Activation of Rho in the injured axons following spinal cord injury

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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 myelin-associated glycoprotein, Nogo and oligodendrocyte myelin glycoprotein. Recent evidence suggests that these proteins inhibit neurite outgrowth by activation of Rho through the neurotrophin receptor p75^{NTR}/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 p75^{NTR}. 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.

Keywords: Rho; myelin; actin cytoskeleton; p75; regeneration

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INTRODUCTION

Rho GTPases 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, and in neurons, RhoA is expressed at higher levels than RhoB and RhoC (Lehmann *et al*,

1999). 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 (Davies, 2000; Schmidt & Hall, 2002). Rho, in its active GTP-bound 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 (McKerracher & Winton, 2002). 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 (Yamashita *et al*, 2002). 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 myelin-derived inhibitors of the neurite outgrowth, act on neurons via p75^{NTR} (Wang *et al*, 2002a). p75^{NTR} in complex with the Nogo receptor is suggested to form a receptor for all the myelin-derived inhibitors found so far (Wang *et al*, 2002b; Wong *et al*, 2002). 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*.

RESULTS

PTD4–Myc–RBD protein detects Rho activity *in situ*

Using the RhoA-binding domain (RBD) of the effector protein Rhotekin, the GTP-bound form of RhoA can be affinity precipitated (Ren *et al*, 1999) and detected *in situ* (Li *et al*, 2002), thus

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allowing us to detect Rho activity *in vivo*. We generated RBD fused with Myc and the amino-terminal 11-amino-acid protein transduction domain from the human immunodeficiency virus protein PTD4 (YARAAARQARA) (Ho *et al*, 2001), so that the protein may gain entry into the cell (Fig 1A). The fused protein PTD4-Myc-RBD was expressed as a recombinant protein in bacteria, as shown in a gel electrophoresis and immunoblot (Fig 1B) analysis. PTD4-Myc fusion proteins with Rap-binding domain of RalGDS (PTD4-Myc-RalGDS) (van Triest *et al*, 2001) and with 20 random amino acids (PTD4-Myc-20aa) were prepared (supplementary Figs 3A,B and 4A,B online) as controls. RBD and Myc tag epitope were also fused with a scrambled version of PTD4 (ARARARYAQAA) and expressed (ScrPTD4-Myc-RBD) (supplementary Fig 5A,B online). We first tested whether PTD4-Myc-RBD works as an indicator of Rho activity in the cells. NIH3T3 cells were serum starved for 24 h, followed by incubation with PTD4-Myc-RBD in the culture medium at a final concentration of 1 μ M for 3 h. Then, the cells were treated with 10% serum, which activates RhoA, for 10 min, and were immunostained with anti-Myc antibody. In the control cells not

treated with serum, we observed a weak and diffuse signal for PTD4-Myc-RBD, showing the presence of the PTD4-fused protein in the cells (Fig 1C). There was no visible difference in the background intensity for the signal among the cells. In contrast, significant colocalization of PTD4-Myc-RBD with F-actin, stained with phalloidin, was observed in the serum-stimulated cells (Fig 1C). Intense signals for PTD4-Myc-RBD were found in the edges of the membrane and along the stress fibres. To quantify the data, we selected five distinct locations under the cell membrane and among the stress fibres in the intermediate zone. In these locations, we measured the intensity of both F-actin and Myc signals (supplementary Fig 1A online, Fig 1E). We found that the marked increase of F-actin signal intensity under the cell membrane after serum stimulation correlated with the increase of Myc signal intensity in the same location ($P < 0.01$). On the contrary, the intensity of Myc signal in the intermediate zone among the stress fibres significantly decreased after serum stimulation ($P < 0.01$). As the average intensity of Myc in the cell (Fig 1E) was not affected by serum stimulation, the increase of Myc intensity under the cell membrane was caused by redistribution of PTD4-Myc-RBD after serum stimulation and its accumulation in the locations where active Rho is present.

