Phosphorylation by Rho Kinase Regulates CRMP-2 Activity in Growth Cones

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Collapsin response mediator protein 2 (CRMP-2) enhances the advance of growth cones by regulating microtubule assembly and Numb-mediated endocytosis. We previously showed that Rho kinase phosphorylates CRMP-2 during growth cone collapse; however, the roles of phosphorylated CRMP-2 in growth cone collapse remain to be clarified. Here, we report that CRMP-2 phosphorylation by Rho kinase cancels the binding activity to the tubulin dimer, microtubules, or Numb. CRMP-2 binds to actin, but its binding is not affected by phosphorylation. Electron microscopy revealed that CRMP-2 localizes on microtubules, clathrin-coated pits, and actin filaments in dorsal root ganglion neuron growth cones, while phosphorylated CRMP-2 localizes only on actin filaments. The phosphomimic mutant of CRMP-2 has a weakened ability to enhance neurite elongation. Furthermore, ephrin-A5 induces phosphorylation of CRMP-2 via Rho kinase during growth cone collapse. Taken together, these results suggest that Rho kinase phosphorylates CRMP-2, and inactivates the ability of CRMP-2 to promote microtubule assembly and Numb-mediated endocytosis, during growth cone collapse.

Axon guidance is essential for the complexity of brain circuitry. Growth cones are thought to be a sensor for guidance molecules during development. Growth cones localize at the tips of axons and dynamically change their morphology in response to attractive and repulsive guidance cues, thus determining the direction of growth (16). Such morphological changes in growth cones are thought to be achieved by cytoskeleton reorganization, cell adhesion, and endocytosis (55, 64). Growth cones consist of actin filaments at the edge and microtubules and neurofilaments at the center. Recently, it was revealed that actin filaments are regulated during growth cone collapse induced by repulsive guidance cues (23). Furthermore, microtubules and endocytosis regulate growth cone morphology (11, 18, 26, 30, 43, 51). However, signal cascades regulating microtubules and endocytosis remain to be clarified.

Recent evidence supports the idea that cytoskeletal components are required for proper axonal path finding, and these components are regulated by members of the Rho family, including RhoA, Rac1, and Cdc42 (32, 36). Rho proteins serve as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound state (35, 42). In their active state, these GTPases bind characteristic sets of effector proteins. The most important effector of RhoA in the growth cone is probably the serine-threonine kinase, Rho-associated kinase (Rho kinase)/ROKα/ROCK1 (5, 9, 74). Rho kinase binds to and is activated by the GTP-bound active form of Rho (2, 40).

Several research groups, including ours, support the idea that Rho kinase is a negative regulator of neurite formation and growth cone motility in neuronal cells downstream of Rho (1, 5, 38, 71). The ephrins, ligands of Eph receptor tyrosine kinases, have also been reported as a repulsive guidance cue to activate the Rho/Rho kinase signaling cascade during growth cone collapse (13, 71). The roles of the Eph family and ephrins in axon guidance have been studied in topographically organized sensory systems such as the retinotectal projection (21, 24, 46, 72). Activation of Eph receptor by, for example, ephrin-A5 causes the turning or collapse of growth cones. Analysis of the underlying signaling cascade has led to the identification of signaling molecules, such as ephexin, a Rho-specific guanine nucleotide exchange factor, which directly binds to Eph receptor and mediates signal from receptor to RhoA (44, 61, 63). In addition, myosin light chain (MLC) has been identified as one of the major substrates of Rho kinase-mediated growth cone collapse (1, 71). However, it is still unknown whether MLC phosphorylation is sufficient to mimic growth cone collapse induced by extracellular signals such as ephrin-A5 (71).

We previously identified collapsin response mediator protein 2 (CRMP-2) as a substrate of Rho kinase in the brain (5).
CRMP-62, the chick CRMP-2 (98% identity), is reported to be required for the growth cone collapse of dorsal root ganglion (DRG) neurons induced by a repulsive guidance cue, semaphorin-3A (Sema3A; also known as collapsin-1) (30). UNC-33, the Caenorhabditis elegans homologue (30% homology), is identified by a mutation resulting in severely uncoordinated movement, abnormalities in axon guidance, and a superabundance of microtubules in neurons (37, 49). These results indicate that CRMP-2 is also a major mediator of growth cone collapse induced by repulsive guidance cues. We and other groups have reported that CRMP-2 is phosphorylated by CdK5 and GSK-3β downstream of Sema3A (10, 14, 68, 73). This phosphorylation of CRMP-2 is essential for Sema3A-induced growth cone collapse (10). However, the exact roles of CRMP-2 phosphorylation remain to be clarified.

