We previously identified Rho-associated protein kinase (Rho-kinase) as a specific effector of Rho. In this study, we identified collapsin response mediator protein-2 (CRMP-2), as a novel Rho-kinase substrate in the brain. CRMP-2 is a neuronal protein whose expression is up-regulated during development. Rho-kinase phosphorylated CRMP-2 at Thr-555 in vitro. We produced an antibody that specifically recognizes CRMP-2 phosphorylated at Thr-555. Using this antibody, we found that Rho-kinase phosphorylated CRMP-2 downstream of Rho in COS7 cells. Phosphorylation of CRMP-2 was observed in chick dorsal root ganglion neurons during lysophosphatidic acid (LPA)-induced growth cone collapse, whereas the phosphorylation was not detected during semaphorin-3A-induced growth cone collapse. Both LPA-induced CRMP-2 phosphorylation and LPA-induced growth cone collapse were inhibited by Rho-kinase inhibitor HA1077 or Y-27632. LPA-induced growth cone collapse was also blocked by a dominant negative form of Rho-kinase. On the other hand, semaphorin-3A-induced growth cone collapse was not inhibited by a dominant negative form of Rho-kinase. Furthermore, overexpression of a mutant CRMP-2 in which Thr-555 was replaced by Ala significantly inhibited LPA-induced growth cone collapse. These results demonstrate the existence of Rho-kinase-dependent and -independent pathways for growth cone collapse and suggest that CRMP-2 phosphorylation by Rho-kinase is involved in the former pathway.

During the development of the nervous system, the nerve growth cones play a central role in axon guidance. They are located at the tip of axons and dynamically change their morphology in response to attractive and repulsive cues to decide the growing direction (1, 2). Such morphological changes of growth cones are considered to be achieved by the reorganization of the cytoskeletons and cell adhesions (3–5). Although recent studies have identified several guidance cue molecules and their receptors (1, 2), the mechanisms of their intracellular signaling are poorly understood.

The Rho family of small GTPases including Rho, Cdc42, and Rac are intracellular signaling molecules that are thought to regulate the cytoskeletons and cell adhesions (6–8). In fibroblasts, Rho activation is required for the formation of stress fibers and focal adhesions (9). On the other hand, Cdc42 and Rac are required for the formation of filopodia and lamellipodia, respectively (9–11). These structures regulated by Rho, Cdc42, and Rac in fibroblasts are similar to those of nerve growth cones (2, 12). Consistently, accumulating evidence suggests that the Rho family GTPases also regulate the neurite and growth cone morphology (13, 14). It is reported that Rho negatively regulates growth cone and neurite formation. Microinjection of RhoA as well as application of LPA, which activates intracellular Rho, induces growth cone collapse and neurite retraction in PC12 and N1E-115 cells (13–15). On the other hand, trituration of Clostridium botulinum C3 transferase (C3), a specific inhibitor of Rho, stimulates neurite outgrowth in DRG neurons (16). A dominant negative form of RhoA and C3 coenzyme also promote filopodia and lamellipodia formation in the N1E-115 cell growth cone (14). Interestingly, in dorsal root ganglion (DRG) neurons, C3 treatment inhibits the lamellipodial spreading of growth cones (16), thereby suggesting that Rho activity is also required for the maintenance of growth cones.

Previously, a novel serine/threonine kinase, Rho-associated kinase (Rho-kinase)/ROKα/ROCK II, was identified as an effector of Rho (17–19). Rho-kinase binds to and is activated by the GTP-bound active form of Rho (19–21). Rho-kinase appears to regulate various cellular responses downstream of Rho (8): stress fiber and focal adhesion formations (17, 20, 21), smooth muscle contraction (22–24), cytokinesis (25), and cell migration (26). Rho-kinase regulates the phosphorylation of myosin light chain (MLC), resulting in actomyosin contractility, by the direct phosphorylation of MLC (22) and by the inactivation of myosin phosphatase through the phosphorylation of myosin-binding subunit (23).

The abbreviations used are: LPA, lysophosphatidic acid; C3, C3 transferase; CRMP-2, collapsin response mediator protein-2; DRG, dorsal root ganglion; MLC, myosin light chain; GST, glutathione S-transferase; CAT, catalytic domain; RB, Rho-binding domain; PH, pleckstrin homology domain; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin.

