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論 文 審 査 委 員	（主査） 教 授 細川　亙 （副査） 教 授 高井　義美　教 授 吉川　秀樹

論 文 内 容 の 要 旨

〔 目　　的 〕

Rho GTPase are a family of highly related proteins that are best characterized for their effects on the actin cytoskeleton. The representatives of the Rho family are Rho, Rac and Cdc42. Several isoforms of Rho have been reported, are in neurons, RhoA is expressed at higher levels than RhoB and RhoC. Rho family members signal to the actin cytoskeleton through a variety of downstream effector proteins that bind specifically to the active form of Rho GTPases. Rho, together with other Rho family members, is responsible for various morphological changes that take place during neuronal development, such as dendrite elaboration, neurite outgrowth and axon guidance. Rho, in its active GTPbound form, rigidifies the actin cytoskeleton, and thereby inhibits axonal elongation and mediates growth cone collapse. Rho has been shown to be a key intracellular effector for growth inhibitory signalling by myelin. The growth inhibition in the central nervous system is a major barrier to axon regeneration. It was shown previously that myelin-associated glycoprotein, a glycoprotein derived from myelin, activates RhoA, and thus inhibits neurite outgrowth from postnatal sensory neurons and cerebellar neurons. The neurotrophin receptor p75 ($p75_{NTR}$), which is expressed in the developing neurons as well as injured neurons, mediates this signal. Subsequent study demonstrates that Nogo and oligodendrocyte myelin glycoprotein, the other myelinderived inhibitors of the neurite outgrowth, act on neurons via$p75_{NTR}$.$p75_{NTR}$ in complex with the Nogo receptor is suggested to form a receptor for all the myelin-derived inhibitors found so far. As these inhibitors may contribute, at least partly, to the lack of regeneration of the central nervous system, these findings are expected to provide us with a molecular target for the treatment of injuries to the central nervous system. However, there has been no direct evidence that Rho is activated in the injured central nervous system in vivo. To address the question, we developed a new tool to visualize Rho activity in situ.

〔 方法ならびに成績 〕

Expression and purification of recombinant proteins. We prepared four recombinant proteins using the

bacterial expression vector pGEX 5x3 plasmid (Amersham Biosciences). Three of them were fused proteins of PTD4 domain and Myc tag epitope with either the RBD of Rhotekin (nucleotides 609-857, accession number NM 009106), Rap1-binding domain of RalGDS (nucleotides 2373-2660, accession number NM 006266) or with 20 random amino acids (NSRAGYAGRTQSCRGNGIRM). The fourth protein consists of RBD, Myc tag and a scrambled version of PTD4 domain (ScrPTD4) (ARARARYAQAA). Escherichia coli strain BL21 transformed with the vectors was treated overnight at 20 1C with 0.1 mM isopropyl-thio-b-D-galactoside to induce protein expression. The protein was purified through a Glutathione-Sepharose 4B column (Amersham Biosciences). The glutathione-S-transferase (GST) moiety was removed by Factor Xa (Amersham Bioscience). Expression of the protein was confirmed by Coomassie blue (CBB) staining and western blotting using monoclonal anti-Myc antibody (Upstate Biotech).

NIH3T3 cells assay. NIH3T3 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) (Sigma,St Louis, MO), penicillin and streptoMycin. Then, the medium was replaced with DMEM without serum for 24 h. After the cells were incubated with or without one of the recombinant proteins for 3 h at the final concentration of 1 mM, they were treated for 10 min with 10% FBS to stimulate RhoA and fixed in 4.0% paraformaldehyde in PBS (50 mM sodium phosphate (pH 7.5) and 150 mM NaCl) for 20 min. Immunocytochemistry was performed by overnight incubation with athi-Myc antibody (Upstate Biotech.), followed by incubation with Alexa fluorTM 488-labelled anti-mouse IgG and Rhodamine-Phalloidin (Molecular Probes).

Neuronal cultures. The cerebella from two P7-P10 animals were combined in 5 ml of 0.025% trypsin, triturated and incubated for 15 min at 37 1C. DMEM containing 10% fetal calf serum was added, and the cells were centrifuged at 800 rpm. Neurons were plated in DMEM containing 10% FBS on poly-L-lysine-or myelincoated chamber slides. The myelin coating was performed as described by Zheng et al (2003) : briefly, the chamber slides were precoated with poly-L-lysine, coated with 1 mg of myelin per chamber, left to dry up and finally coated with laminin. Using this technique, 98% of the cells were positive for neuron-specific b-tubulin III. For assays, plated cells were serum starved for 36 h and incubated with 1 mM of one of the recombinant proteins for the final 3 h of serum atarvation. Where indicated, the Nogo peptide (residues 31-55 of the extracellular fragment of Nogo) (Alpha Diagnostics) at the final concentration of 10 mM was added to the culture medium and was incubated for 10 min. The cells were fixed in 4% (wt/vol) paraformaldehyde, and were immnostained with anti-Myc antibody and polyclonal anti-p75 antibody (Promega). Alexa fluorTM 488- labelled anti-mouse and Alexa fluorTM 568-labelled anti-rabbit IgG (Molecular Probes) were used as secondary antibodies. Selected cultures were preincubated with 10 mg/ml of P75/Fc chimaera (R&D Systems) for 2 h before the Nogo peptide addition.