When PTD4-Myc-RalGDS or PTD4-Myc-20aa proteins were used for the assay, a diffuse signal for Myc was observed without serum stimulation, and remained unchanged after the addition of serum (supplementary Figs 3C and 4C online; Fig 1D). By quantitative analysis, we found that Myc signal did not correlate with the increase of F-actin signal intensity under the cell membrane and there were no significant changes in the Myc signal distribution in the cells. Again, the total Myc intensity in the

Fig 1 | PTD4-Myc-RBD as a tool for detecting Rho activity. (A) Structure of PTD4-Myc-RBD. RBD of Rhotekin (residues 609–857). (B) Purification of PTD4-Myc-RBD. PTD4-Myc-RBD was produced as a GST-fused protein, and the GST moiety was removed. Coomassie blue staining (left) and immunoblot with the anti-Myc antibody (right) are shown. (C) PTD4-Myc-RBD detects the active form of Rho. Serum-starved NIH3T3 cells were either treated or not treated with serum. The cells were stained with anti-Myc antibody (Myc) and phalloidin (F-actin). Note the colocalization of PTD4-Myc-RBD with F-actin in the serum-stimulated cells. In the control cells not treated with serum, only a weak and diffuse signal for PTD4-Myc-RBD was observed, showing the presence of the PTD4-fused protein in the cells. RBD: PTD4-Myc-RBD. Scale bar, 10 μ m. (D) PTD4-Myc-20aa does not detect the active form of Rho. Serum-starved NIH3T3 cells were either treated or not treated with the serum. The cells were stained with anti-Myc antibody (Myc) and phalloidin (F-actin). The PTD4-Myc-20aa signal is not colocalized with F-actin and shows no detectable changes in the distribution after 10% FBS addition. 20aa: PTD4-Myc-20aa. Scale bar, 10 μ m. (E) Quantitative analysis of PTD4-Myc-RBD intensity in NIH3T3 cells. Myc and F-actin (supplementary Fig 1A online) were measured in five distinct locations under the cell membrane and in the intermediate zone among the stress fibres. Note the increase of Myc staining under the cell membrane after serum stimulation (left) together with the increase of F-actin signal intensity in the same locations (supplementary Fig 1A online). In the intermediate zone, there was a significant decrease in Myc signal intensity after stimulation (middle), and the total intensity of Myc signal was not affected by serum stimulation (right). Black columns, minus FBS; grey columns, plus FBS. * $P < 0.01$.

cells was not affected by serum stimulation (supplementary Figs 4D and 5D online). No signal was observed in the cells treated with ScrPTD4-Myc-RBD (supplementary Fig 5C online). These results show that PTD4-Myc-RBD may be used as an efficient tool to detect the active form of Rho *in situ*.

Activation of Rho in cerebellar neurons by Nogo

Several myelin-derived proteins, which prevent axonal regeneration, have been identified in the adult central nervous system. Recent studies have shown that these proteins inhibit neurite outgrowth from some postnatal neurons by activating Rho. This signal transduction is mediated by p75^{NTR}. These findings prompted us to examine the activity of Rho in neurons. The Nogo peptide was shown to inhibit neurite outgrowth of cerebellar granule neurons (CGNs) (Yamashita & Tohyama, 2003). CGNs from P7–P10 rats were labelled with 1 μ M PTD4-Myc-RBD for 3 h, followed by treatment with 10 μ M Nogo peptide (residues 31–55 of the extracellular fragment of Nogo) (Fournier *et al*, 2001) for 10 min. Figure 2A shows the signal for PTD4-Myc-RBD in CGNs, which appears punctate on the neurites after stimulation with the Nogo peptide. In Nogo-untreated cells, only a weak and diffuse signal is seen in the neurites. The same cells were stained with an anti-p75^{NTR} antibody, and the distribution was assessed. p75^{NTR} expression on the cell bodies was rather diffuse but that on the neurites showed fine speckled staining. Consistent with the data that show activation of Rho by p75^{NTR} (Wang *et al*, 2002b; Yamashita *et al*, 2002), the vast majority of puncta for p75^{NTR} immunoreactivity was colocalized with PTD4-Myc-RBD after Nogo stimulation. The extracellular domain of p75^{NTR} acts as a dominant-negative form that inhibits the signal (Wang *et al*, 2002b). When CGNs were preincubated with 10 μ g/ml p75-Fc for 2 h before Nogo stimulation, the PTD4-Myc-RBD signal in the neurites remained diffuse and no colocalization with p75^{NTR} signal could be observed (supplementary Fig 1B online). When CGNs were plated on myelin, the signal for PTD4-Myc-RBD was more robust than that in the cells plated on poly-L-lysine (Fig 2A, supplementary Fig 1C online). In the case of PTD4-Myc-RaIGDS and PTD4-Myc-20aa proteins, we observed only a diffuse signal in the neurites without any change after the Nogo peptide stimulation (supplementary Figs 6 and 7 online). We did not detect any signal for ScrPTD4-Myc-RBD protein (supplementary Fig 8 online). These results suggest that Rho is activated by Nogo in a spatially restricted manner.

