学位論文内容の要旨

博士の専攻分野の名称 博士（医学）　　氏 名 ネパル　プラバ

学 位 論 文 題 名

Studies on the role of a mitochondrial protein GC1 in the regulation of Ras-PI3K signaling-mediated endocytosis (Ras-PI3Kシグナルを介したエンドサイトーシス制御機構におけるミトコンドリアタンパク質GC1の役割に関する研究)

[Background and Objectives] The small GTPase Ras plays a key role in a variety of cellular functions through a spatiotemporal control of effector activation. The GTP-bound, active form of Ras interacts with the Ras-binding domain (RBD) of the effector molecules, which include phosphoinositide 3-kinase (PI3K), c-Raf serine threonine kinase, and Ral guanine nucleotide dissociation stimulator (RalGDS). Using the bimolecular fluorescence complementation (BiFC) technique, our laboratory has previously reported that different from the complexes of Ras-Raf and Ras-RalGDS, the Ras-PI3Kp110γ (Class IB PI3K catalytic subunit gamma isoform) complex specifically translocates from the plasma membrane to endosomes upon epidermal growth factor (EGF) stimulation, which resulted in the activation of Ras-PI3K complex on the endosome. In addition, colleagues in our laboratory have revealed that Ras-PI3K signaling from the endosomes promotes clathrin-independent endocytosis and endosomal maturation. The question to be addressed is what gives rise to the localization of the Ras-PI3K complexes in the endosome. Our laboratory has identified a 28-amino-acid-long sequence that is specifically found in RBD of PI3K, but not in those of Raf and RalGDS, and revealed that this peptide is involved in the translocation of the Ras-PI3K complex to the endosome. The peptide was named RAPEL after Ras-PI3K endosomal localization. Through the screening of RAPEL-binding factors with the use of yeast two-hybrid system and immunoprecipitation followed by mass spectrometry, 48 potential binding proteins to RAPEL have been isolated. To my surprise, among these factors, several mitochondrial proteins have been identified. In this study, I focused on one of these mitochondrial membrane proteins, glutamate carrier 1 (GC1), a glutamate-proton symporter, and aimed to evaluate its role in endocytosis.

[Materials and Methods] The interaction of GC1 with RAPEL was evaluated by immunoprecipitation and immunoblotting. The coding sequence of GC1 was obtained from a cDNA library of 293T cells and those of truncation mutants were prepared by PCR with the use of primer sets for respective fragments. These coding sequences were subcloned into the restriction enzyme recognition sites of expression vectors for the proteins tagged with epitopes, including fluorescent proteins. Such expression vectors or others that had been developed in our laboratory were transfected into cells according to the manufactures' recommendations. Immunofluorescence analysis was performed by a common procedure. The cells either expressing fluorescent protein-tagged proteins or being subjected to immunofluorescence were observed under wide-field epi-fluorescence microscopy or confocal microscopy. The localization of GC1 in the inner and outer mitochondrial membranes was evaluated by mega-mitochondria method using valinomycin, a reagent that triggers mitochondrial swelling and makes it possible to distinguish the inner and outer membrane of mitochondria by confocal microscopy. Knockdown experiments were carried out by introducing small interfering RNA (siRNA) against the GC1 into cells. The knockdown efficacy was determined at a mRNA level and a protein level by quantitative PCR (48 hours after transfection) and immunoblotting (after 72 hours), respectively. Endocytic activity of cells were evaluated by quantifying the uptake of fluorescently labeled dextran and transferrin, which are incorporated into cells through clathrin-independent and clathrin-mediated endocytosis, respectively. Endosomal acidification was examined by labeling and tracking acidic vesicles using fluorescent probe, LysoTracker® Red DND-99. The acquired images were quantified and analyzed by using MetaMorph software. Other biochemical analyses, including immunoprecipitation, were performed as described elsewhere.