Animal experiments. Male Wistar rats (200 g body weight) were anaesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), and wide bilateral laminectomy at the thoracic level (Th12) was performed. Then, dorsal 3/4 of the spinal cord was transected using microscissors. At 14 h after the transection, 8 mg/kg body weigtht of PTD4-Myc-RBD, PTD4-Myc-RalGDS or ScrPTD4-Myc-RBD, or 3 mg/kg body weight of PTD4-Myc-20aa, was administered via the tail vein. The rats were killed 24 h after the injury and perfused with 4% paraformaldehyde. The spinal cord was postfixed with 4% paraformaldehyde, dipped into 30% sucrose for a day, frozen on solid CO_2, mounted and serial longitudinal sections were prepared. The sections were air-dried, blocked with 5% FBS and 0.2% Triton X-100 for 1 h, and incubated overnight with rabbit polyclonal anti-Myc antibody (Upstate) and either monoclonal anti-p75 antibody (Chemicon) or monoclonal anti-neuron-specific b-tubulin III antibody (Covance). After extensive washing with 0.02M PBS, the secondary antibody reaction was carried out as described above.

Affinity precipitation of active RhoA. NIH3T3 cells and CGNs were incubated with 1 mM PTD4-Myc-RBD,

and treated with 10% FBS or 10 mM Nogo peptide. The cells were then lysed in a lysis buffer as described in Yamashita et al (2002). A total of 8 mg/kg body weight of PTD4-Myc-RBD was administered via the tail vein of the rats with or without spinal cord injury. At 24 h after injury, the rats were killed, and their spinal cords were removed and sonicated on ice in short bursts in the same lysis buffer. The cell lysates were finally clarified by centrifugation at 13,000 g at 4 1C for 10 min, and the supernatants were incubated with 20 mg of GST-Rho-binding domain of Rhotekin beads at 4 1C for 45 min. The beads were washed four times with washing buffer (Yamashita et al, 2002). Bound Rho proteins and total Rho proteins from the cell lysate were detected by western blotting using a monoclonal antibody against RhoA (Santa Cruz Biotechnology).

Quantitative analysis of Myc signal intensity in spinal cord. We analysed at least three sections per rat, six rats for each experimental condition. The area of corticospinal tract in the rostral stump, approximately 3-6 mm rostrally from the injury site, was studied. Using Macsope analysis software (Mitani Corp.), we measured the intensity of fibres longer than 15 mm. Double immunohistochemical analysis showed that fibres shorter than 15 mm were negative for neuron-specific b-tubulin III, demonstrating that they were not neurons, whereas fibres longer than 15 mm were positive for neuron-specific b-tubulin III. As the focus of our study is to analyse the activity of RhoA in the neurons, we selected a subset of fibres longer than 15 mm. The statistical analysis was carried out by t-test.

NIH3T3 cells. Using Macsope software, we examined five cells for each experimental condition. In each cell, we selected five distinct locations under the cell membrane and in the intermediate zone among the stress fibres. In every location, we measured the intensity of both Myc and F-actin. The average intensity of Myc signal in the cell was calculated as well. Finally, the data obtained from serum-stimulated and unstimulated cells were analysed by t-test.

〔総　括〕

Axons of the adult central nervous system have very limited ability to regenerate after injury. This inability may be, at least partly, attributable to myelin-derived proteins, such as myelinassociated glycoprotein, Nogo and oligodendrocyte myelin glycoprotein. Recent evidence suggests that these proteins inhibit neurite outgrowth by activation of Rho through the neurotrophin receptor p75NTR/Nogo receptor complex. Despite rapidly growing knowledge on these signals at the molecular level, it remained to be determined whether Rho is activated after injury to the central nervous system. To assess this question, we establish a new method to visualize endogenous Rho activity in situ. After treatment of cerebellar granular neurons with the Nogo peptide in vitro, Rho is spatially activated and colocalizes with p75NTR. Following spinal cord injury in vivo, massive activation of Rho is observed in the injured neurites. Spatial regulation of Rho activity may be necessary for axonal regulation by the inhibitory cues.

論文審査の結果の要旨

現在、Nogo、myelin associated protein や oligodendrocytemyelin glycoprotein などのミエリン関連物質は中枢神経再生を阻害していると考えられている。最近、P75NTR/Nogo receptor complex を介した Rho の活性化がミエリン関連物質の神経再生阻害に関与していることが判明した。そういった分子レベルのシグナル伝達の解明は急速に進歩しているが、未だ生体内において、中枢神経損傷時に実際に Rho が活性化されているかは明らかになっていない。本研究ではこの疑問を解明するために、生体内において Rho の活性化を可視化する方法を発明した。すなわち、活性化型 Rho とのみ結合する Rhotekin と、細胞内にタンパク導入するための PTD4 を融合させたタンパクを作成し生体内に投与し、この融合タンパクに対する抗体を用いて活性化型 Rho の局在を探知するというものである。実際のラット

の脊髄損傷モデルに融合タンパクを投与すると本研究で作成した融合タンパクは、脊髄損傷部位に局在しまた、P75NTR の局在とも一致していた。よって中枢神経損傷時、生体内で Rho は活性化されていることが明らかとなった。以上の研究結果は神経軸索再生に関する基礎研究として重要な結果を示したと考えられ、学位の授与に値するものと認める。