We recently identified two molecules, tubulin heterodimer and Numb, as CRMP-2-interacting molecules (28, 34). CRMP-2 shows much higher affinity to tubulin heterodimers than to the polymerized tubulin (microtubules). CRMP-2 copolymerizes with tubulin dimers into microtubules and promotes tubulin polymerization in vitro. Furthermore, the overexpression of CRMP-2 facilitates the rate of axonal growth, whereas the mutant lacking the activity of the microtubule assembly inhibits axonal growth. Given the enriched localization of CRMP-2 in growing axons, it is likely that the CRMP-2–tubulin complex concentrated in the distal part of the axon promotes microtubule assembly and axon formation (28). CRMP-2 participates in Numb-mediated endocytosis and regulates L1 recycling at the growth cone, followed by axon elongation (56). Thus, CRMP-2 basically has positive effects on axon growth. These results raise the possibility that modification, such as phosphorylation, of CRMP-2 regulates the CRMP-2 activity in axon growth or growth cone dynamics, including growth cone collapse.

Here, we report that phosphorylation by Rho kinase diminishes the CRMP-2 activity with respect to binding to tubulin dimer and Numb in neurons. Such phosphorylation by Rho kinase is observed during ephrin-A5–induced growth cone collapse. These results suggest that phosphorylation of CRMP-2 by Rho kinase enhances growth cone collapse by inhibiting the ability of CRMP-2 to associate with microtubules and Numb.

MATERIALS AND METHODS

Materials and chemicals. cDNA encoding human CRMP-2 was obtained as described previously (5). CRMP-2 was subcloned into pB-GEX (rearranged pGEX) (Novagen, Piscataway, NJ). Native bovine CRMP-2 was purified from bovine brain extracts by a method described previously (5). His-tagged CRMP-2 and GST-tagged CRMP-2 (CRMP-2-GST) were purified following the procedures recommended by Invitrogen Corp.

Phosphorylation assay. The phosphorylation assay of the samples was carried out as described previously (52). In brief, the kinase reaction for Rho kinase was performed in 50 μl of a reaction mixture (102 mM PIPES at pH 6.8, 1 mM EDTA, 1 mM dithiothreitol, 1.55 mM MgSO4, 100 μM γ-[32P]ATP [1 to 20 GBq/μmol], 100 nM purified GST-Rho kinase catalytic domain [RhoK-cat]) for 30 min at 30°C. Rho-K cat was produced in Sf9 cells with a baculovirus system and purified on glutathione-Sepharose 4B beads (Amersham Biosciences Corp.). GSK-3β and CdK5 were obtained from Upstate Biotech (Charlottesville, VA). Then, the reaction mixtures were boiled in sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for estimation of the stoichiometry. The radiolabeled bands were visualized by an image analyzer (BAS 2000; Fujifilm, Tokyo, Japan).

In vitro binding assay. We first immobilized 0.5 μM phosphorylated or nonphosphorylated CRMP-2 onto glutathione-Sepharose 4B beads for 60 min at 4°C. To examine the interaction of CRMP-2 and tubulin heterodimers, the immobilized CRMP-2 beads with 1.0 μM tubulin heterodimer for 120 min at 4°C. After removal of the supernatant of the CRMP-2–tubulin mixture, the beads were washed three times with 100 mM PIPES, pH 6.8, 1 mM EDTA, 0.5 mM MgSO4. Then the samples were boiled in SDS sample buffer and subjected to SDS-PAGE and immunoblot analysis with the indicated antibodies. To examine the interaction of CRMP-2 with other molecules, the immobilized CRMP-2–tubulin beads with 1.0 μM tubulin heterodimer were further washed three times with washing buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1.0% NP-40, 1 mM EDTA, 100 mM NaCl, 100 mM MgSO4). Then the samples were boiled in SDS sample buffer and subjected to SDS-PAGE and immunoblot analysis with the indicated antibodies.

Surface plasmon resonance measurements. Anti-α-tubulin monoclonal antibody (DM1A) was covalently coupled to a CMS sensor chip in a Biacore 3000 system (Biacore, Tokyo, Japan) according to the manufacturer's instructions. Purified tubulin heterodimer (0.5 μM) was captured at a flow rate of 10 μl/min for 3 min. The level of resonance units raised by the addition of anti-tubulin antibodies was about 1,800 to 2,000 in each condition. Binding of 8, 4, 2, 1, and 0.5 μM phosphorylated or nonphosphorylated His-tagged CRMP-2 in PEM buffer. This mixture was incubated at 37°C for 10 min and centrifuged at 100,000 × g for 30 min at 37°C. The pellet and supernatant were subjected to SDS-PAGE.

