Myelin-associated Glycoprotein Inhibits Microtubule Assembly by a Rho-kinase-dependent Mechanism*

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Myelin-associated glycoprotein (MAG) and Nogo are potent inhibitors of neurite outgrowth from a variety of neurons, and they have been identified as possible components of the central nervous system myelin that prevents axonal regeneration in the adult vertebrate central nervous system. The activation of RhoA and Rho-kinase is reported to be an essential part of the signaling mechanism of these proteins. Here, we report that the collapsing response mediator protein-2 (CRMP-2) is phosphorylated by a Rho-kinase-dependent mechanism downstream of MAG or Nogo-66. The overexpression of the nonphosphorylated form of CRMP-2 at threonine 555, which is the phosphorylation site for Rho-kinase, counteracts the inhibitory effect of MAG on the postnatal cerebellar neurons. Additionally, the expression of the dominant negative form of CRMP-2 or knockdown of the gene using small interference RNA (siRNA) mimics the effect of MAG in vitro. Consistent with the function of CRMP-2, which promotes microtubule assembly, microtubule levels are down-regulated in the cerebellar neurons that are stimulated with MAG in vitro. Reduction in the density of microtubules is also observed in the injured axons following the spinal cord injury, and this effect depends on the Rho-kinase activity. Our data suggest the important roles of CRMP-2 and microtubules in the inhibition of the axon regeneration by the myelin-derived inhibitors.

Several myelin-derived proteins have been identified as components of the central nervous system myelin that prevents axonal regeneration in the adult vertebrate central nervous system. To date, three major inhibitors that are expressed by oligodendrocytes and myelinated fiber tracts have been identified (1). These are Nogo, myelin-associated glycoprotein (MAG),2 and oligodendrocyte-myelin glycoprotein. All these proteins act on neurons through the p75 receptor (p75) (2–4) in common with the Nogo receptor. One potential clue to understanding the signal transduction mechanism downstream of p75 is the role of MAG in axon elongation. It is suggested that dynamic actin filaments allow the enhanced transport and/or polymerization of microtubules, thereby resulting in enhanced process elongation (12). Therefore, dynamic properties of microtubules, which are regulated by diverse cellular factors, appear to be important for the regulation of axon elongation.

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2 The abbreviations used are: MAG, myelin-associated glycoprotein; DRG, dorsal root ganglia; GST, glutathione S-transferase; siRNA, small interference RNA; PBS, phosphate-buffered saline; ANOVA, analysis of variance; MLC, myosin light chain; CRMP-2, collapsing response mediator protein-2.

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Eagle’s medium containing 10% fetal calf serum was added, and the cells were centrifuged at 1000 rpm. Neurons were plated in Sato media (15) on poly-L-lysine-coated chamber slides. For outgrowth assays, plated cells were incubated for 24 h, fixed in 4% (w/v) paraformaldehyde, and immunostained with a monoclonal antibody (TuJ1) recognizing the neuron-specific β-tubulin III protein. Then, the length of the longest neurite or the total process outgrowth for each β-tubulin III-positive neuron was determined. Where indicated, IgG-Fc (25 μg/ml, Athens Research and Technology), MAG-Fc (25 μg/ml), or glutathione S-transferase (GST)-fused protein of Nogo-66 was added to the medium after plating. Myc-tagged CRMP-2 plasmid, the deletion form of CRMP-2 plasmid (14), and the mutant CRMP-2 T555A (Thr-555) (15) was replaced with Ala-555 (16) were used. CRMP-2 siRNA (5’-ACTCTTCCTCCTCGTGTACAT-3’) and a scramble siRNA (5’-CAGTCGCGTTTGCGACTGG-3’), as a control, were synthesized by Japan Bio Service (Saitama, Japan). Transfection of plasmids or siRNA in primary culture was performed after 12-h incubation employing nucleofection reagent (Amaxa, Koln, Germany), according to the manufacturer’s instructions. 24 h after transfection, the cells were replated for an additional 4 h. To determine the transfected cells, cells were permeabilized and immunostained with the anti-Myc antibody (1:1000, Sigma) or polyclonal anti-CRMP-2 antibody (1:500). Where indicated, 10 μM Y27632 (Calbiochem) or Fasudil (provided by Asahi Kasei) was added to the cultures.