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Several groups including ours have found that Rho-kinase is involved in LPA-induced neurite retraction of neuronal cells downstream of Rho (27, 28) and have identified MLC as one of the major substrates of Rho-kinase-mediated neurite retraction (28). However, the activated MLC could not completely mimic the Rho-kinase-induced neurite retraction, raising the possibility that other Rho-kinase substrates are also involved in the Rho-kinase-induced neurite retraction.

In light of these observations, we here searched for novel substrates of Rho-kinase in brain and identified collapsin response mediator protein-2 (CRMP-2). We also identified Thr-555 as the site of phosphorylation in CRMP-2 by Rho-kinase. Monitoring this phosphorylation, we found the existence of Rho-kinase-dependent and -independent pathways for growth cone collapse.

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals—**Anti-CRMP-2 monoclonal antibody was kindly provided by Dr. Y. Ibara (Tokyo University, Tokyo, Japan). Semaphorin-3A (Sem3A) was produced as described (29). LPA was purchased from Sigma. HA1077 was provided by Asahi Chemical Industry (Shizuka, Japan). Y-32885 was synthesized as described (30). Human fetal brain cDNA library was kindly provided by Dr. H. Saya (Kumamoto University, Kumamoto, Japan). pEF-BOS-myc-RhoAV14, -RhoAN19, -Rac1V12, -Cdc42V12, -Rho-kinase, -Rho-kinase catalytic domain (CAT), and -RB/PHTP were constructed as described (20, 26). Other materials and chemicals were obtained from commercial sources.

**Plasmid Constructs—**The cDNA of human CRMP-2 was amplified by polymerase chain reaction from human fetal brain cDNA library with primers 5′-AGATCTATGTCTTATCAGGGGAAGAAAAA-3′ and 5′-AG-ATCCTCTAGCCCAGGCTGGTGATGT-3′. The mutant CRMP-2 T555A was generated with a Mutazyme kit (Stratagene, La Jolla, CA) by changing Thr-555 into Ala-555. The cDNA fragments of CRMP-2 and CRMP-2 T555A were subcloned into pEF-BOS-HA vector.

**Purification of a Rho-kinase Substrate, p70—**Bovine brain cytosol fraction (20 mg of protein) prepared as described (31, 32) was loaded onto the Mono Q HR5/5 column (Amersham Pharmacia Biotech) pre-equilibrated with 10 mM Tris-HCl at pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 7 mM MgCl2, 10 μM GGTP, and purified glutathione S-transferase (GST)-dominant active form of Rho-kinase (GST-CAT) (1 μg of protein) for 10–60 min at 30 °C. GST-CAT was digested by 20% SDS in a mixture with a baculovirus system (33) and purified on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) (19). Then the reaction mixtures were boiled in SDS sample buffer and subjected to SDS-PAGE. The radiolabeled bands were visualized by an image analyzer (BAS 2000; Fuji, Tokyo, Japan). To confirm the site of phosphorylation of CRMP-2 by Rho-kinase, HA-CRMP-2 and HA-CRMP-2 T555A were incubated with p70 and immunoprecipitated from COS7 cells transiently transfected with each expression vector as described (32).

**Peptide Sequencing—**Peptide sequencing of p70 was carried out as described (34). In brief, partially purified p70 was resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and stained with 0.1% Ponceau S in 1% acetic acid. The band corresponding to p70 was digested by lysyl endopeptidase, AcChE, and proteases I and endopeptidase, Asp-N. The obtained peptides were fractionated by C18 column chromatography (Shiseido, Japan) and subjected to amino acid sequencing for identification.