Cosedimentation assay. Tubulin (10 μM) was first assembled with 1 mM GTP, 10% dimethyl sulfoxide, and 20 μM Taxol for 30 min at 37°C. The assembled microtubules were mixed with 5 μM bovine serum albumin (BSA) or 5 μM phosphorylated or nonphosphorylated His-tagged CRMP-2 in PEM buffer. This mixture was incubated at 37°C for 10 min and centrifuged at 100,000 × g for 10 min at 37°C. The pellet and supernatant were subjected to SDS-PAGE.

Cell culture and transfection. Vero cells and NIE-115 cells were seeded on 13-mm round glass coverslips at 1.5 × 104 cells/mm2 for immunostaining and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in an atmosphere of 5% CO2 at 37°C. For transfection, Vero and NIE-115 cells were plated on glass coverslips at 104 cells/mm2 for immunostaining and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37°C. The cells were transfected with your plasmid and incubated for 48 h before fixation and analysis.

Data analysis. Statistical analysis was performed with the software package StatView (version 4.5) (SAS Institute, Cary, NC). The significance of differences was evaluated by Student’s t test.
FIG. 1. An in vitro tubulin or microtubule binding assay using phosphorylated or nonphosphorylated CRMP-2-GST. (A) GST and CRMP-2-GST (0.5 μM) were phosphorylated by Rho kinase catalytic domain (RhoK-cat) in the presence (+) or absence (−) of ATP. GST or CRMP-2-GST immobilized on beads was incubated with 1 μM tubulin in PEM buffer for 1 h at 4°C. GST and CRMP-2-GST results were analyzed using a Coomassie brilliant blue (CBB)-stained gel (right panel). Purified CRMP-2-GST contained degradation products, as confirmed by immunoblotting (IB) with anti-GST and anti-CRMP-2 antibody. An asterisk indicates RhoK-cat. An arrowhead and an arrow indicate the intact protein of CRMP-2-GST and GST, respectively. Phosphorylated CRMP-2-GST or the bound tubulin was analyzed by immunoblotting with anti-phospho-CRMP-2 antibody and anti-α-tubulin antibody (left panels). (B) Biacore sensorgram of tubulin capture with anti-tubulin antibody. Purified tubulin heterodimer (0.5 μM) was captured with the anti-α-tubulin antibody immobilized over the sensor chip at a flow rate of 10 μl/min for 3 min. The sensorgram in the sample of nonphosphorylated CRMP-2 [ATP (−)] is identical to that obtained with phosphorylated CRMP-2 [ATP (+)] (data not shown). The increase in RU (resonance units) results from the binding of tubulins to anti-tubulin antibodies. (C) Biacore sensorgram of CRMP-2 binding to tubulin. Binding of 8, 4, 2, 1, and 0.5 μM phosphorylated [ATP (+)] or nonphosphorylated [ATP (−)] His-CRMP-2 to a tubulin heterodimer-loaded sensor chip surface was examined. (D) Cosedimentation analysis of phospho-CRMP-2 or non-phospho-CRMP-2. His-CRMP-2 (5 μM) was phosphorylated by Rho kinase (RhoK-cat) in the presence (+) or absence (−) of ATP. His-CRMP-2 was mixed with 10 μM microtubules stabilized by Taxol (+) or left unixed (−). After a 10-min incubation at 37°C, mixtures containing microtubules were centrifuged at 37°C. The quantity of His-CRMP-2 in supernatant (S) or pellet (P) was shown by Coomassie brilliant blue gel staining results (upper panel). The samples were analyzed by immunoblotting using anti-GST antibody to confirm the identity of this calcium phosphate method with chick DRG neurons. The mean level of ectopic CRMP-2 expression is maximally fivefold that of the endogenous levels 3 days after transfection. For the analysis of axon length, we cultured DRG neurons without NGF. Spontaneous axon elongation was observed, probably because of the trophic factors secreted from contaminated neurotrophic cells or neurons.