Western Blot Analysis—Cells grown in 3.5-cm tissue culture dishes were collected in cold PBS by gentle scraping, washed three times, and then resuspended in lysis buffer containing 1% Nonidet P-40, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM NaF, and protease inhibitor mixture (Roche Applied Science), followed by rocking for 10 min at 4 °C. After a 15-min centrifugation, the lysates were normalized for protein concentration using a Bio-Rad DC protein assay kit. Equal amounts of protein were then boiled in sample buffer for 5 min and subjected to SDS-PAGE. The proteins were transferred onto a polyvinylidene difluoride membrane and blocked for 1 h at room temperature in PBS containing 5% skim milk and 0.05% Tween 20. Membranes were blotted overnight with polyclonal anti-phospho CRMP-2 antibody (16) (1:10000), polyclonal anti-CRMP-2 antibody (1:10000), polyclonal anti-phospho-cofilin antibody (1:10000, Chemicon), polyclonal anti-cofilin antibody (1:10000, Chemicon), polyclonal anti-phospho-MLC antibody (1:500), polyclonal anti-MLC antibody (1:300), polyclonal anti-Glu-tubulin antibody (1:1000, Chemicon), or anti-β-tubulin III antibody (1:1000, Covance). For detection, an ECL chemiluminescence system (Amersham Biosciences) and horseradish peroxidase-conjugated secondary antibodies (1:1000, Cell Signaling Technology) were used.

Immunofluorescence—To extract soluble cytoplasmic proteins before staining, neurons were fixed and extracted for 30 min in a solution containing 0.1 M phosphate buffer (PB), 10 mM EGTA, and 2 mM MgCl2 (pH 6.9), as well as 4% paraformaldehyde, and 0.2% Triton X-100. For other immunostaining, cells were fixed with 4% paraformaldehyde in 0.1 M PB for 30 min. Fixed neurons were washed with PBS and incubated in PB containing 5% fetal calf serum, 0.1% Triton X-100, and 0.1% bovine serum albumin for 30 min to block nonspecific antibody binding. Both primary antibodies and secondary antibodies were diluted with the blocking buffer. Microtubules were stained with anti-β-tubulin III antibody.

Neurons were viewed with an inverted light microscope equipped with epifluorescence optics and a dry condenser for phase-contrast optics. To quantify fluorescence, cells were imaged using fixed exposure time and camera settings in each experiment.

In Situ Rho GTPase Activity Assay—The construct consisting of the Rho GTPase binding domain of Rhotekin (nucleotides 609–857) fused in the pGEX vector (RBD-GST) was kindly provided by Dr. M. A. Schwartz. In situ Rho GTPase activity assay using recombinant RBD-GST was performed as described previously (17). Animals were fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.4) at room temperature, and the spinal cords were then dissected and fixed in 4% paraformaldehyde in 0.1 M PB for 1 h at room temperature, rinsed in PBS. Cryostat sections (20 μm) taken from the rostral and caudal stump 10–15 mm from the transection site were incubated with blocking solution containing 5% bovine serum albumin and 0.1% Triton X-100 in PBS for 1 h, followed by overnight incubation with RBD-GST (15 μg/ml) at 4 °C overnight. After rinsing, sections were fixed again in 2% paraformaldehyde in 0.1 M PB for 10 min at room temperature, and rinsed again. To quantify the spatial distribution of RhoA, sections were double stained with an anti-neurofilament antibody (1:200, Chemicon) in combination with an anti-GST monoclonal antibody (diluted 1:100 in blocking solution, Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight. For cultured neurons, the cells were fixed in 4% (w/v) paraformaldehyde and were incubated with the anti-GST monoclonal antibody and the anti-neurofilament antibody. Fluorescence-conjugated antibodies (diluted 1:1000) were used as the secondary antibodies.

Affinity Precipitation of the GTP-bound Form of RhoA—Rat spinal cords were removed and sonicated on ice in the short bursts in the lysis buffer containing 50 mM Tris, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl2, with leupeptin and aprotinin, each at 10 μg/ml. The lysates were clarified by centrifugation at 13,000 × g at 4 °C for 10 min, and the supernatants were incubated with the 20 μg of GST-Rho binding domain of Rhotekin beads (Upstate Biotechnology) at 4 °C for 45 min. The beads were washed four times with washing buffer (50 mM Tris, pH 7.5, containing 1% Triton X-100, 150 mM NaCl, 10 mM MgCl2, 10 μg/ml each of leupeptin and aprotinin). Bound Rho proteins were detected by Western blotting using a monoclonal antibody against RhoA (Santa Cruz Biotechnology).