[Results] I revealed that GC1 interacted with RAPEL by an immunoprecipitation method and its localization in the mitochondria was confirmed by an immunofluorescence analysis as well as live-cell imaging for cells expressing fluorescent protein-tagged GC1. GC1-positive mitochondria are localized in close proximity with

Ras-PI3K complex-positive endosomes. These results strengthened a hypothesis that endosomes may interact with mitochondria through the binding between GC1 and Ras-PI3K complex. However, to interact with the cytosolic or endosomal PI3K, GC1 needs to be localized at the mitochondrial outer membrane. As far as I am aware, there are no reports about its localization in the outer mitochondrial membrane. Therefore, I evaluated submitochondrial localization of GC1 with mega-mitochondria method and demonstrated that GC1 was localized not only in the inner membrane, but also in the outer membrane of mitochondria, further suggesting the direct interaction of GC1 with RAPEL. Assay with truncated mutants of GC1 revealed that its N-terminal half was responsible for RAPEL binding whereas the C-terminal half was for mitochondrial localization. Live-cell imaging demonstrated that GC1 negatively regulates the translocation of the Ras-PI3K complex to the endosome. Transferrin and dextran uptake assays demonstrated that GC1 positively and negatively regulates clathrin-mediated and -independent endocytosis, respectively. Given that GC1 functions as a symporter of glutamate and proton and Ras-PI3K signaling is reported to be involved in endosomal maturation, I hypothesized that GC1 might play a role in endosomal maturation. In fact, assay with the fluorescent probe for acidic organelles in living cells revealed that GC1 negatively regulates endosome acidification and delayed endosome maturation. These results together demonstrated that GC1 plays two-sided roles in the regulation of endocytosis.

[Discussion] In this study, I revealed that the mitochondrial protein GC1 interacts with RAPEL and negatively regulates endosomal translocation of the Ras-PI3K complex from the plasma membrane, clathrin-independent endocytosis, and endosomal maturation. Another important finding is that GC1 positively regulates clathrin-mediated endocytosis. The negative role of GC1 in the regulation of endosomal maturation might be accounted for by the function of GC1 as a proton transporter; i.e., possibly from cytosol to mitochondria; a local decrease in cytosolic proton due to pumping proton into the mitochondria by activated GC1 to a decrease in proton transport into the endosome, which results in the delay in endosome maturation. However, many questions remain to be addressed. For example, it is unknown whether the interaction of GC1 with RAPEL is direct or indirect. In addition, the direction of proton/glutamate flux at the endosome-mitochondria contact sites are yet to be investigated. Since clathrin-mediated pathway is reported to be independent of Ras-PI3K signaling, it is possible that GC1 plays a role in the "pathway switching" between clathrin-mediated and clathrin-independent endocytosis.

GC1 has been reported to be associated with neurological disorder like epilepsy, some types of which are related with the dysfunction of endocytosis: namely, mutation in dynamin, a key regulator of clathrin-mediated endocytosis. Therefore, it might be possible that GC1 might participate in the pathogenesis of epilepsy not only through glutamate transport, but also through the regulation of clathrin-mediated endocytosis, albeit in part, although the detailed molecular mechanism has yet to be determined. In addition, GC1 is revealed to upregulate proliferation and migration of colorectal cancer cells with K-Ras mutation by promoting glutamine synthesis. Given that endocytosis is reported to suppress cancer cell proliferation and invasion, it might be possible for GC1 to promote cancer cell malignancy thorough the regulation of membrane trafficking. Thus, it might be worth exploring the involvement of GC1 in pathophysiological conditions, including endocytosis-related diseases in the future.

[Conclusion] In this study, I have demonstrated that:

- GC1, a mitochondrial membrane protein, interacts with RAPEL of PI3K.
- GC1 negatively regulates translocation of Ras-PI3K complex from the plasma membrane to the endosome.
- GC1 negatively regulates clathrin-independent endocytosis and endosomal acidification.
- GC1 positively regulates clathrin-mediated endocytosis but this mechanism is still unknown.

To the best of my knowledge, there are no reports so far on the role of GC1 in the endocytic process; therefore, this is the first study carried out to demonstrate its role in endocytosis. I hereby provide a novel function of GC1 as a regulator of clathrin-independent endocytosis through regulating the Ras-PI3K signaling.