**Identification of the Phosphorylation Site of CRMP-2 by Rho-kinase—**The site of phosphorylation of CRMP-2 by Rho-kinase was identified as described (26, 35). In brief, CRMP-2 (77 μg of protein) was phosphorylated by GST-CAT (70 μg of protein), and the reaction product was digested with Asp-N. The obtained peptides were fractionated by C18 reverse-phase column chromatography and subjected to amino acid sequencing. The fraction obtained from each Edman degradation cycle was measured as 3P in a Beckman liquid scintillation counting system. The criterion for the collapsed growth cones was a total loss of filopodia and lamellipodia. For immunofluorescence analysis, the cells were stimulated by 1 μM LPA or 5 units/ml Sema3A for 30 min at 37 °C and then cultured in serum-free medium without NGF for 4 h. The pretreatment with Rho-kinase inhibitor, HA1077 or Y-32885, was performed for 1 h before collapse assay. For immunoblot analysis, the cells were stimulated by 5 μM LPA or 5 units/ml Sem3A for the indicated time at 37 °C in serum-free medium without NGF. The cells were treated with 10% (w/v) trichloroacetic acid. The resulting precipitates were boiled in SDS sample buffer and subjected to SDS-PAGE. The radiolabeled bands were visualized by an image analyzer (BAS 2000; Fuji, Tokyo, Japan). To confirm the site of phosphorylation of CRMP-2 by Rho-kinase, HA-CRMP-2 and HA-CRMP-2 T555A were incubated with p70 and immunoprecipitated from COS7 cells transiently transfected with each expression vector as described (32).

**RESULTS**

**Identification of CRMP-2 as a Novel Rho-kinase Substrate in Bovine Brain—**To search for Rho-kinase substrates in brain, we separated bovine brain cytosolic proteins by Mono Q column chromatography and subjected the fractions to phosphorylation assay using a dominant active form of Rho-kinase (GST-CAT). Among the several proteins detected in this assay, one with a mass of about 70 kDa (p70) was recognized as a major band phosphorylated by GST-CAT (70 μg of protein), and the reaction product was digested with Asp-N. The obtained peptides were fractionated by C18 reverse-phase column chromatography and subjected to amino acid sequencing. The fraction obtained from each Edman degradation cycle was measured as 3P in a Beckman liquid scintillation counting system. The criterion for the collapsed growth cones was a total loss of filopodia and lamellipodia. For immunofluorescence analysis, the cells were stimulated by 1 μM LPA or 5 units/ml Sem3A for the indicated time at 37 °C in serum-free medium without NGF. The cells were treated with 10% (w/v) trichloroacetic acid. The resulting precipitates were boiled in SDS sample buffer and subjected to SDS-PAGE. The radiolabeled bands were visualized by an image analyzer (BAS 2000; Fuji, Tokyo, Japan). To confirm the site of phosphorylation of CRMP-2 by Rho-kinase, HA-CRMP-2 and HA-CRMP-2 T555A were incubated with p70 and immunoprecipitated from COS7 cells transiently transfected with each expression vector as described (32).

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**Identification of the Phosphorylation Site of CRMP-2 by Rho-kinase—**The site of phosphorylation of CRMP-2 by Rho-kinase was identified as described (26, 35). In brief, CRMP-2 (77 μg of protein) was phosphorylated by GST-CAT (70 μg of protein), and the reaction product was digested with Asp-N. The obtained peptides were fractionated by C18 reverse-phase column chromatography and subjected to amino acid sequencing. The fraction obtained from each Edman degradation cycle was measured as 3P in a Beckman liquid scintillation counting system. The criterion for the collapsed growth cones was a total loss of filopodia and lamellipodia. For immunofluorescence analysis, the cells were stimulated by 1 μM LPA or 5 units/ml Sem3A for the indicated time at 37 °C in serum-free medium without NGF. The cells were treated with 10% (w/v) trichloroacetic acid. The resulting precipitates were boiled in SDS sample buffer and subjected to SDS-PAGE. The radiolabeled bands were visualized by an image analyzer (BAS 2000; Fuji, Tokyo, Japan). To confirm the site of phosphorylation of CRMP-2 by Rho-kinase, HA-CRMP-2 and HA-CRMP-2 T555A were incubated with p70 and immunoprecipitated from COS7 cells transiently transfected with each expression vector as described (32).

