主論文の要旨

Rho–Rho-Kinase Regulates Ras-ERK Signaling Through SynGAP1 for Dendritic Spine Morphology

（Rho–Rho-KinaseはRas-ERKシグナル経路を活性化することで
スパインの形態を制御する）

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【Introduction】

Glutamate is the most abundant excitatory neurotransmitter and acts through ionotropic receptors, including AMPA receptors (AMPA-R) and NMDA receptors (NMDA-R). When glutamate binds to AMPA-R at the postsynaptic membranes, Na^+ flows into the postsynaptic cells and subsequently induces depolarization. NMDA-R is thought to play important roles in controlling long-term potentiation (LTP), which is one of the most investigated synaptic plasticity and is important for establishing memory, learning, activity-dependent development, and other higher brain processes. Under the depolarized state induced by AMPA-R activation, glutamate stimulates the influx of Ca^{2+} into postsynaptic cells through NMDA-R, which is followed by activating CaMKII and then promoting AMPA-R trafficking via presynaptic sites or intracellular pools to the postsynaptic density for LTP. Ras-ERK signaling is also required for AMPA-R trafficking and surface delivery following LTP induction. One of the key features of LTP is structural synaptic plasticity, such as dendritic spine enlargements. Upon stimulation of NMDA-R, CaMKII is thought to activate RhoA and its effector Rho-kinase/ROCK for spine enlargement, presumably through promoting actin polymerization. The Ras-ERK pathway is also involved in spine enlargement during NMDA-dependent LTP. However, the regulatory interaction between RhoA-Rho-kinase and Ras underlying spine enlargement and LTP is still largely unknown.

We previously developed a novel phosphoproteomic method called phosphatase inhibitor and kinase inhibitor substrate screening (PIKISS) that could comprehensively identify Rho-kinase substrates. The data obtained from the phosphoproteomics were registered on the online database named KANPHOS (kinase-associated neural phosphosignaling), which we developed. KANPHOS provides the phosphorylated proteins and sites identified by the abovementioned approach as well as those previously reported in the literature. We hypothesized that the KANPHOS data contained Rho-kinase substrate candidates that regulated Ras activity, performed pathway analysis, and identified SynGAP1 as a candidate. SynGAP1 (Synaptic Ras-GTPase-activating protein) converts the GTP-bound active form of Ras to the GDP-bound inactive form. SynGAP1 is involved in synaptic plasticity, AMPA-R trafficking to the postsynaptic membrane and ERK activity.

【Methods and Results】

To examine whether Rho-kinase directly phosphorylates SynGAP1, we performed an *in vitro* kinase assay using SynGAP1 fragments. The plasmids encoding GST-SynGAP1-NT, -NTMD or -CT were transfected into COS7 cells, and the GST-fusion proteins were purified by glutathione affinity beads. GST-SynGAP1-NT, -NTMD or -CT was incubated with Rho-kinase-CAT in the presence of [γ-^{32}P]ATP. SynGAP1-CT was efficiently phosphorylated by Rho-kinase, whereas SynGAP1-NT and -NTMD were weakly phosphorylated (Fig. 1a, b). The KANPHOS data predicted that Rho-kinase phosphorylated SynGAP1 at Ser842 in the C-

terminal disorganized domain. To examine whether Rho-kinase phosphorylates SynGAP1 at Ser842, we generated anti-pS842 SynGAP1 antibody. We next examined whether Rho-kinase phosphorylated SynGAP1 in intact cells. The plasmids harboring Myc-SynGAP1 were transfected into HeLa cells, and the cells were treated with DMSO or Y-27632 for 1 hour and then stimulated with okadaic acid for 1 hour. Immunoblot analysis with anti-p842 SynGAP1 and anti-Myc antibodies revealed that okadaic acid induced SynGAP1 phosphorylation at Ser842 and Y-27632 prohibited it (Fig. 1c, d). These results indicate that Rho-kinase phosphorylates SynGAP1 at Ser842.

Because SynGAP1 is a negative regulator of Ras and the binding of 14-3-3 usually affects the binding partner's activity, we next investigated whether Rho-kinase regulates Ras activity through SynGAP1 phosphorylation. We investigated whether Rho-kinase regulates Ras activity through SynGAP1 phosphorylation. HA-Rho-kinase-CAT or RB/PH (TT) was expressed with Myc-SynGAP1 in HeLa cells. The cell lysates were incubated with GST- Raf1-RBD to precipitate active Ras, which was followed by immunoblot analysis using anti-Ras and phosphorylated ERK. Coexpression of Rho-kinase-CAT with SynGAP1 increased active Ras and ERK1/2 phosphorylation, but neither were stimulated with that of RB/PH (TT) (Fig. 2a-c). Under the same conditions, Rho-kinase-CAT did not show the stimulatory effect of SynGAP1-S842A on Ras activity and ERK phosphorylation (Fig. 2d-f), suggesting that Rho-kinase phosphorylates SynGAP1 at Ser842, thereby stimulating Ras-ERK signaling.