Image Analysis and Statistics—For quantification of CRMP-2 siRNA effects on the level of endogenous proteins in transfected neurons, cell images were collected using identical intensity settings. Fluorescence intensity of transfected cells and nontransfected cells was measured. Data are presented as CRMP-2 fluorescence of the cell normalized to fluorescence of nontransfected cells. We regarded efficient knockdown of CRMP-2 when the threshold was <20%.

To quantify polymerized microtubules in the axon, neurons under various conditions were fixed and extracted at the same time and stained with an anti-β-tubulin III antibody. Fluorescence images were taken with a 20× objective. The distal region of the axon ~50 μm long was selected for microtubule density measurement according to the method described by He et al. (18). Briefly, the total fluorescence intensity of the selected axon segment was measured. The background measurement was then subtracted to obtain the adjusted total fluorescence intensity. The final results were represented as average fluorescence intensity per micron length of axon.

To analyze Glu-tubulin distribution in the axons, we measured length of Glu-tubulin-positive fibers in each section of the spinal cords. The area of corticospinal tract in the rostral stump, ~3–6 mm rostrally from the injury site, was studied. Double immunohistochemical analysis showed that the fibers shorter than 25 μm were negative for neuron-specific β-tubulin III, demonstrating that they were not neurons, whereas the fibers longer than 25 μm were positive for neuron-specific β-tubulin III. As the focus of our study was to analyze Glu-tubulin in the
Microtubule Disassembly by MAG and Nogo

**FIGURE 1. Phosphorylation of CRMP-2 is induced by Nogo-66 and MAG-Fc.** A. Nogo-66 induces CRMP-2 phosphorylation. The representative figure (n = 3) shows the phosphorylation of CRMP-2 (p-CRMP2) by GST-Nogo-66 in cultured cerebellar neurons. Dissociated cerebellar neurons were treated with or without GST-Nogo at the indicated concentrations for 10 min, and the lysates were electrophoresed and blotted with anti-phosphorylated CRMP-2 (p-CRMP2), anti-phosphorylated cofilin (p-cofilin), or anti-phosphorylated MLC (p-MLC) antibody. The second panel shows the total CRMP-2 (CRMP2). B, time course of CRMP-2 phosphorylation. GST-Nogo was added at a concentration of 30 nM. C, quantitation of relative phospho-CRMP-2 density (percentage when compared with the control). Nogo, 10 min after the addition of Nogo (30 nM). Data are mean ± S.E. of three independent experiments. The asterisk indicates statistical significance, p < 0.01, when compared with the control (Student’s t test). D, CRMP-2 phosphorylation depends on Rho-kinase. The representative figure (n = 3) shows the phosphorylation of CRMP-2 (p-CRMP2) by GST-Nogo-66 in cultured cerebellar neurons. Up-regulated phosphorylation of CRMP-2 is abolished when Rho-kinase inhibitors were added to the culture. Y, Y-27632 (10 μM); HA, Fasudil (10 μM). E, MAG-Fc induces CRMP-2 phosphorylation. Dissociated cerebellar neurons were treated with MAG-Fc or Fc, as a control protein (−), for the indicated concentrations for 30 min. The representative figure (n = 3) shows the phosphorylation of CRMP-2 (p-CRMP2) by MAG-Fc in cultured cerebellar neurons. MAG-Fc induces CRMP-2 phosphorylation. Dissociated cerebellar neurons were treated with MAG-Fc or Fc, as a control protein (−), for the indicated concentrations for 30 min. The representative figure (n = 3) shows the phosphorylation of CRMP-2 (p-CRMP2) by MAG-Fc in cultured cerebellar neurons. Phosphorylation of CRMP-2 induced by MAG-Fc was compared with the control (n = 3 independent experiments). The asterisk indicates statistical significance, p < 0.05 (ANOVA).