**Peptide Sequencing—**Peptide sequencing of p70 was carried out as described (34). In brief, partially purified p70 was resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and stained with 0.1% Ponceau S in 1% acetic acid. The band corresponding to p70 was digested by lysyl endopeptidase, AcChE, and proteases I and endopeptidase, Asp-N. The obtained peptides were fractionated by C18 column chromatography (Shiseido, Japan) and subjected to amino acid sequencing for identification.
phosphorylated in a GST-CAT-dependent manner (Fig. 1A, fractions 8–12). p70 was eluted in peaks between 200 and 300 mM NaCl and further purified by Mono S column chromatography (Fig. 1B). To clarify the molecular identity of p70, the purified protein was subjected to amino acid sequencing as described under “Experimental Procedures.” Six peptide sequences derived from p70 were determined: KQIGENLIVP, KSSAEVIAQARK, KMDENQFVAV, KVFNLYPR, KIVLED, and KAIEALAELRXVP. These sequences were almost identical to that of bovine CRMP-2 (Fig. 2). Furthermore, the antibody raised against CRMP-2 (amino acids 1–572) cross-reacted with p70 (Fig. 5). Recently, CRMP-2 homologues have been identified from various species (29, 39–43). CRMP-62, the chick CRMP-2 (98% identity), is reported to be required for the growth cone collapse of DRG neurons induced by Sema3A (also known as collapsin-1) (29). UNC-33, the C. elegans homologue (30% homology), is identified by a mutation resulting in severely uncoordinated movement, abnormalities in axon guidance, and superabundance of microtubules in neurons (44, 45).

Identification of the in Vitro CRMP-2 Phosphorylation Site by Rho-kinase—Next, we determined phosphorylation sites of CRMP-2 by Rho-kinase. The purified CRMP-2 was phosphorylated by GST-CAT in the presence of \[^{32}P\]ATP in vitro and digested with endoproteinase, Asp-N. The digested peptides were separated by C18 column chromatography. One radioactive peak was obtained (Fig. 3A). The phosphorylated peptide in the fraction was subjected to amino acid sequencing. The sequence obtained from this fraction was DNIPRRT-TQRIVAPPGGR, corresponding to amino acids 548–565 of CRMP-2. The fraction obtained from each Edman degradation cycle was measured for \[^{32}P\] in a liquid scintillation counter. As a result, Thr-555 was found to be phosphorylated by GST-CAT (Fig. 3B). To rule out the possibility that GST-CAT phosphorylates not only Thr-555 but also Thr-554, we produced a CRMP-2 mutant (HA-CRMP-2 T555A) in which Thr-555 was replaced by Ala. HA-CRMP-2 or HA-CRMP-2 T555A was expressed in COS7 cells, and the cell lysates were immunoprecipitated with anti-HA antibody. The resulting immunoprecipitate was subjected to the phosphorylation assay (Fig. 4). HA-CRMP-2 was phosphorylated by GST-CAT, whereas HA-CRMP-2 T555A was not. Taken together, these results indicate that Thr-555 is the major site of CRMP-2 phosphorylation by Rho-kinase in vitro.

Production of a Site- and Phosphorylation State-specific Antibody for CRMP-2 at Thr-555—To examine in vivo CRMP-2 phosphorylation by Rho-kinase, we prepared a rabbit polyclonal antibody that specifically recognizes CRMP-2 phosphorylated at Thr-555 (anti-pT555). We used a phosphopeptide corresponding to amino acids 550–560 of CRMP-2 in which Thr-555 is phosphorylated as an antigen. The specificity of this antibody was examined by immunoblot analysis (Fig. 5). Two pmol of purified CRMP-2 (p70) in the fraction 11 from the Mono S column (Fig. 1B) containing increasing amounts of the phosphorylated CRMP-2 was loaded on the gel. Anti-pT555 bound to the phosphorylated CRMP-2 in a dose-dependent manner but did not react with the unphosphorylated form. The binding of anti-pT555 to CRMP-2 phosphorylated by Rho-kinase was inhibited by preincubation of the antibody with the antigen phosphopeptide (data not shown). These results indicate that...
anti-pT555 specifically recognizes the phosphorylation of CRMP-2 at Thr-555.

**Rho/Rho-kinase-dependent Phosphorylation of CRMP-2 at Thr-555 in COS7 Cells**—Next, we examined whether Rho-kinase can phosphorylate CRMP-2 at Thr-555 in the cells, using anti-pT555 and COS7 cells. Because endogenous CRMP-2 was undetectable in COS7 cells by immunoblot analysis using anti-CRMP-2 antibody. The immunoprecipitates were subjected to the phosphorylation assay, separated by SDS-PAGE, and detected by autoradiography (left). The amount of proteins in each lane was normalized by immunoblot analysis with anti-HA antibody (right). The results are representative of three independent experiments.