**RESULTS**

We previously showed that CRMP-2 binds to the tubulin dimer and enhances microtubule formation (28). We examined whether phosphorylation of CRMP-2 affects its tubulin binding band as CRMP-2 (lower panel). An asterisk indicates RhoK-cat. An arrowhead and an arrow indicate His-CRMP-2 and tubulin, respectively.
activity in vitro. Purified CRMP-2-GST was phosphorylated by Rho kinase catalytic domain (RhoK-cat) (A) or Cdk5 and/or GSK-3β (B) in the presence (+) or absence (−) of ATP. GST and CRMP-2-GST immobilized on beads were incubated with extracts of rat brain (P6 and P7) for 1 h at 4°C. CRMP-2-GST and the bound proteins were analyzed by immunoblotting (IB) using anti-GST antibody, anti-phospho-CRMP-2 antibody, anti-α-tubulin antibody, anti-Numb antibody, anti-Rho-GDI antibody, or anti-actin antibody. Input, immunoreactive bands of brain lysate (5% in total lysate). The lane labeled “beads only” shows the results for beads and brain lysates without GST proteins.

FIG. 2. An in vitro binding assay using phospho- or non-phospho-CRMP-2-GST and rat brain lysate. (A and B) GST and CRMP-2-GST (0.5 μM) were phosphorylated by Rho kinase catalytic domain (RhoK-cat) (A) or Cdk5 and/or GSK-3β (B) in the presence (+) or absence (−) of ATP. GST and CRMP-2-GST immobilized on beads were incubated with extracts of rat brain (P6 and P7) for 1 h at 4°C. CRMP-2-GST and the bound proteins were analyzed by immunoblotting (IB) using anti-GST antibody, anti-phospho-CRMP-2 antibody, anti-α-tubulin antibody, anti-Numb antibody, anti-Rho-GDI antibody, or anti-actin antibody. Input, immunoreactive bands of brain lysate (5% in total lysate). The lane labeled “beads only” shows the results for beads and brain lysates without GST proteins.

We next examined whether phosphorylation of CRMP-2 affects its binding activity to microtubules in vitro (Fig. 1D) by use of a standard cosedimentation assay. Phosphorylated CRMP-2 or nonphosphorylated CRMP-2 was incubated with microtubules polymerized by Taxol at 37°C, and these mixtures were centrifuged to sediment the filamentous microtubules and the interacting molecules. BSA, a control protein, did not cosediment with microtubules (data not shown). Some nonphosphorylated CRMP-2 cosedimented with Taxol-stabilized microtubules, as reported previously (28), but not on the beads coated with phosphorylated CRMP-2 (Fig. 1A). The perturbation of the association of tubulin and CRMP-2 by phosphorylation was also confirmed by using the Biacore system (Fig. 1B and C). CRMP-2 bound to the retained tubulin in the Biacore sensor chip in a dose-dependent manner, whereas phosphorylated CRMP-2 did not (Fig. 1C). These results indicate that CRMP-2 phosphorylated by Rho kinase does not bind to the tubulin heterodimer.

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We next examined the effect of phosphorylation on the interaction of CRMP-2 with other molecules. We previously found that Numb binds to CRMP-2 directly and is localized at the axonal growth cone in neurons (56). The Numb and CRMP-2 complex mediates the endocytosis of L1 in the axonal growth cones, and Numb-mediated endocytosis of L1 is necessary for axon growth (56). We examined the interaction of phosphorylated CRMP-2 with partner proteins, including Numb, by a GST pulldown assay using rat brain lysate (Fig. 2A and B). Phosphorylated or nonphosphorylated CRMP-2 was immobilized on GST beads and incubated with postnatal rat brain lysate. An association of Numb with the beads coated with CRMP-2-GST was observed but not with the beads coated with phosphorylated CRMP-2-GST (Fig. 1C). These results indicate that CRMP-2 phosphorylated by Rho kinase loses the ability to bind to Numb as well as tubulin (Fig. 2A). A small amount of tubulin was retained by phosphorylated CRMP-2 under these conditions, presumably because the high concentration of tubulin (approximately 13 μM) in brain lysates increased the efficiency of tubulin binding to phosphorylated CRMP-2 (Fig. 2A). Rho-GDI (a cytosolic and abundant protein used as a negative control) did not associate with CRMP-2. In addition, we identified actin as a CRMP-2-interacting molecule (Fig. 2A). Actin bound to phosphorylated CRMP-2 as well as to nonphosphorylated CRMP-2. These results indicate that CRMP-2 phosphorylation at the C terminus affects the interaction with certain proteins, including Numb and tubulin, but not with actin.

