主論文の要旨

Identification of PTPRσ-interacting proteins by proximity-labelling assay

（近接標識アッセイによるPTPRσ相互作用タンパク質の同定）

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【Introduction】

After a spinal cord injury, reactive astrocytes secrete chondroitin sulfate (CS) proteoglycans, which form a physical barrier preventing the worsening of the lesion site. However, this wall also prevented the simultaneous regeneration of the neuron axons and left the patients paralyzed for life. PTPRσ is a class IIa receptor protein tyrosine phosphatase (RPTP) which is expressed on neuronal membranes. In 2011, Coles et al. showed that PTPRσ can act as a key molecular switch when it binds to different types of glycosaminoglycans heperan sulfate (HS) or chondroitin sulfate. Whether the neurons can regenerate successfully or not after a spinal cord injury depends on which type of glycosaminoglycans acts on the PTPRσ. Specifically, HS can induce a “healthy” state of an axon tip called the growth cone, while CS on the other hand turns the growth cone into an “abnormal” state called the dystrophic endball. Previous work has shown that autophagosome accumulation is the cause of dystrophy. However, little is known regarding the downstream signaling pathway of PTPRσ. Therefore, it is crucial to determine an intracellular pathway to link between PTPRσ and autophagy.

To identify the intracellular substrates for PTPRσ, we performed the proximity-dependent biotin identification assay (BioID), which is developed by Dr. Roux. This method allows us to capture the biotinylated PTPRσinteracting proteins by streptavidin pulldown and to identify the substrates of PTPRσ by mass spectroscopy. We successfully identified cortactin, Lipirin-α and Trio, which were known substrates for PTPRσ. Our results indicate that BioID is a reliable way of detecting fast and transient protein-protein interactions.

【Materials and Methods】

We subcloned PTPRσ into an MCS-BioID2-HA plasmid, a gift from Dr. Roux. The plasmid was transfected into HEK293T cells to make the PTPRσ-BioID stable cell lines, which were maintained in biotin free DMEM/ITSA until use. After the 16 hours of biotin treatment, cells were lysed and the supernatant was subjected to the SDS-PAGE and the streptavidin pull-down assay. For identification of the PTPRσ substrates, the proteins were trypsinized, and the peptides were analyzed by LC-MS. GO enrichment analysis was performed on the PANTHER classification system.

We co-transfected v-src and PTPRσ-BioID to HEK293T cells to validate the phosphatase activity of PTPRσ by western blotting with an anti-phospho-tyrosine antibody (4G10). The phosphorylation of cortactin was also determined by western blotting with an anti-phospho-cortactin antibody. For the Immunoprecipitation assay, HEK293T cells were co-transfected with Flag-cortactin and PTPRσ-HA. The cell lysate was pulled down with an anti-Flag antibody and then blotted with an anti-HA antibody.

【Results】

To screen the substrates for PTPRσ, we constructed a fusion protein of PTPRσ with a biotin ligase attaching to the phosphatase domain (Fig. 1A). The wild-type PTPRσ and PTPRσ-BioID were both properly processed by furin cleavage (Fig. 1B). Furthermore, PTPRσ-BioID successfully dephosphorylated v-src induced phospho-tyrosine in the HEK293T cells (Fig. 1C, top panel). Cortactin, a known substrate of PTPRσ, was phosphorylated and dephosphorylated by v-src and PTPRσ-BioID, respectively (Fig. 1C, lower panels). Together, we showed that PTPRσ-BioID can be properly processed and retain its phosphatase activity.

The streptavidin pulldown assay workflow was outlined in Fig 2A. To minimize the non-specific biotinylation, we established a stable cell line, which expressed PTPRσ-BioID constitutively. The stable cell line was initially biotin starved for 48 hours, then was supplemented with biotin for 24 hours. The PTPRσ-BioID induced biotinylation was confirmed by streptavidin (Fig. 2B and Fig. 2C).

The general strategy for identifying the PTPRσ substrates was outlined in Fig 3A. After the streptavidin pull-down, proteins were trypsinized into peptides and the protein identities were identified with the LC-MS/MS and the Mascot search. Three independent analysis were performed to ensure reproducibility, and the 99 overlapping protein candidates were listed (Fig. 3B, 3C and Table 1).

Gene Ontology (GO) enrichment analysis suggested that these proteins related to cell adhesion and actin cytoskeleton organization in the cytoplasm were frequently identified as candidates for the PTPRσ substrates, which was consistent with its suggested physiological roles (Fig.4A to 4C). Importantly, we found that Liprin α-1 and Trio, two previously known PTPRσ binding partners, were also listed as the top and the 10th protein in our list. This suggested that the BioID assay was a reliable method to capture interactors for RPTP. Our previous work showed that PTPRσ dephosphorylated cortactin at the Y421 position. To support this result, the biotinylated proteins from the stable cell lines were subjected to western blotting with an anti-cortactin antibody. The cortactin was successfully blotted as shown in Fig. 4D. Additionally, we found that PTPRσ was co-immunoprecipitated with cortactin, suggesting that these two proteins directly interacted with each other (Fig. 4E).

【Conclusion】

In this study, we conducted a proximity-dependent biotin identification assay to identify the substrate for PTPRσ and verified the candidate substrates by western blotting and co-immunoprecipitation methods. BioID could be a powerful tool, which can be used to identify fast and transient protein-protein interactions such as enzymatic or phosphatase activities.