Rho is activated in the axons after spinal cord injury

The aim of our study was to determine Rho activity after spinal cord injury *in vivo*. Rat spinal cord injury at the Th12 level was made by transection of the dorsal 3/4 of the spinal cord. At 14 h after injury, 8 mg/kg body weight of PTD4-Myc-RBD was administered via the tail vein, and the animals were killed 10 h later. We made longitudinal sections of the spinal cords. PTD4-Myc-RBD was successfully incorporated into the cells in the uninjured as well as the injured spinal cord (Fig 3A), showing that the protein can cross the blood–brain barrier. In the sham-operated animals, PTD4-Myc-RBD is mainly localized in cell bodies in the grey matter, whereas the signal in the white matter is weak (Fig 3A). However, after the injury, we observed intensively stained fibres in the white matter, in which the expression of p75^{NTR} is induced (Fig 3A). The Myc signal was significantly more

Fig 2 | Nogo-mediated activation of Rho in CGN. (A) Rho is spatially activated by the Nogo peptide. CGNs were either treated or not treated with the Nogo peptide (10 μ M) for 10 min, and immunostained with the anti-Myc antibody and the anti-p75 antibody. Representative single optical sections for p75^{NTR} (left), PTD4-Myc-RBD (middle) and overlay images (right) are shown. Note the patchy distribution of p75^{NTR} and its association with PTD4-Myc-RBD on the neurites (arrowheads in the upper row merged image) after the Nogo peptide treatment, suggesting activation of Rho in a spatially restricted manner. In the control cells, the neurites were stained only diffusely. The smaller panel for each condition in the upper two rows shows the details of an area depicted by a frame in the main panel. Scale bar, 10 μ m. (B) PTD4-Myc-RBD does not block Rho activation in the cells. NIH3T3 cells (NIH3T3) and CGNs were exposed to PTD4-Myc-RBD, and either treated or not treated with 10% FBS or 10 μ M Nogo peptide, respectively. PTD4-Myc-RBD was administered to the rats with or without spinal cord injury. Active fraction of RhoA that was affinity precipitated from lysates was detected together with total RhoA by western blotting using monoclonal antibody against RhoA (Santa Cruz). The panels for active RhoA indicate that RhoA activation occurs after FBS or the Nogo peptide addition or after spinal cord injury in the presence of PTD4-Myc-RBD.

intensive in the injured animals than in the sham-operated ones when quantifying the signal intensity in the corticospinal tract rostrally to the injury site (Fig 3B). There were no major changes in the intensity and distribution of Myc signal in the grey matter.

It is also noted that p75^{NTR} immunoreactivity was markedly upregulated after the injury in both grey and white matter. Double immunostaining with anti-Myc- and anti-neuron-specific β -tubulin III revealed colocalization and correlation in the signal intensity of both signals (supplementary Fig 2 online). Experiments

Fig 3 | Activation of Rho following spinal cord injury. (A) Representative longitudinal sections of spinal cords of injured and sham-operated animals that received PTD4-Myc-RBD intravenously. Images of white matter were taken from the rostral stump from the area of the corticospinal tract 3 mm from the transection site and the corresponding site in sham-operated rats. Images of grey matter were taken from the base of the dorsal horn 3 mm rostrally from the transection site. The sections of injured and sham-operated spinal cord were immunostained with the anti-Myc antibody and the anti-p75 antibody. p75^{NTR} immunoreactivity is induced in the fibres in the white matter after the injury as well as in the neurons in the grey matter. In the white matter, a clear increase in the Myc staining showing close association with the p75^{NTR} signal can be observed. RBD +: PTD4-Myc-RBD; RBD -: no administration of PTD4-Myc-RBD. Scale bar, 20 µm. (B) Quantitative analysis of PTD4-Myc-RBD signal intensity in the white matter of injured and sham-operated (intact) animals. The intensity of the Myc signal in the fibres was significantly higher in the injured than in the sham-operated animal. The data are mean ± s.e.m. *P < 0.01.

with PTD4-Myc-RaIGDS and PTD4-Myc-20aa proteins (supplementary Figs 9A and 10A online) showed no significant difference in the Myc signal intensity between injured and sham-operated animals (supplementary Figs 9B and 10B online). No Myc signal was detected in the spinal cord after ScrPTD4-Myc-RBD administration (supplementary Fig 11 online). These results demonstrate that Rho is activated extensively, at least in the neurites, after spinal cord injury.