CRMP-2 can be phosphorylated by Cdk5 at Ser-522, and by recognizing this priming phosphorylation, GSK-3β phosphorylates CRMP-2 at Ser-518 and Thr-514 (10, 14, 68, 73). Since these phosphorylation sites are close to the phosphorylation site of Rho kinase, we examined whether these phosphorylations affect the association of CRMP-2 and interacting molecules. Similarly to Rho kinase results, the phosphorylation by
Cdk5 and GSK-3\(\beta\) also prevents the binding of tubulin dimers and Numb but not that of actin (Fig. 2B). These results indicate that CRMP-2 phosphorylation at the C terminus by Rho kinase, Cdk5, and GSK-3\(\beta\) has similar effects on CRMP-2 function.

Next we examined the localization of CRMP-2 at DRG neuron growth cones. As reported previously, CRMP-2 is accumulated at the distal part of axons and growth cones (39, 54, 58). To examine the intracellular localization of phosphorylated CRMP-2 and interacting molecules, we performed an indirect immunofluorescence study using anti-phospho-CRMP-2 antibody (Fig. 3A). CRMP-2 immunoreactivity was mainly localized at the center of the growth cones and partially colocalized with microtubules and actin, as reported previously (56, 75). However, phosphorylated CRMP-2 was localized on punctate structures all around the growth cone area and some was seen even in the filopodia of the growth cones, which was stacked with actin filaments. Because phosphorylated CRMP-2 was present in all areas of growth cone, its immunoreactivity was overlapped with the filamentous image of microtubules. Localization of phosphorylated CRMP-2 itself did not appear to occur in the form of microtubule-like bundles or filaments, however, as was observed in experiments with anti-CRMP-2 antibody (Fig. 3B).

Subcellular localization of CRMP-2 in chick DRG growth cones was also examined by electron microscopic immunocytochemistry. As shown in immunofluorescence images (Fig. 3), microtubules were stuck and distributed in parallel. Immunolabeling in the electron micrograph was found predominantly on the filamentous image of microtubules but rarely in the background (Fig. 4A). In the grazing section of growth cones, the immunolabeling was also localized close to the edge, near the membranous areas, in addition to being localized at microtubules (Fig. 4B). This suggests that CRMP-2 is present in the vicinity of the plasma membrane of growth cones. For more-precise localization, we employed the freeze-etching immunoreplica method, which illustrates different morphological views, in particular, the membrane cytoskeletal complex (the so-called membrane undercoat). Freeze-etched images showed a few clathrin-coated pits and the cortical actin filaments in the cytoplasmic surface of the apical distal membrane (membrane undercoat) of growth cones. The high-power view of the cytoplasmic surface of the membrane provides evidence that CRMP-2 was localized on clathrin-coated pits and actin filaments (Fig. 4D and E) and actin filaments (Fig. 4G). More than 75% of the clathrin-coated pits showed immunolabeling of CRMP-2 (76.5%; \(n = 17\)). Because AP-2, which is a component of clathrin-coated pits, associates with Numb, CRMP-2 is thought to bind to clathrin-coated pits through Numb (56). In contrast, the immunolabeling against phosphorylated CRMP-2 was found only on actin filaments (Fig. 4H) and not on microtubules or clathrin-coated pits (0%, \(n = 15\); Fig. 4C and F). These observations are morphological counterparts of the molecular interaction scheme derived from biochemical experiments on the basis of the idea that CRMP-2 associates with Numb on clathrin-coated pits, microtubules, or actin filaments, whereas phosphorylated CRMP-2 interacts only with actin filaments in growth cones.

We then examined the functional relevance of the relationship between phosphorylation at Thr-555 and CRMP-2 activity in neurons (Fig. 5A). We first characterized two CRMP-2 mutants, one in which the Rho kinase phosphorylation site (Thr-555) is replaced by Asp (CRMP-2 T555D), which is expected to mimic the phosphorylated form (3), and another in