**LPA Stimulation of DRG Neurons Induced CRMP-2 Phosphorylation by Rho-kinase**

RhoA (RhoA\(^{V14}\)) induced the CRMP-2 phosphorylation, whereas that of a dominant negative RhoA (RhoAN\(^{19}\)) did not (Fig. 6, top). The expression of wild-type Rho-kinase (Rho-kinase) or a dominant active Rho-kinase (Rho-kinase CAT) also induced CRMP-2 phosphorylation. Furthermore, the coexpression of a dominant active RhoA (RhoAV\(^{14}\)) further enhanced the Rho-kinase-induced CRMP-2 phosphorylation. In addition, HA-CRMP-2 T555A coexpressed with Rho-kinase CAT was not recognized by anti-pT555. Taken together, these results indicate that CRMP-2 was phosphorylated at Thr-555 by Rho-kinase downstream of Rho in COS7 cells. Interestingly, the expression of a dominant active Rac1 (Rac1\(^{V12}\)) or a dominant active Cdc42 (Cdc42\(^{V12}\)) also resulted in a small increase in the level of HA-CRMP-2 phosphorylation (Fig. 6, upper panel). This result raises the possibility that CRMP-2 is also phosphorylated downstream of Rac1 and Cdc42 in COS7 cells.

**LPA Stimulation of DRG Neurons Induced CRMP-2 Phos-**
phorylation by Rho-kinase—CRMP-2 is reported to be highly expressed in the developing nerve system (39, 41, 42) and implicated in Sema3A-induced growth cone collapse (29). In addition, the C. elegans homologue of CRMP-2, UNC-33, is thought to control the guidance and outgrowth of neuronal axons (44, 45). Recently, we found that CRMP-2 plays a critical role in the axon formation of primary cultured hippocampal neurons.2 On the other hand, recent studies reported that Rho is involved in the regulation of growth cone morphology (16, 46). These observations raise the possibility that the CRMP-2 phosphorylation by Rho-kinase is involved in the regulation of the growth cone morphology, especially growth cone collapse. To address this issue, we monitored the phosphorylation of CRMP-2 in chick DRG neurons during growth cone collapse induced by LPA or Sema3A (Fig. 7). DRG neurons cultured for 24 h were stimulated with 5 μM LPA or 5 units/ml Sema3A for 1, 3, 10, or 30 min. The cell lysates were resolved by SDS-PAGE and immunoblotted with anti-pT555 (top) or anti-CRMP-2 antibody (bottom). The multiple bands of CRMP-2 probably represent differentially phosphorylated forms. The results are representative of three independent experiments. B. DRG neurons pretreated with or without Rho-kinase inhibitor, 10 μM HA1077 or 1 μM Y-32885, for 1 h were stimulated by 5 μM LPA for 3, 10, or 30 min. The cell lysates were resolved by SDS-PAGE and immunoblotted with anti-pT555 (top) or anti-CRMP-2 antibody (bottom). The relative levels of CRMP-2 phosphorylation were calculated with those of untreated control cells as 100 units. The values shown are means ± S.E. of triplicates. **, p < 0.01, significance of difference from the nonstimulated culture analyzed by Student’s t test.

FIG. 7. LPA-induced CRMP-2 phosphorylation at Thr-555 by Rho-kinase in chick DRG neurons. A, primary cultured chick DRG neurons cultured for 24 h were stimulated with 5 μM LPA or 5 units/ml Sema3A for 1, 3, 10, or 30 min. The cell lysates were resolved by SDS-PAGE and immunoblotted with anti-pT555 (top) or anti-CRMP-2 antibody (bottom). The multiple bands of CRMP-2 probably represent differentially phosphorylated forms. The results are representative of three independent experiments. B, DRG neurons pretreated with or without Rho-kinase inhibitor, 10 μM HA1077 or 1 μM Y-32885, for 1 h were stimulated by 5 μM LPA for 3, 10, or 30 min. The cell lysates were resolved by SDS-PAGE and immunoblotted with anti-pT555 (top) or anti-CRMP-2 antibody (bottom). The relative levels of CRMP-2 phosphorylation were calculated with those of untreated control cells as 100 units. The values shown are means ± S.E. of triplicates. **, p < 0.01, significance of difference from the nonstimulated culture analyzed by Student’s t test.