**RESULTS**

Phosphorylation of CRMP-2 Is Induced by Nogo-66 and MAG-Fc—The neurite outgrowth of the postnatal cerebellar granule neurons or dorsal root ganglion neurons is inhibited in vitro by MAG or Nogo-66. For immunohistochemistry, fresh frozen tissues were obtained from an uninjured spinal cord and from ones at 0, 30 min, 2 h, and 12 h after injury. After deep anesthesia with sodium pentobarbital, the rats were decapitated and the spinal cords were dissected out, embedded in Tissue Tek OCT, and immediately frozen on dry ice at −80 °C. A series of parasagittal sections was cut at 20 μm on a cryostat and mounted on APS-coating Super-frost-Plus slides (Matsunami, Osaka, Japan). The sections were fixed in 4% (w/v) paraformaldehyde for 1 h at room temperature, washed three times with PBS, and blocked in PBS containing 5% bovine serum albumin, 0.1% Triton X-100 for 1 h at room temperature. To extract soluble cytoplasmic proteins before staining, the sections were fixed and extracted for 1 h in a solution containing 0.1 M PB, 10 mM EGTA, and 2 mM MgCl₂ (pH 6.9), as well as 4% paraformaldehyde, and 0.2% Triton X-100. The sections were incubated with primary antibodies overnight at 4 °C and washed three times with PBS, followed by incubation with fluorescein-conjugated secondary antibodies (1:1000, Molecular Probes) for 1 h at room temperature. Monoclonal antibody (TuJ1) recognizing the neuron-specific β-tubulin III protein (1:1000, Covance), polyclonal anti-Glu-tubulin antibody (1:500, Chemicon), anti-neurofilament antibody (1:200, Chemicon), or polyclonal anti-CRMP-2 antibody was used as the primary antibody. Texas Red-conjugated phallolidin (Sigma) was used to label the F-actin structure. Samples were examined under a fluorescence microscope. Control experiments of immunocytochemistry were performed by leaving out the primary antibodies.
66. This effect depends on the activation of RhoA and Rho-kinase through p75 (4). To explore the signal transduction of MAG or Nogo-66, we focused on CRMP-2, because CRMP-2 is phosphorylated at Thr-555 and inactivated by Rho-kinase in response to lysosphosphatic acid (16). To examine whether Nogo-66 induces phosphorylation of CRMP-2, we employed the cerebellar neurons from postnatal days 7 (P7) rats. These neurons expressed abundant levels of CRMP-2 in vitro as assessed by Western blot (Fig. 1A, second panel) and are responsive to MAG as well as Nogo-66 (4, 7). We then treated the cultured cells for 10 min with the GST-Nogo-66 protein, which is a fusion protein corresponding to the extracellular fragment of Nogo (10–300 nM) (19), and assessed the phosphorylation state by the antibody that specifically recognizes phosphorylated CRMP-2 at Thr-555 (16). The addition of Nogo-66 resulted in a significant dose-dependent phosphorylation of CRMP-2 (Fig. 1A). The enhancement of phosphorylation was observed within 10 min (Fig. 1, B and C). This phosphorylation of CRMP-2 is Rho-kinase dependent, because the inhibitors of Rho-kinase—Y-27632 and Fasudil—attenuated the phosphorylation of CRMP-2 (Fig. 1D). Additionally, MAG-Fc at a concentration of 15 μg/ml significantly up-regulated the phosphorylation level of CRMP-2 at 30 min (Fig. 1E). Time-course experiments show that the phosphorylation level was enhanced at least until 60 min after the addition of MAG-Fc (Fig. 1, F and G). These results demonstrate that MAG and Nogo-66 induce phosphorylation of CRMP-2 by a Rho-kinase-dependent mechanism in the postnatal cerebellar neurons.

Rho-kinase phosphorylates various substrates. For example, Rho-kinase directly phosphorylates myosin light chain (MLC) (20), and the phosphorylated MLC induces contraction of the actomyosin filaments and retraction of the neurites (21). However, we did not detect changes in the phosphorylation state of MLC in these neurons by the GST-Nogo-66 (Fig. 1A) and MAG-Fc treatments (Fig. 1F).