![Fig. 3. Localization of CRMP-2 or phoso-CRMP-2 in chick DRG neuron growth cones. (A) Chick DRG neuron growth cones were triple stained with anti-CRMP-2 antibody (red), anti-unique \(\beta\)-tubulin antibody (green), and Alexa-649-phalloidin (blue) (upper panels) or anti-phospho-CRMP-2 antibody (red), anti-unique \(\beta\)-tubulin antibody (green), and Alexa-488-phalloidin (blue) (lower panels). Arrowheads indicate the colocalization of phosphorylated CRMP-2 or CRMP-2 and actin filaments. (B) Graphs plot the fluorescence intensity of immunolabeled CRMP-2 (red) and unique \(\beta\)-tubulin (green) or phosphorylated CRMP-2 (red) and unique \(\beta\)-tubulin (green) in the dotted line shown in each growth cone image. Bar, 10 \(\mu\)m.](http://mcub.asm.org/)
which Thr-555 is replaced by Ala (CRMP-2 T555A) and is not phosphorylated by Rho kinase (5). Because ectopic green fluorescent protein (GFP)-tagged CRMP-2 was diffusely distributed, it was difficult to examine the localization of ectopic CRMP-2 in detail at the growth cones. Although we do not know why GFP mutants are located diffusely, this is presumably because neurons express CRMP-2 at levels much higher than other proteins (approximately 1% in total proteins). In contrast, in the cells expressing CRMP-2 at lower levels, we observed the clear colocalization of GFP-CRMP-2 and microtubules. When GFP-CRMP-2 wild type (WT) was expressed in Vero fibroblasts, 72.7% of GFP-tagged CRMP-2-expressing cells (n = 22) showed clear localization along the mitotic spindle, as previously described (28, 34) (Fig. 5A). Colocalization of the mutant CRMP-2 T555A with microtubules was also observed in 95.2% of the transfected cells (n = 21). However, the mutant CRMP-2 T555D was diffusely distributed, and only 9.5% of GFP-CRMP-2 T555D-expressing cells showed the colocalization with the mitotic spindle (n = 21).

Using these mutant constructs, we examined the functional relevance of phosphorylation at Thr-555 and CRMP-2 activity in terms of neurite formation (Fig. 5B). N1E-115 neuroblastoma cells were transfected with CRMP-2 T555D and CRMP-2 T555A. N1E-115 cells have a round morphology in the presence of serum, whereas these cells can differentiate and extend neurites in serum-free medium. In the presence of serum, only about 5% of the cells extended neurites. Under these conditions, expression of CRMP-2 WT increased the numbers of the cells bearing neurites, in contrast to the results seen with control cells expressing GST (P < 0.01), as described previously (28) (Fig. 5C). The expression of CRMP-2 T555A increased the percentage of cells bearing neurites (P < 0.01), but that of the T555D mutant only slightly increased the percentage (P > 0.2). This neurite formation in N1E-115 is known to require the assembly of the microtubules (28). The morphology of neurites induced by the ectopic expression of CRMP-2 WT or T555A was similar to that induced by the serum deprivation, and the neurites had microtubules that were detected with anti-α-tubulin antibody (28) (Fig. 5B).

We next examined the effects of these constructs on axon elongation in DRG neurons. Dissociated DRG neurons were transfected with the plasmids used in Fig. 5B. After transfection, neurons were cultured for 3 days in NGF-deprived medium. Under these conditions, some neurons transfected with control plasmids maintained the long axon. In neurons transfected with CRMP-2 WT, the percentage of the neurons bearing a long axon (>1,300 μm) was increased, in contrast to the control cells expressing GST (P < 0.01; Fig. 5D). CRMP-2 T555A had an effect on axon elongation similar to that seen with CRMP-2 WT (P < 0.01), but that of T555D was weak (P < 0.05). These observations indicate that the dephosphorylated form of CRMP-2 has the ability to promote axon elongation, presumably through the interaction with tubulin and Numb, and that the phosphorylated form of CRMP-2 loses most of ability to support neurite formation in N1E-115 and DRG neurons (Fig. 5C and D). Compared to control results, however, the mutant T555D has at least a weak ability to enhance neurite outgrowth or axon elongation (Fig. 5C and D). Although we do not know why T555D mutants have these positive effects on axon elongation, we think that the mutant T555D may not completely mimic the phosphorylated states, even though it increases the negative charge at the phosphorylation site, as previously reported (15, 25, 47). In fact, 9.5% of GFP-CRMP-2 T555D-expressing cells show the colocalization with mitotic spindles under the conditions in which 72.7% of GFP-CRMP-2 WT colocalized with spindles (Fig. 5A). Thus, it appears that the T555D mutant still keeps some activity to interact with tubulin and/or microtubules, thereby promoting neurite elongation. Alternatively, this implies the existence of other mechanisms that enhance neurite elongation, which are regulated independently of phosphorylation by Rho kinase at Thr-555.