FIG. 8. Effects of Rho-kinase inhibitors on LPA- or Sema3A-induced growth cone collapse. A, DRG neurons were pretreated with or without Rho-kinase inhibitor, 10 μM HA1077 or 1 μM Y-32885, for 1 h and then incubated with control buffer, 1 μM LPA or 5 units/ml Sema3A. After the 30-min stimulation, the cultures were fixed and doubly labeled by anti-neurofilament antibody (green) and tetramethylrhodamine B isothiocyanate-phalloidin (red). Scale bar, 10 μm. B, the time course of the growth cone collapse induced by LPA or Sema3A in the presence or absence of the Rho-kinase inhibitors. The values shown are means ± S.E. of triplicates. *, p < 0.05; **, p < 0.01, significance of difference from the growth cone precultured without Rho-kinase inhibitor analyzed by Student’s t test.

The addition of 5 μM LPA induced rapid phosphorylation of endogenous CRMP-2 at Thr-555 (Fig. 7A). The phosphorylation of CRMP-2 was increased during the first 3 min and then incubated with control buffer, 1 μM LPA or 5 units/ml Sema3A. After the 30-min stimulation, the cultures were fixed and doubly labeled by anti-neurofilament antibody (green) and tetramethylrhodamine B isothiocyanate-phalloidin (red). Scale bar, 10 μm. B, the time course of the growth cone collapse induced by LPA or Sema3A in the presence or absence of the Rho-kinase inhibitors. The values shown are means ± S.E. of triplicates. *, p < 0.05; **, p < 0.01, significance of difference from the growth cone precultured without Rho-kinase inhibitor analyzed by Student’s t test.

2 N. Inagaki, K. Chihara, N. Arimura, C. Ménager, N. Matsuo, T. Nishimura, M. Amano, and K. Kaibuchi unpublished data.
CRMP-2 phosphorylation by Rho-kinase

Fig. 9. Effects of a dominant negative Rho-kinase on LPA-induced growth cone collapse. A, DRG neurons were transfected with Myc-RB/PH(TT) or a control (lacZ) 3 h after plating, cultured for 20 h, and then stimulated with 1 μM LPA or 5 units/ml Sema3A for 30 min. The stimulated neurons were fixed and doubly labeled by anti-Myc antibody (red) and anti-neurofilament antibody (green) or by anti-β-galactosidase antibody (red) and anti-neurofilament antibody (green). Scale bar, 10 μm. B, the population of the collapsed growth cones expressing Myc-RB/PH(TT) or β-galactosidase was calculated. The values shown are means ± S.E. of triplicates. **, p < 0.01, significance of difference from the growth cones expressing β-galactosidase analyzed by Student’s t test.

CRMP-2 phosphorylation by Rho-kinase.

Inhibition of Rho-kinase Blocks LPA-induced, but Not Sema3A-induced, Growth Cone Collapse—We next examined whether Rho-kinase mediates LPA- or Sema3A-induced growth cone collapse. In the control cultures, exposure to LPA or Sema3A for 3, 10, or 30 min increased the percentage of collapsed growth cones (Fig. 8). However, in the HA1077- or Y-27632-treated cultures, LPA-induced growth cone collapse was completely inhibited from 10 to 30 min. On the other hand, Sema3A-induced growth cone collapse was a little inhibited for 30 min (Fig. 8). Next, we examined the effects of a dominant negative Rho-kinase on LPA-induced or Sema3A-induced growth cone collapse. In neurons transfected with the control vector, exposure to LPA or Sema3A increased the percentage of the collapsed growth cones from about 20% to about 70%. The population of the collapsed growth cones was higher than that of untransfected neurons (Fig. 9B). The reason for the increase in the collapsing rate in transfected neurons is unclear, and the possibility of the effects of gene overexpression cannot be ruled out. The expression of a dominant negative Rho-kinase, RB/PH(TT), completely blocked LPA-induced growth cone collapse (Fig. 9). However, the Sema3A-induced growth cone collapse was insensitive to RB/PH(TT) (Fig. 9). In COS7 cells, CRMP-2 was phosphorylated by the expression of a dominant active Rac1 or Cdc42 (Fig. 6). We have also found that CRMP-2 was phosphorylated at Thr-555 by myotonic dystrophy kinase-related Cdc42-binding kinase β, an effector of Cdc42 (47), at a lower level than Rho-kinase in COS7 cells (data not shown). However, RB/PH(TT), which is a specific inhibitor of Rho-kinase and has no effect on the activity of myotonic dystrophy kinase-related Cdc42-binding kinase β (48), inhibited LPA-induced growth cone collapse. Thus, we conclude that the LPA-stimulated phosphorylation of CRMP-2 was induced by Rho-kinase. We consider that slight inhibition of Sema3A-induced growth cone collapse by HA1077 and Y-27632 (Fig. 8) may be due to the inactivation of other kinases than Rho-kinase by the drugs, because Sema3A-induced growth cone collapse was not inhibited by a more Rho-kinase-specific inhibitory molecule RB/PH(TT) (30, 48). Taken together, these results suggest that Rho-kinase mediates LPA-induced growth cone collapse but does not play a central role in Sema3A-induced growth cone collapse.

