R6 units are almost identical to those of R3, but their spatial arrangement is unique and unprecedented. These homologous R5 and R6 units are positioned side by side and related by an almost perfect translation, without any bends or twists at the junction. R5 and R6 are connected by a short linker, and they make intimate contact through hydrophobic interactions. Electron tomographic imaging also indicated the same type of arrangement of reelin repeats. A four-repeat fragment, R3-6, was subjected to electron microscopy, and was shown to form a rod-like structure (Fig. 2(c)). The longest dimension of R3-6 is almost twice that of R5-6, and the other two-dimensions are almost the same as those of R5-6. Presumably, all four repeats in R3-6 are arranged similarly to those in R5-6.

Subsequently, we attempted to identify the receptor-biding site on reelin. Structural studies of other lipoprotein receptor-ligand systems elucidated the importance of lysine residues of the ligands in the interaction. We designed a series of point mutants, in each of which a single lysine residue was changed to alanine. On the basis of the structure of R5-6, we selected lysine residues located at the molecular surface and close to the boundary between R5 and R6. Finally, we found that Lys-2360 and -2467 are involved in receptor-binding. In addition, the mutation of Lys-2467 to alanine, in the context of either R5-6 or full-length reelin, abolished their ability to induce Dab-1 phosphorylation in neurons, which means that this residue is essential for the signaling. We are now trying to crystallize the reelin fragment in complex with its receptor so as to confirm the involvement of the two lysine residues in the interaction and to further analyze the binding mechanism on the basis of the three-dimensional structure at an atomic level.

Terukazu Nogi*, Norihisa Yasui and Junichi Takagi

Institute for Protein Research, Osaka University

* E-mail: nogi@protein.osaka-u.ac.jp

References

[1] *T. Nogi, N. Yasui, M. Hattori, K. Iwasaki and J. Takagi: EMBO J.* ***25*** *(2006) 3675.*
[2] *N. Yasui, T. Nogi, T. Kitao, Y. Nakano, M. Hattori and J. Takagi: Proc. Natl. Acad. Sci. USA* ***104*** *(2007) 9988.*