As we reported previously, CRMP-2 is phosphorylated by Rho kinase during LPA-induced growth cone collapse (5). However, its physiological role in axon guidance is still unknown. Thus, we examined the physiological role of the phosphorylation of CRMP-2 by Rho kinase in growth cone collapse induced by repulsive guidance cues (Fig. 6). As some groups have reported, several repulsive axon guidance cues stimulate Rho/Rho kinase signaling to induce growth cone collapse (74). Among them, ephrin-A5 induces the phosphorylation of MLC through Rho kinase (71). We then examined whether ephrin-A5 induces CRMP-2 phosphorylation by Rho kinase at Thr-555. DRG neurons cultured for 24 h were serum-starved for 4 h and then stimulated by ephrin-A5 for 3, 10, and 30 min; these stimuli induced growth cone collapse. The addition of ephrin-A5 induced rapid phosphorylation of endogenous CRMP-2 at Thr-555 (Fig. 6A). The phosphorylation increased up to about sixfold more than the basal level during the first 3 min (Fig. 6A and B). To examine whether ephrin-A5-induced phosphorylation of CRMP-2 at Thr-555 was mediated by Rho kinase, DRG neurons were stimulated by ephrin-A5 in the presence of Rho kinase inhibitor (Y-27632 or HA1077) for 1 h (69). Y-27632 and HA1077 inhibited the ephrin-A5-induced phosphorylation of CRMP-2 (Fig. 6A and B). These results indicate that CRMP-2 is phosphorylated at Thr-555 by Rho kinase during ephrin-A5-induced growth cone collapse of DRG neurons, as well during as during that induced by LPA, as reported previously (5). We then examined the localization of phosphorylated CRMP-2 after the stimulation with ephrin-
The expression of CRMP-2 T555D increased the number of collapsed growth cones and slightly inhibited ephrin-A5-induced growth cone collapse (51). However, the molecular mechanisms involving CRMP-2 in growth cone collapse have not been elucidated. We previously reported that Rho kinase phosphorylates CRMP-2 at Thr-555 during LPA-induced, but not Sema3A-induced, growth cone collapse in DRG neurons (5) and that overexpression of CRMP-2 in hippocampal neurons enhances axon formation by its association with tubulin heterodimer and Numb (28, 34, 39, 56). CRMP-2 functions as a carrier of tubulin heterodimers, which delivers tubulin dimers to the assembly plus ends of nucleating sites or growing microtubules (28). CRMP-2 associates with Numb and regulates L1 endocytosis at axonal growth cones in hippocampal neurons (56). Although these reports revealed that CRMP-2 functions in axon elongation, the molecular mechanisms involving CRMP-2 and its interacting molecules in growth cone collapse remain unresolved. And we also reported the involvement of phosphorylation at Thr-514 by GSK-3β in neuronal polarity and that Thr-522 was phosphorylated by Cdk5 downstream of Sema3A (68, 73) but have not investigated the meaning of these phosphorylation events with respect to the growth cone collapse. In the present study, we found that phosphorylation by Rho kinase inhibits the ability of CRMP-2 to bind tubulin and Numb. These interactions are necessary for growth cone advance and axon growth. Therefore, we suggest that ephrin-A5 stimulation dissociates CRMP-2 from interacting

DISCUSSION

CRMP-2 was first reported as a mediator of Sema3A-induced growth cone collapse (31). However, the molecular mechanisms involving CRMP-2 in growth cone collapse have not been elucidated. We previously reported that Rho kinase phosphorylates CRMP-2 at Thr-555 during LPA-induced, but not Sema3A-induced, growth cone collapse in DRG neurons (5) and that overexpression of CRMP-2 in hippocampal neurons enhances axon formation by its association with tubulin heterodimer and Numb (28, 34, 39, 56). CRMP-2 functions as a carrier of tubulin heterodimers, which delivers tubulin dimers to the assembly plus ends of nucleating sites or growing microtubules (28). CRMP-2 associates with Numb and regulates L1 endocytosis at axonal growth cones in hippocampal neurons (56). Although these reports revealed that CRMP-2 functions in axon elongation, the molecular mechanisms involving CRMP-2 and its interacting molecules in growth cone collapse remain unresolved. And we also reported the involvement of phosphorylation at Thr-514 by GSK-3β in neuronal polarity and that Thr-522 was phosphorylated by Cdk5 downstream of Sema3A (68, 73) but have not investigated the meaning of these phosphorylation events with respect to the growth cone collapse. In the present study, we found that phosphorylation by Rho kinase inhibits the ability of CRMP-2 to bind tubulin and Numb. These interactions are necessary for growth cone advance and axon growth. Therefore, we suggest that ephrin-A5 stimulation dissociates CRMP-2 from interacting

A5. Because ephrin-A5 treatment makes the growth cone collapse, the relative amount of increased phosphorylation of CRMP-2 could not be measured in spreading growth cones (data not shown). However, higher immunolabeling of phos-
molecules by phosphorylation and enhances growth cone collapse.