We next focused on the molecule LIM kinase, which is activated by Rho-kinase as well as by p21-activated kinase. In mammalian cells, LIM kinase phosphorylates cofilin, which is an actin depolymerization factor, at Ser-3 (22). Cofilin is essential for axon growth in Drosophila neurons (23). However, both GST-Nogo-66 (Fig. 1A) and MAG-Fc (Fig. 1F) did not induce phosphorylation of cofilin at Ser-3 in the cerebellar neurons. We did not observe any significant increase in the phosphorylation state during a period from 1 min to 2 h (data not shown). These data suggest the important role of CRMP-2 in the MAG or Nogo-66-mediated neurite inhibition.
CRMP-2 Abolishes the Effect of MAG-Fc—Because the phosphorylation of CRMP-2 by Rho-kinase prevents the association of CRMP-2 with tubulin dimer (16), the Nogo and the MAG signals appear to have a negative effect on the CRMP-2 function. Thus, the next hypothesis that we tested was whether the inactivation of CRMP-2 is involved in the effect of Nogo-66 and MAG in the neurons; we employed the neurite growth assay to test this. GST-Nogo-66 (100 nM; data not shown) or MAG-Fc (25 μg/ml) inhibited the neurite outgrowth of the cerebellar neurons from the P7 rats (Fig. 2, A and B), as previously reported (7). The same results were obtained irrespective of whether total process outgrowth or length of the longest neurite was measured (data not shown). We transfected cerebellar neurons with Myc-tagged wild-type CRMP-2 (14) when the plated cells initiated the growth of neurites and found that the overexpression of CRMP-2 dramatically facilitated the neurite outgrowth (Fig. 2, A and B). However, GST-Nogo-66 (data not shown) or MAG-Fc did not significantly inhibit the neurite outgrowth of the neurons that overexpressed the wild-type CRMP-2. CRMP-2 is phosphorylated at Thr-555 by Rho-kinase (16). We then transfected the cells with a CRMP-2 in which Thr-555 was replaced by Ala (T555A). However, we did not observe any significant promotion of neurite outgrowth by the CRMP-2 T555A expression (Fig. 2, A and B). The difference in the neurite promotion effect between the wild-type CRMP-2 and CRMP-2 T555A might be due to the difference in the expression level, because the expression of CRMP-2 T555A was significantly lower than that of the wild-type CRMP-2 (Fig. 2C). As we transfected the cells with the plasmids or siRNA after establishing the cell polarity (these cells had tau-positive axons), the effect we observed was neurite elongation. The knock down of CRMP-2 expression by siRNA (24) resulted in the inhibition of neurite outgrowth, whereas scrambled siRNA showed no effect (Fig. 2, A and B). As we transfected the cells with the plasmids or siRNA after establishing the cell polarity (these cells had tau-positive axons), the effect we observed was neurite elongation and not axonal formation. These results suggest that the inactivation of CRMP-2 downstream of Rho-kinase is involved in the inhibitory effect of MAG or Nogo-66 on neurite elongation, although we could not rule out the possibility that overexpression of CRMP-2 coun-

![Image of figure 3](https://example.com/figure3.png)
teracts other signaling pathways downstream of MAG or Nogo-66, thus abolishing the effect.

Regulation of Neurite Microtubule Levels through the Rho-kinase Pathway—Accumulated evidence indicates that axon growth involves the regulation of axonal microtubules (25). Inactivation of CRMP-2 by MAG-Fc or GST-Nogo-66 presumably results in the inhibition of microtubule assembly. To directly visualize microtubule polymers in the neurites, we extracted and fixed the neurons simultaneously to eliminate tubulin monomers and then stained for polymerized microtubules, as previously described (18, 26). We employed the dissociated P6–10 DRG neurons (Fig. 3, A–D) or postnatal cerebellar neurons (Fig. 3, E–H) for immunocytochemistry. Under control conditions, the polymerized microtubules showed a distribution along the axon (Fig. 3, A and E). Treatment with MAG-Fc (Fig. 3, A and E) or GST-Nogo-66 (data not shown) significantly reduced the density of the polymerized microtubules throughout the neurites. However, in the presence of Y-27632, the density of the polymerized microtubules in the neurons treated with MAG-Fc was restored to the control level, although Y-27632 itself had no effect on the density of the polymerized microtubules (Fig. 3, A, C, E, and G). We did not observe any significant change in the total tubulin levels in the neurites due to the treatment with MAG-Fc or the Rho-kinase inhibitor (Fig. 3, B, D, F, and H). The tubulin modification cycle involves two enzymes, namely, tyrosine ligase and a tubulin carboxypeptidase, and generates two major forms of tubulin, namely, tyrosinated tubulin and Glu-tubulin. Stable microtubules are composed largely of Glu-tubulin. To evaluate the effects of MAG stimulation on microtubule dynamics, we assessed the levels of detyrosinated Glu-tubulin, as a marker for stable microtubules. Quantitative Western blots revealed that the level of Glu-tubulin in the postnatal cerebellar neurons was reduced in response to MAG-Fc treatment (Fig. 3I); this effect of MAG-Fc was diminished in the presence of Y-27632. Total β-tubulin III level remained unchanged by any treatment. These results suggest that MAG-Fc inhibits the CRMP-2 activity, thereby negatively regulating microtubule levels downstream of the Rho-kinase pathway.