CRMP-2 Mutant T555A Partially Inhibits LPA-induced Growth Cone Collapse—To examine the roles of CRMP-2 phosphorylation by Rho-kinase in growth cone morphology, we expressed the CRMP-2 mutant T555A, in which the Rho-kinase phosphorylation site was mutated, in DRG neurons. The expression of wild-type HA-CRMP-2 had no effect on LPA-induced growth cone collapse (Fig. 10). On the other hand, the expression of the mutant HA-CRMP-2 T555A partially but significantly inhibited LPA-induced growth cone collapse (Fig. 10). These results suggest that the phosphorylation of CRMP-2 by Rho-kinase is at least partly involved in LPA-induced growth cone collapse.

DISCUSSION

In the present study, we identified CRMP-2 as a novel Rho-kinase substrate in brain. CRMP-2 was recognized as a major band phosphorylated in the bovine brain cytosol fractions. Rho-kinase phosphorylated CRMP-2 at Thr-555 in vitro and in COS7 cells. Furthermore, we demonstrated that CRMP-2 is phosphorylated by Rho-kinase in DRG neurons during LPA-induced growth cone collapse.

Recent studies have identified various axon guidance mole-
cues and their receptors (1, 2). However, little information is available about the intracellular mechanisms responsible for axon guidance and growth cone regulation. In this study, we examined whether Rho-kinase is involved in growth cone collapse induced by LPA and SemA3A. Interestingly, while both LPA and SemA3A induced growth cone collapse of DRG neurons, only the former stimulated CRMP-2 phosphorylation by Rho-kinase. Furthermore, the inhibition of Rho-kinase activity by Rho-kinase inhibitors prevented LPA-induced growth cone collapse completely but not SemA3A-induced collapse. These results clearly indicate that there are Rho-kinase-dependent and -independent pathways for growth cone collapse. LPA-induced growth cone collapse occurs via the former, while SemA3A-induced collapse involves the latter.

Current reports have shown that Rho-family GTPases are involved in growth cone collapse induced by repulsive guidance cues. A constitutively active form of RhoA inhibited myelin-induced growth cone collapse of motor neurons (49). In addition, retinal neurons treated with C3 extended neurites on myelin and myelin-associated glycoprotein substrates (50). Thus, myelin and myelin-associated glycoprotein are good candidates for extracellular guidance cues to activate the Rho-kinase signaling pathway. Rac1 is implicated in SemA3A-induced DRG and motor neuron growth cone collapse completely but not SemA3A-induced collapse. These results suggest that CRMP-2 is involved in the axonogenesis. It is known that the Rho-Rho-kinase signaling pathway is activated by the extracellular signals and prevents neurite elongation. This raises the possibility that the Rho-kinase signaling pathway may link extracellular signals to CRMP-2-mediated axonogenesis.

In conclusion, the present study identified CRMP-2 as a novel substrate of Rho-kinase in brain. We also showed that the CRMP-2 phosphorylation by Rho-kinase plays a partial role in the regulation of growth cone morphology. The molecular mechanism of the growth cone regulation by Rho-kinase and CRMP-2 remains an important issue for future investigations.

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neurons. These results suggest that CRMP-2 is involved in the axonogenesis. It is known that the Rho-Rho-kinase signaling pathway is activated by the extracellular signals and prevents neurite elongation. This raises the possibility that the Rho-kinase signaling pathway may link extracellular signals to CRMP-2-mediated axonogenesis.

In conclusion, the present study identified CRMP-2 as a novel substrate of Rho-kinase in brain. We also showed that the CRMP-2 phosphorylation by Rho-kinase plays a partial role in the regulation of growth cone morphology. The molecular mechanism of the growth cone regulation by Rho-kinase and CRMP-2 remains an important issue for future investigations.
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