Essential Role of Sphingosine Kinase 2 in the Regulation of Cargo Contents in the Exosomes from K562 Cells

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Ｋ５６２細胞由来エキソソームの積荷量調節におけるスフィンゴシンキナーゼ２の重要な役割

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Exosomes are membrane-bound vesicles with a size of 50-100 nm that are released from many types of cells into the extracellular space. Exosomes are considered important means of cell-to-cell communication by transferring internal cargo molecules such as proteins, soluble factors, microRNA, and mRNA to recipient cells, thereby playing a key role in intercellular communications such as antigen presentation, cancer progression, and spreading of neurodegenerative diseases.

Exosomes are generated by the fusion of multivesicular endosomes (MVEs) with the plasma membranes. Although mechanism underlying cargo sorting into intralumenal vesicles (ILVs) of MVEs destined for degradation by the fusion with lysosomes is well known, *i.e.*, the one using endosomal sorting complex required for transport (ESCRT) machinery, it remained unclear as to how selective cargo molecules were sorted into exosomal ILVs. Recently an important discovery was made to show the importance of ceramide in the generation of exosomal ILVs. It has been shown through mass spectrometric analysis that secreted proteolipid protein 1 (PLP1)–containing exosomes purified from cell culture medium are enriched in ceramide and that neutral sphingomyelinase inhibitor causes a reduction of exosome secretion. They also showed that addition of a bacterial sphingomyelinase to giant unilamellar vesicles (GUVs) containing domains with different degrees of fluidity resulted in inward budding formation specifically from the "raft"-like lipid phase. However the mechanism of cargo sorting into exosomal ILVs remains unclear.

sphingosine 1 phosphate(S1P) is a lipid mediator that is implicated in the regulation of a wide variety of important cellular events through a specific receptors of a family of GTP-binding protein (G-protein)-coupled receptor, termed $S1P_{1-5}$ receptors and triggers diverse cellular processes, including cell angiogenesis, cardiac development, immunity, cell motility, neurotransmitter release and endosome maturation. S1P is a phosphorylated product of sphingosine catalyzed by sphingosine kinase (SphK). Two isoforms of mammalian SphK (SphK1 and SphK2) have been cloned and characterized.

Very recently report has been shown that S1P signaling is involved in vesicular trafficking especially multivesicular formation towards exosome release, it has been revealed that sustained activation of (S1P) receptor on CD63-positive MVEs in an intracrine manner is essential for cargo sorting into exosomal ILVs.

Although Sphk2 has a high degree of homology to Sphk1, especially in the previously identified conserved domains identified in Sphk1, it is much larger (65.6 kDa for mSPHK2, versus 42.4 kDa for mSPHK1). Furthermore, its differential tissue expression, temporal developmental expression, cellular localization, and in vitro kinetic properties in response to increasing ionic strength and detergents are completely different from Sphk1, suggesting that it most likely has a different function and regulates levels of S1P in a different manner than Sphk1, which is known to play a prominent role in regulating cell growth and survival.

In the present study we have shown the importance of SphK2, in the regulation of cargo content in exosomes released from human myeloid leukemia K562 cells.

I. SphK2 has been shown to localize with N-Rh-PE-positive late endosomes in the cells.

SphK1 was colocalized with some of cellular vesicles, which was stained with an early endosomal marker, early endosomal antigen 1 (EEA1) as previously reported. On the other hand, SphK1 showed poor colocalization with late endosomal markers N-Rh-PE and LysoTracker. The other isozyme SphK2 was colocalized with exosomal MVE marker N-Rh-PE. We have previously shown that GFP fusion to these enzymes itself did not cause any apparent changes in cellular distribution as compared with non-fused SphK1 or SphK2.

These results are consistent with our previous observations that SphK2 is associated with exosomal MVEs and constantly supplying S1P, which is a prerequisite for exosomal cargo sorting and MVE maturation in HeLa cells.

II. SiRNA-mediated knockdown of Sphk2 but not SphK1 resulted in a reduction of cargo content in purified exosomes.

SphK1- and SphK2-siRNAs were validated for their ability to inhibit the expression of HA-hSphK1 and HA-hSphK2 expressed in HEK293 cells. Each SphK isozyme-specific siRNA specifically inhibit the expression of the respective isozyme, while having a minimal effect on the expression of the other counterpart, assuring that the siRNA system works properly. When cells were treated with the siRNAs, SphK2-siRNA caused a robust reduction of exosomal cargo contents compared with control siRNA treatment. SphK1-siRNA had little or no effect on the cargo content.

To confirm that SphK2 exerts its role in the regulation of cargo contents in the exosomes in an activity-dependent manner, the ability of an activity-negative mutant SphK2(G248D) to rescue the phenotype seen in SphK2-knockdown cells was tested and compared to the wild-type counterpart. SphK2-siRNA caused a strong reduction of transferrin receptor, HSP70 and flotillin 2.

Importantly, expression of siRNA-resistant wild-type SphK2 almost completely rescued the phenotype, however, expression of activity-negative mutant, SphK2(G248D), could not rescue it. These results strongly suggest that kinase activity of SphK2 is important and that S1P signal plays an important role in the cargo contents in exosomes. The present results that SphK2 knockdown showed reduction in cargo contents in exosomes suggest two possibilities, i.e., inhibition of cargo sorting into exosomal vesicle of MVEs or inhibition of exosome formation. Judging from previous studies that knockdown of SphK2 in HeLa cells caused a reduction of cargo contents in exosomes

without changing the number of exosomes as analyzed by protein-lipid double labeling assay, it may be likely that SphK2 knockdown causes inhibition of cargo sorting into exosomal vesicles in K562 cells.

III. **To strengthen the importance of kinase activity of SphK2 in this phenomenon, further pharmacological experiments were conducted.**

As inhibitors of SphK, N, N-dimethylsphingosine (DMS) and 2-(p-Hydroxyanilino)-4-(p-chlorophenyl) thiazole (HACPT) were widely used to modulate SphK1 and SphK2 activities. When K562 cells were treated with HACPT, the cargo contents in exosomes was strongly reduced in consistence with gene-silencing results. Unexpectedly, DMS treatment increased the cargo contents. So far, we have presented evidence to show that SphK2 is required for the regulation of the cargo contents in exosomes. In this context, DMS action may elicit up-regulation of S1P signal on MVEs but not its inhibition.

IV. **Also the effects of DMS on the kinase activity of SphK2 were studied in a purified in vitro system.**

DMS inhibited the SphK2 activity in a dose-dependent manner when the substrate sphingosine was at 100 µM, which is consistent with a previous report. Since the cellular concentrations of sphingosine were estimated to be around 100 nM as reported before, SphK2 activity was also measured with the lower concentrations of sphingosine (0.2 µM). Under these conditions there were doublet bands in the absence of DMS. The upper band existed in the absence of sphingosine and was therefore not studied further in the present study. The lower band could be detected only in the presence of sphingosine and corresponded to the position of authentic S1P. Surprisingly, under these lower substrate concentrations DMS caused stimulation of the activity (1.5-fold increase) in the DMS concentrations up to 10 µM, and then caused inhibition over the higher concentrations. These results suggest that DMS treatment of cells may cause SphK2-selective activation in certain circumstances, which may account for the phenomenon that seems contradictory.

Collectively, our present results strongly suggest that SphK2 (through receptor-mediated S1P signaling) plays an important role in exosomal MVE maturation.