Activation of RhoA in the Injured Axons—It has been reported that RhoA is activated in neurons and glial cells that are present around the injury site within 1.5 h following the spinal cord injury (27, 28). During

**FIGURE 4. Rho activation following the spinal cord injury.** A, in the neurons, Rho is spatially activated by MAG-Fc. DRG neurons were either treated or not treated with MAG-Fc (25 μg/ml) for 30 min and immunostained for the detection of active RhoA. The representative single optical sections for active RhoA (upper) and the neuronal marker (lower) are shown. Note the distribution of active RhoA in the distal shaft and the growth cones of the neurites treated with MAG-Fc (right). In the control cells (left), the neurites were only faintly stained. B, relative average fluorescence intensity (%) of the immunoreactivity for the detection of active RhoA at the growth cones. Significant difference is observed between the control cells and the MAG-Fc-treated cells. NGF, NGF (50 ng/ml); MAG, MAG-Fc (25 μg/ml). Data are mean ± S.E. The asterisks indicate statistical significance. *, p < 0.01 (Student’s t test). C, representative transverse sections of the injured spinal cords (n = 3). Images of the white matter in the dorsal columns were obtained from the rostral stump at a distance of 15 mm from the transection site. Intense signals for GST-RBD in the neurofilament-positive axons are found after the injury (SCI 2h) but not in the axons of sham-operated controls (control). D, relative average fluorescence intensity (%) for the detection of active RhoA in the axons. Significant difference is observed between the axons of sham-operated controls (control) and those of injured spinal cords (SCI 2h). Data are mean ± S.E. (n = 3). Asterisks indicate statistical significance. *, p < 0.01 (Student’s t test). E, RhoA is activated after the spinal cord injury. The spinal cords were removed from the rats with or without spinal cord transection. The active fraction of RhoA was detected by employing the pull-down assay, and the detection of total RhoA was conducted by Western blotting using a monoclonal antibody against RhoA. control, sham-operated rats; SCI 2h, 2 h after the spinal cord injury. Note the activation of RhoA in the tissue around the site of injury. F, quantification of relative GTP-RhoA density (percentage when compared with the control). control, sham-operated rats; SCI 2h, 2 h after the spinal cord injury. Data are mean ± S.E. of three independent experiments. The asterisk indicates statistical significance, p < 0.01, when compared with the control (Student’s t test).
the spinal cord injury in rats with the Y-27632 treatment. Data are mean microtubule-positive axons in the white matter (percentage when compared with the control) under each condition (spinal cord injury (Fig. 4, observed intensely stained fibers in the white matter at 2 h after the application of Y-27632 to the lesion sites does not affect the staining pattern in the spinal cord at 2 h postaxotomy (SCI 2h). Local application of Y-27632 to the lesion sites does not affect the staining pattern in the spinal cord at 2 h postaxotomy (Y-27632 + SCI 2h). C, quantification of the relative length of microtubule-positive axons in the white matter (percentage when compared with the control) under each condition (n = 3 independent experiments). SCI 2h + Y-27632, at 2 h after the spinal cord injury in rats with the Y-27632 treatment. Data are mean ± S.E. Asterisks indicate statistical significance, p < 0.01 (ANOVA).

FIGURE 5. Decreased microtubule levels in the axons after the spinal cord injury. A, the microtubule levels are decreased at 2 h after the spinal cord injury by a Rho-kinase-dependent mechanism. The sections were extracted and fixed simultaneously to eliminate tubulin monomers and then were stained for polymerized microtubules. The polymerized microtubules show a distribution along the axon in the white matter of the control spinal cord (control). The polymerized tubulin levels are dramatically reduced in the adjacent areas at 2 h after the spinal cord injury (SCI 2h). Loss of polymerized tubulin was almost completely recovered to the control level by the application of gel foams, which were pre-soaked with Y-27632 to the lesion sites (Y-27632 + SCI). B, immunohistochemistry for neuron-specific β-tubulin III protein using TuJ1 antibody. Neuron-specific β-tubulin III is present in the axons in the white matter of the spinal cord (control). The immunoreactivity is not changed significantly in the adjacent areas at 2 h after the spinal cord injury (SCI 2h). Local application of Y-27632 to the lesion sites does not affect the staining pattern in the spinal cord at 2 h postaxotomy (Y-27632 + SCI 2h). C, quantification of the relative length of microtubule-positive axons in the white matter (percentage when compared with the control) under each condition (n = 3 independent experiments). SCI 2h + Y-27632, at 2 h after the spinal cord injury in rats with the Y-27632 treatment. Data are mean ± S.E. Asterisks indicate statistical significance, p < 0.01 (ANOVA).