To examine whether Rho-kinase regulates the Ras-ERK pathway through SynGAP1 phosphorylation in neurons, we prepared DIV21 striatal neurons and then chemically induced LTP using a standard protocol that selectively activated synaptic NMDARs (Chemical LTP). In this method, magnesium in the media was withdrawn in conjunction with glycine perfusion. With spontaneous glutamate release from axonal terminals, glycine strongly and specifically stimulates synaptic NMDA-R. The striatal neurons were treated with DMSO or Y-27632 for 1 hour and then incubated with glycine. To induce chemical LTP for 10 min or 60 min, which was followed by morphology analysis of dendritic spines using anti-SynGAP1 and PSD95 antibodies (Fig. 3a). SynGAP1 is localized at dendritic spines and forms a large macromolecule complex with PSD95 and NMDA-R. We found that treatment with glycine induced a sustained increase in dendritic spine size at 10 min and 60 min (Fig. 3b). We also found that glycine treatment showed a decrease in SynGAP1 colocalized with PSD95 at the spines (Fig. 3c). However, pretreatment with Y-27632 inhibited the glycine-induced increases in spine size and dispersion of Syngap1 from the spines (Fig. 3b, c).

We next examined whether chemical LTP induction modulated the interactions among SynGAP1, PSD95 and 14-3-3ζ. After treatment with glycine, SynGAP1 was immunoprecipitated from the cell lysates with the anti-SynGAP1 antibody, and the immunoprecipitants were subjected to immunoblot analysis with the anti-PSD95 and anti-14-3-3ζ antibodies (Fig. 3d-f). The amounts of PSD95 immunoprecipitated with SynGAP1 progressively decreased during

chemical LTP, whereas those of 14-3-3ζ increased. These changes were reversed by pretreating the neurons with Y- 27632 (Fig. 3e, f).

We also tested whether SynGAP1 was phosphorylated during chemical LTP using the anti-p842 SynGAP1 antibody but could not detect phosphorylation. This failure may be due to the low sensitivity of the antibody and expression levels of SynGAP1. However, we assume that chemical LTP induction stimulates SynGAP1 phosphorylation by Rho-kinase because treatment with glycine increased the interaction of SynGAP1 with 14-3-3ζ and pretreatment with Y-27632 inhibited the interaction.

We further examined whether chemical LTP induction affected Ras-ERK signaling. After treatment with glycine, we measured the Ras activity and the phosphorylation of MYPT1 and ERK. The results showed that glycine treatment stimulated Ras activity and the phosphorylation of MYPT1 and ERK in a time-dependent manner, and these effects were prevented by pretreatment with Y-27632 (Fig. 3g-j).

【Conclusion and Discussion】

In this study, we found that Rho-kinase phosphorylated SynGAP1 at Ser842 and induced the association of SynGAP1 with 14-3-3ζ, thereby activating Ras-ERK signaling. The stimulation of NMDA-R by glycine treatment for LTP induction stimulated SynGAP1 phosphorylation, Ras-ERK activation, spine enlargement and delocalization of SynGAP1 from the spines in striatal neurons, whereas these effects were prevented by pretreatment with a Rho-kinase inhibitor (Y-27632). The Rho-kinase-mediated phosphorylation of SynGAP1 appeared to induce its dissociation from PSD95 but its association with 14-3-3. Based on our findings with previous findings, we propose the mechanism by which the RhoA-Rho-kinase signal regulates Ras-ERK signaling during LTP induction (Fig. 4). Upon NMDA-R stimulation, the influx of Ca^{2+} to neurons is induced to activate CaMKII, thereby stimulating RhoA-Rho-kinase. Rho-kinase phosphorylates SynGAP1 and induces its association with 14-3-3ζ, resulting in inactivation of SynGAP1 activity and its dissociation from PSD95, which is anchored to NMDA-R. Eventually, Ras-ERK signaling is activated, leading to postsynaptic insertion of AMPA-R and spine enlargement, as previously reported. On the other hand, the RhoA-Rho-kinase pathway appears to modulate actin polymerization, resulting in spine enlargement (Fig. 4).