DISCUSSION

Evidence for extensive activation of Rho in the fibres of the injured central nervous system neurons strengthens the view that the effects of myelin-derived inhibitors of axonal regeneration may be mediated by Rho (Wang *et al*, 2002b; Yamashita *et al*, 2002; Yamashita & Tohyama, 2003). Indeed, inhibition of Rho as well as Rho kinase, an effector of Rho, facilitates regeneration after axonal injury in the spinal cord (Dergham *et al*, 2002; Fournier *et al*, 2003) and in the optic nerve (Lehmann *et al*, 1999). The myelin-derived inhibitors of axon outgrowth act on neurons through p75^{NTR} (Wang *et al*, 2002b; Wong *et al*, 2002; Yamashita *et al*, 2002). Activation of Rho could not be observed during the early stage after spinal cord injury (until 72 h) in the injured spinal cord of mice lacking functional p75^{NTR} (Dubreuil *et al*, 2003).

Although p75^{NTR} itself does not mediate the process of guanine nucleotide exchange, p75^{NTR} has an ability to release RhoA from Rho GDI (Yamashita & Tohyama, 2003). The release of Rho from Rho GDI is an important step allowing the activation by guanine nucleotide exchange factors and membrane association of the GTP-bound form of Rho. Interestingly, a recent report suggests a role of Rho GDI in spatial and temporal activation of the downstream pathway of Rac1 (Del Pozo *et al*, 2002), which is the other member of Rho family. Although Rho GDI associates with Rac1 and blocks effector binding, the release of Rac1 from Rho GDI at specific regions where integrin localizes allows Rac1 to bind its effectors. Thus, Rho GDI is suggested to confer spatially restricted regulation of Rho GTPases-effectors interaction. These findings are in good correlation with our observation that Rho was spatially activated by Nogo in CGNs, which was colocalized with p75^{NTR}. As neurons show highly asymmetrical structures, regulation of Rho activity in a spatially restricted manner in neurons may be crucial for proper axonal guidance or motility. It will be interesting to test where Rho is activated in the growth cone of neurons by repulsive or attractive molecules, which repel or attract axons, respectively.

Our study shows that the PTD4-Myc-RBD protein is useful for visualizing Rho activity *in situ*. Somewhat unexpectedly, the intensity of the protein signal in the state of basal RhoA activity was minimal, at least in NIH3T3 cells, CGNs and the spinal cord of rats. At the protein doses and time periods of incubation with the protein we used, we do not expect PTD4-Myc-RBD to block Rho function, as we were able to detect Rho activation in the presence of PTD4-Myc-RBD in the NIH3T3, CGNs and the spinal cord (Fig 2B). Therefore, PTD4-Myc-RBD may be used as an efficient and easy tool to visualize Rho activity also in other systems.

METHODS

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 (NSRAGYAGRTQSCRGNIGRM). 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 °C with 0.1 mM isopropyl-thio- β -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 Biosciences). 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 μ M, 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 anti-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 °C. 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 myelin-coated 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 β -tubulin III. For assays, plated cells were serum starved for 36 h and incubated with 1 μ M of one of the recombinant proteins for the final 3 h of serum starvation. Where indicated, the Nogo peptide (residues 31–55 of the extracellular fragment of Nogo) (Alpha Diagnostics) at the final concentration of 10 μ M was added to the culture medium and was incubated for 10 min. The cells were fixed in 4% (wt/vol) paraformaldehyde, and were immunostained 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 μ g/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 weight 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 CO₂, 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 β -tubulin III antibody (Covance). After extensive washing with 0.02 M PBS, the secondary antibody reaction was carried out as described above.

Affinity precipitation of active RhoA. NIH3T3 cells and CGNs were incubated with 1 μ M PTD4–Myc–RBD, and treated with 10% FBS or 10 μ M 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 13,000g at 4 °C for 10 min, and the supernatants were incubated with 20 μ g of GST–Rho-binding domain of Rhotekin beads at 4 °C 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 Macscope analysis software (Mitani Corp.), we measured the intensity of fibres longer than 15 μ m. Double immunohistochemical analysis showed that fibres shorter than 15 μ m were negative for neuron-specific β -tubulin III, demonstrating that they were not neurons, whereas fibres longer than 15 μ m were positive for neuron-specific β -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 μ m. The statistical analysis was carried out by *t*-test.

NIH3T3 cells. Using Macscope 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.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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