Decreased Microtubule Levels in the Injured Axons following the Spinal Cord Injury—Our data demonstrated that MAG-Fc as well as Nogo-66 phosphorylated CRMP-2, presumably resulting in its inactivation, which down-regulated the microtubule levels in the neurons in vitro. It is believed that MAG and Nogo contribute to inhibiting the regeneration of the injured central nervous system (1). Consistent with this notion, RhoA was activated in the injured axons following the spinal cord injury in vivo. Therefore, we assessed whether our in vitro observations actually occurred in vivo. To test this hypothesis, we examined polymerized tubulin levels following the spinal cord injury. Fresh frozen sections were obtained at 2 h after complete transection of the rat thoracic spinal cord. Immunostaining for neuron-specific β-tubulin III protein using TuJ1 antibody showed that neuron-specific β-tubulin III was present in the axons in the white matter (Fig. 5B). No significant change was observed in the β-tubulin levels in the axons in the adjacent areas at 2 h after the spinal cord injury; this demonstrated that axon degeneration has not occurred during this period. Next, we extracted and fixed the sections simultaneously to eliminate tubulin monomers and then stained for polymerized microtubules, similarly to that performed for in vitro staining. Under control conditions, the polymerized microtubules showed a distribution along the axons in the white matter (Fig. 5A). However, the polymerized tubulin levels were dramatically reduced in the axons in the adjacent areas at 2 h after the spinal cord injury (Fig. 5, A and C). These changes were observed in the portions that were located rostral as well as caudal to the site of the spinal cord injury. To examine whether Rho-kinase activation is involved in the abovementioned effect, we applied gel foams that were presoaked with the vehicle (PBS) or Y-27632, to the lesion sites and examined the spinal cords at 2 h postaxotomy by immunostaining. Loss of polymerized tubulin was almost completely recovered to the control level by the treatment with Rho-kinase inhibitor and not by the control vehicle (Fig. 5, A and C). These results demonstrate that the loss of polymerized
microtubules depends on the RhoA/Rho-kinase activation after the spinal cord injury. Next we immunostained the sections with polyclonal anti-Glu-tubulin antibody to measure the polymerized forms of microtubules. Fresh frozen sections were obtained at 30 min, 2 h, and 12 h after the spinal cord injury. Under control conditions, Glu-tubulin was expressed in a line along the axons in the white matter (Fig. 6A, arrowheads). However, the Glu-tubulin staining pattern changed significantly in the axons in the adjacent areas at 30 min, 2 h, and 12 h after the spinal cord injury (Fig. 6). The Glu-tubulin signal was quantified by measuring the length of the Glu-tubulin-positive staining following the spinal cord injury (Fig. 6B). In comparison with the control, the average signal length was significantly shorter after the spinal cord injury. Double immunohistochemical analysis showed that the signals shorter than 25 μm were negative for neuron-specific β-tubulin III; this demonstrated that they were not neurons. On the other hand, the fibers longer than 25 μm were positive for neuron-specific β-tubulin III (data not shown). Indeed, the decrease in the average length after the spinal cord injury was due to a relative decrease in the staining in the neurites. This change in the Glu-tubulin staining pattern was almost completely recovered to the control level by the treatment with Rho-kinase inhibitor at 2 h following the spinal cord injury (Fig. 6A and B). These findings demonstrate that the number of polymerized microtubules was decreased in the axons after the spinal cord injury by a Rho-kinase-dependent mechanism.

Finally, we determined the expression of CRMP-2 in the spinal cord to assess the possible involvement of CRMP-2 in the adult rat spinal cord injury. The CRMP-2 expression was found in both white and gray matter (Fig. 7A). Double staining using Tuj1 antibody showed that CRMP-2 was expressed in the axons in the white matter and the somata of Tuj1-positive neurons in the gray matter. The staining pattern for F-actin or neurofilaments was not changed at 2 h after the spinal cord injury (Fig. 7B). Notably, the level of phospho-CRMP-2 was increased significantly at 2 h following the spinal cord injury (Fig. 7, C and D). Enhanced phosphorylation of CRMP-2 returned to the control level by the treatment with Y-27632 (Fig. 7, C and D), suggesting that phosphorylation of CRMP-2 is dependent on the activity of Rho-kinase. These findings support the notion that CRMP-2 inactivation may play a role in the microtubule disassembly after the spinal cord injury.

