審 査 の 結 果 の 要 旨

氏 名　　邱詠玟

[Introduction]

Alzheimer disease (AD) is the most common progressive neurodegenerative disorder, pathologically characterized by the deposition of the amyloid-β peptide (Aβ) as senile plaques in the brain. Several lines of evidence indicate that the abnormal aggregation of Aβ affects the pathological process of AD by triggering neurotoxicity and neurodegeneration, suggesting the important role of Aβ in the etiology of AD. Aβ is generated through the sequential cleavage of amyloid precursor protein (APP) by β-site APP cleaving enzyme 1 (BACE1) and γ-secretase. After the first cleavage by BACE1, γ-secretase cleaves the stub of APP at different positions, producing Aβ with various C-terminal lengths, such as Aβ40 and Aβ42, the latter being more hydrophobic and the initial as well as predominant species in the senile plaques. γ-Secretase is an intramembrane-cleaving protease complex comprising four subunits, namely, the catalytic subunit presenilin 1 (PS1) or presenilin 2 (PS2), anterior pharynx-defective 1 (Aph-1), Nicastrin (Nct), and presenilin enhancer 2 (Pen2). Several genetic studies have demonstrated that familial AD-linked autosomal-dominant mutations in PS1 and PS2 increases Aβ42 production, indicating the involvement of γ-secretase in the pathogenesis of AD via generating the heterogeneity of Aβ species. As described, whereas the aberrant metabolism of Aβ has been implicated in the etiology of AD, the precise regulatory mechanism of Aβ generation remains unclear. To obtain a better understanding of the molecular mechanism of Aβ production, we established a genetic screening based on the CRISPR/Cas9 system to identify novel regulators of Aβ production.

[Results]

1. <u>Identification of CIB1 as a negative regulator of Aβ production</u>

Murine neuroblastoma Neuro2a (N2a) cells were infected with a lentiviral guide-RNA (gRNA) library. The resulting cell lines were screened to identify those showing changes in Aβ production. The gRNA sequences in the obtained cell lines were amplified and read using a next-generation sequencer (NGS) to identify candidate genes. Through this screening, we discovered 13 candidates from 3 cell lines with changes in Aβ levels. Regarding these 13 candidate genes, we analyzed the effects of each candidate on Aβ levels by disrupting each gene individually and finally identified calcium and integrin-binding protein 1 (CIB1) as a negative regulator of Aβ production. We further confirmed that the upregulated Aβ levels in *Cib1*-knockout (KO) monoclonal cell lines can be restored to basal condition by overexpressing mouse CIB1 in these cells, strongly suggesting that CIB1 has the ability to negatively regulate Aβ production.

2. <u>Direct interaction between CIB1 and γ-secretase</u>

We first analyzed the expression levels of the proteins related to the Aβ production machinery. However, no significant difference was found in the expression levels of APP, BACE1, and the γ-secretase components in *Cib1*-KO cells. Since CIB1 has been previously identified as a PS2-binding protein, we next hypothesized the possibility of CIB1 in regulating γ-secretase activity through their interaction. To clarify this, we performed immunoprecipitation using the anti-PS2 C terminus antibody. After assembly in the endoplasmic reticulum, the γ-secretase complex is transported to the plasma membrane and activated. During this process, PS undergoes endoproteolysis to generate the N- and C-terminal fragments (i.e., NTF and CTF, respectively), and Nct is fully glycosylated as mature Nct (mNct). When we overexpressed PS2 in PS1/PS2 double-knockout (DKO) mouse embryonic fibroblasts (MEFs), sufficient active γ-secretase with fragmented PS and mNct was detected. Notably, together with the components of γ-secretase, endogenous CIB1 was coimmunoprecipitated, indicating the interaction between CIB1 and the γ-secretase complex. This result suggested the possibility that CIB1 regulates Aβ production by controlling the γ-secretase through their direct interaction.

3. CIB1 regulates the subcellular localization of γ-secretase

To assess whether the direct binding of CIB1 to the γ-secretase affects its intrinsic enzymatic activity, we carried out an *in vitro* assay using detergent-solubilized membrane fractions derived from *Cib1*-disrupted cells. The γ-secretase activity was preserved in this membrane fraction to produce Aβ species upon coincubation with the recombinant γ-secretase substrate C99. However, we observed no difference in the *de novo* generation of Aβ levels, suggesting the intrinsic enzymatic activity of γ-secretase was not affected by the downregulation of CIB1.

Previous reports indicated that CIB1 regulates the intracellular translocation of its binding partners. Intriguingly, the endo-lysosomal localization of the γ-secretase enhances its activity to overproduce Aβ, and may contribute to the pathogenesis of AD. Thus, we hypothesized that CIB1 regulates the Aβ production by affecting the subcellular localization of γ-secretase. To test this hypothesis, the cell surface biotinylation assay was performed using *Cib1*-disrupted cells. We found that the levels of mNct at the cell surface significantly decreased upon KO of *Cib1* without changes in the total level of mNct. In contrast, we did not observe significant differences in the cell surface expression levels of other membrane proteins, including APP and BACE1. This suggested that the disruption of *Cib1* specifically decreased the surface localization of γ-secretase. Since the colocalization of internalized APP and γ-secretase facilitates the Aβ generation, CIB1 would assist the translocation of the γ-secretase to the plasma membrane and reduce the encounter of γ-secretase with its substrate, thereby suppressing the Aβ production.

4. *CIB1* mRNA level decreased in AD patients

To investigate the relationship between CIB1 and AD pathology in humans, we referred to a single-cell RNA-seq analysis of AD patients. The level of *CIB1* transcripts in excitatory neurons significantly decreased in the early stage of AD compared with control without pathology, suggesting that the downregulation of CIB1 is involved in the pathogenesis of AD.

[Summary and Discussion]

In this study, we established an innovative approach using the CRISPR/Cas9 system and successfully identified CIB1 as a negative regulator of Aβ production. In addition, we demonstrated that CIB1 regulates Aβ production via controlling the subcellular localization of γ-secretase. However, the precise mechanism whereby CIB1 regulates the γ-secretase activity is not well understood. As one possibility, CIB1 might promote the membrane localization by directly shuttle γ-secretase from the cytosol to the plasma membrane. Additionally, CIB1 could retain γ-secretase at an early endosome by interacting with other vesicles transporting proteins. It is also possible that CIB1 suppresses the further internalization of γ-secretase by competing for the binding with the molecules helping γ-secretase internalization. Whereas further molecular and cellular studies will be needed to clarify the mechanistic role of CIB1 in the intracellular trafficking of γ-secretase, our data demonstrated CIB1 is involved in the development of AD.

よって本論文は博士（薬科学）の学位請求論文として合格と認められる。