学位論文（要約）

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）

2019 年 9 月
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【Background and Objectives】

Ras proteins are small GTPases that regulate a variety of signal transduction processes through the activation of different effector molecules. The Ras subfamily consists of three members: homolog of Harvey rat sarcoma virus (H-Ras), homolog of Kirsten rat sarcoma virus (K-Ras), and Ras derived from neuroblastoma (N-Ras). Ras proteins cycle between two states, a GTP-bound active form and a GDP-bound inactive form. The activation of Ras is mediated by guanine nucleotide exchange factors (GEFs). 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). Our laboratory has previously reported that a complex formed between H-Ras and p110γ (Class IB PI3K catalytic subunit gamma isoform), when visualized by the bimolecular fluorescence complementation (BiFC) assay, is specifically localized in early endosomes upon epidermal growth factor (EGF) stimulation. In addition, this translocation of the Ras-PI3K complex from the plasma membrane to the endosome eventually promotes endocytosis, namely clathrin-independent endocytosis. Moreover, our laboratory has recently identified the amino acid sequence in PI3K that is responsible for endosomal translocation of the Ras-PI3K complex. This sequence has been named RAPEL after Ras-PI3K endosomal localization. Screening of RAPEL-binding factors with the yeast two-hybrid method and mass spectrometry of immunoprecipitates has identified 48 candidates, in which four mitochondrial proteins are interestingly included. These results suggest the possibility that the mitochondrial proteins can interact with RAPEL and participate in the spatiotemporal regulation of the Ras-PI3K complex, either directly or indirectly. Among these factors, I focused on the mitochondrial membrane protein glutamate carrier 1 (GC1), a glutamate-proton symporter, and aimed to evaluate its role in the regulation of endocytosis.

【Methods】

Binding of GC1 to RAPEL was examined by co-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 amplifying respective fragments. The obtained coding sequences were subcloned into expression

vectors for the proteins tagged with epitopes, including fluorescent proteins. The cells either expressing fluorescent-tagged proteins or being subjected to immunofluorescence were observed under wide-field epi-fluorescence microscopy or confocal microscopy. The submitochondrial localization of GC1 (i.e. at the inner or outer mitochondrial membranes) was evaluated by mega-mitochondria method using valinomycin, a reagent that triggers mitochondrial swelling and enables to distinguish the inner and outer membranes of mitochondria by confocal microscopy. Knockdown experiments were carried out by introducing small interfering RNA (siRNA) against the GC1 into cells. The mRNA level was evaluated by quantitative PCR while the protein level was analyzed using immunoblotting. The endocytic activities were investigated by quantifying the uptake of fluorescently labeled dextran and transferrin, which are incorporated into cells through clathrin-independent and -dependent endocytosis, respectively. Dynamics of the Ras-PI3K complex was evaluated by a BiFC method. Endosomal acidification was examined by staining and tracking acidic vesicles using the fluorescent probe LysoTracker® Red DND-99.

【Results】

I revealed that GC1 interacted with RAPEL of PI3K and was localized in the mitochondria. GC1-positive mitochondria were localized in close proximity with Ras-PI3K complex-positive endosomes. Assay with truncated forms of GC1 demonstrated that the N-terminal half of GC1 were responsible for its RAPEL binding whereas the C-terminal half were for mitochondrial localization. As far as I am aware, there are no reports that demonstrated GC1 localization in the outer mitochondrial membrane. Therefore, submitochondrial localization of GC1 was evaluated by a mega-mitochondria method. GC1 was localized not only at the inner membrane as reported previously, but also at the outer membrane of mitochondria, further raising the possibility of direct interaction of GC1 with RAPEL. As compared with control cells, GC1 depletion increased endosomal localization of the Ras-PI3K complex and promoted clathrin-independent endocytosis. The rescue experiments with exogenous expression of GC1 in GC1-knockdown cells further confirmed that GC1 negatively regulates endosomal localization of the Ras-PI3K complex and clathrin-independent endocytosis. Unexpectedly, GC1 knockdown suppressed the uptake of

transferrin. Given the GC1 function as a cotransporter of glutamate and proton, I hypothesized that GC1 might have a regulatory role in endosomal maturation process. To examine this possibility, the endosomal acidification in GC1-overexpressing cells and in GC1 knockdown cells was evaluated by using the fluorescent proton sensor. GC1 was found to negatively regulate endosome acidification. The delay in endosome maturation was further confirmed by the rescue experiment. These results together demonstrated that GC1 plays a role in the negative and positive regulation for clathrin-independent and clathrin-dependent endocytosis, respectively.

【Discussion】

In this study, I revealed that mitochondrial protein GC1 interacts with RAPEL in the endosome and negatively regulates endosomal translocation of the Ras-PI3K complex from the plasma membrane, clathrin-independent endocytosis, and endosomal maturation. In contrast, GC1 positively regulates clathrin-dependent endocytosis. The negative role of GC1 in the regulation of endosomal maturation might be explained by GC1 function as a transporter of proton possibly from cytosol to mitochondria. GC1 activation in proximity to the endosome leads to a local decrease in the cytosolic proton concentration, less proton transport into the endosome, and the delay in endosome maturation. However, there remains some questions to be addressed. For example, the direction of proton/glutamate flux at the endosome-mitochondria contact sites are yet to be investigated. In addition, to evaluate the role of GC1 in the regulation of endocytosis, experiments by using GC1 mutants that cannot bind to RAPEL should be performed. Furthermore, once critical amino acid residue(s) for glutamate/H^+ transport will be identified, requirement for transport activity of GC1 in the regulation of endocytosis will be clarified. Visualization of spatiotemporal dynamics of the local glutamate and proton concentrations by using fluorescent protein-based biosensors can elucidate these issues. Since clathrin-dependent pathway is reported to be independent of Ras-PI3K signaling-mediated endocytosis, it might be possible that GC1 regulates “switching” from clathrin-independent pathway to clathrin-dependent one. GC1 has been reported to be associated with neurological disorder like epilepsy, some types of which are related to dysfunction of endocytosis. In addition, GC1 is revealed to upregulate proliferation and migration of colorectal cancer cells with

K-Ras mutation by promoting glutamine synthesis. Moreover, endocytosis is reported to suppress cancer cell proliferation and invasion. Therefore, it might be possible that GC1 plays a causative role in pathogenesis of cancer, albeit in part, through the regulation of trafficking pathways. It might be worth exploring the involvement of GC1 in pathophysiological conditions, including those involved in endocytosis-related diseases in the future.

【Conclusion】

In this study, I have demonstrated that GC1 interacts with RAPEL of PI3K and is localized in the outer membrane of mitochondria. GC1 negatively regulates the Ras-PI3K complex translocation from the plasma membrane to the endosome and subsequent clathrin-independent endocytosis by suppressing endosomal acidification. GC1 is a positive regulator for clathrin-dependent endocytosis. This is the very first study carried out to demonstrate the role of GC1 in the regulation of endocytosis; however, the molecular mechanism through which GC1 regulates endocytosis has yet to be determined. Specifically, requirement for glutamate transport activity in the regulation of endocytosis is totally unknown. I, here, provide evidence for a novel function of GC1 as a regulator of clathrin-independent endocytosis through the regulation of Ras-PI3K signaling.