**DISCUSSION**

In this study, we addressed the question whether MAG or Nogo-66 regulates neurite outgrowth. We have demonstrated that CRMP-2 was phosphorylated (presumably inactivated) downstream of RhoA and Rho-kinase in the postnatal cerebellar neurons by MAG-Fc or Nogo-66. Consistent with the function of CRMP-2 that binds to tubulin heterodimers and promote microtubule assembly, we detected decreased level of the microtubules in the neurites of the DRG neurons by *in vitro*
stimulation. Decreased polymerization of microtubules was also observed in the injured axons after the spinal cord injury.

**Activation of RhoA following Spinal Cord Injury**—To date, three major inhibitors of axon regeneration, Nogo, MAG, and oligodendrocyte-myelin glycoprotein, which are expressed by oligodendrocytes, and myelinated fiber tracts have been identified. A Nogo-66-binding protein, designated as the Nogo receptor, was observed to bind all these proteins, and it was considered as the functional binding partner (5, 19, 31, 32). Rho appears to be a key intracellular effector located downstream from the receptor for these proteins. In neurons, myelin and MAG inhibit growth that is abolished by the botulinus toxin C3, which inactivates RhoA (33). More specifically, there is direct evidence regarding the activation of RhoA by MAG-Fc in the cerebellar granule neurons; this has been demonstrated by the affinity precipitation of the GTP-bound form of RhoA (4). This activation is also observed in postnatal DRG neurons and in neurons in the injured spinal cord (27, 28). Following spinal cord injury, RhoA is activated around the injury site as early as 2 h after the injury (Fig. 4), when the axon growth is probably not occurring. Because several factors such as tumor necrotizing factor elicit RhoA activation, very early RhoA activation in vivo may not depend on the three axon growth inhibitors alone. Therefore, RhoA may play some roles other than the inhibition of axon regeneration during the early period following the spinal cord injury. In fact, we have previously reported that RhoA is involved in axon degeneration (29) after the spinal cord injury. The precise function of RhoA, which might be widespread, in central nervous system injury should be explored in the future.

**Microtubules and Axon Elongation**—During neurite outgrowth, microfilaments and microtubules constitute the major cytoskeletal components of neurons. Microfilaments are composed of filamentous actin and a complex set of actin binding proteins. Microtubules are the prominent components of the neurite shaft and the central region of the growth cone. Axon elongation is initiated with the growth cone advancement via actin polymerization at the leading edge. Subsequent microtubule polymerization and bundling are the major events that mediate the formation of axons (9). Microtubules are essential for the formation and maintenance of neurite extension, because their polymerization and subsequent stabilization leads to the overall extension and consolidation of the growing neurites. The exposure of neurons to tubulin polymerization inhibitors such as colchicines or nocodazol not only inhibits growth cone advancement but also induces neurite retraction (34). Although the cytoskeletal mechanisms underlying growth cone motility during the axon growth and guidance have been extensively studied, the mechanism by which the extracellular signals control the...
growth cone cytoskeleton, particularly the microtubules, remains to be elucidated.

The dynamic properties of microtubules are regulated by diverse cellular factors. For example, tau and MAP1b may act as stabilizers by preferentially binding to the microtubule lattice (35), whereas OP18/stathmin-like proteins act as destabilizers (6). In addition, CRMP-2 has emerged as a crucial regulator of microtubule dynamics during neurite/axon growth (13). Rho-kinase has been shown to phosphorylate CRMP-2, thereby resulting in the prevention of the association of CRMP-2 with tubulin dimer (16). We observed that growth cone collapse elicited by ephrin-A5 is inhibited by CRMP-2 in which Thr-555 was replaced by Ala (36); this suggests a common molecular mechanism underlying the axon guidance and inhibitory molecules. Lysophosphatic acid-phosphorylated proteins should be evaluated in the future. The overexpression of the axonal factors. For example, tau and MAP1b may act as stabilizers by acting on the microtubules, particularly the microtubules, remains to be elucidated. The role of actin filaments in the growth cone for neurite elongation remains to be elucidated.

We included CRMP-2, a new protein that causes rapid transduction of MAG or Nogo-66 in neurons. Because disappearance of microtubules was observed following the spinal cord injury, the reorganization of microtubules may be drastic and important for the development of the pathophysiology. Proper regulation of microtubules might be effective for the regeneration of the injured central nervous system.

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