In previous reports, we showed that CRMP-2 enhances microtubule assembly and, thereby, axon formation (28). And here we report that the phosphorylation by Rho kinase prevents the association with tubulin dimer-microtubules. This regulation of CRMP-2 activity seems to cause the reduction of microtubule assembly. The plus ends of microtubules in growth cones exhibit a property termed dynamic instability, wherein they cycle through periods of growth and shrinkage (6, 27, 53, 65). The increase of microtubule assembly is required for the growth cone advance (27, 28). In fact, some groups showed that the polymerization and capturing of microtubules in one direction and the shrinkage of microtubules in the other direction are early steps in guidance of axonal growth cones, suggesting that the regulation of microtubule dynamic instability is closely related to the morphological changes of growth cones (11, 60, 66, 76). Therefore, the enhancement of microtubule assembly by CRMP-2 is critical for the growth cone dynamics. The canceling of the interaction of CRMP-2 and tubulin dimer and/or microtubules by phosphorylation is thought to prevent the proper microtubule formation. Taken together, these data indicate that the negative regulation of CRMP-2 activity appears to disrupt the normal microtubule dynamics, followed by the collapse of growth cone morphology.

Knockdown of CRMP-2 in hippocampal neurons inhibits Numb-mediated L1 endocytosis and axon growth (56). Here we report that phosphorylated CRMP-2 could not associate with Numb. Thus, it is possible that CRMP-2 phosphorylation also inhibits Numb-mediated L1 endocytosis. However, two groups have reported that growth cone collapse triggered by Sema3A or ephrins was accompanied by enhanced endocytosis (26, 41); they observed the fluorescence-labeled dextran uptake or reorganization of signaling molecules neuropilin 1.
and GSK-3β. Sema3A is thought to activate Cdk5 and GSK-3β. These activations cause phosphorylation at Ser-522, Ser-518, and Thr-514. Ephrin-A5 stimulation activates the Rho/Rho kinase signaling pathway and subsequently induces the phosphorylation of CRMP-2 at Thr-555 by Rho kinase. The binding activity of CRMP-2 to tubulin is decreased by the phosphorylation by Cdk5, GSK-3β, and Rho kinase. Nonphosphorylated CRMP-2 binds to tubulin heterodimers to promote microtubule assembly or Numb-mediated endocytosis, thereby enhancing axon elongation and branching. In contrast, the phosphorylated form cannot associate with interacting molecules and loses the positive effect on axon elongation, thereby causing arrest of axon growth and growth cone collapse.

FIG. 7. Model schema for the phosphorylation of CRMP-2 by Rho kinase, Cdk5, and GSK-3β. Sema3A is thought to activate Cdk5 and GSK-3β. These activations cause phosphorylation at Ser-522, Ser-518, and Thr-514. Ephrin-A5 stimulation activates the Rho/Rho kinase signaling pathway and subsequently induces the phosphorylation of CRMP-2 at Thr-555 by Rho kinase. The binding activity of CRMP-2 to tubulin is decreased by the phosphorylation by Cdk5, GSK-3β, and Rho kinase. Nonphosphorylated CRMP-2 binds to tubulin heterodimers to promote microtubule assembly or Numb-mediated endocytosis, thereby enhancing axon elongation and branching. In contrast, the phosphorylated form cannot associate with interacting molecules and loses the positive effect on axon elongation, thereby causing arrest of axon growth and growth cone collapse.

CRMP-2 is implicated in Sema3A-induced growth cone collapse (31). The phosphorylation of CRMP-2 at Thr-555 by Rho kinase was observed during LPA-induced growth cone collapse but not during Sema3A-induced collapse (5). In retinal ganglion cells, ephrin-A5-induced growth cone collapse is mediated by Rho kinase (13, 71). In the present study, we found that Rho kinase phosphorylates CRMP-2 at Thr-555 in response to ephrin-A5. It has been reported that activation of Fyn, Cdk5, and GSK-3β is involved in Sema3A signaling (22, 62) (Fig. 7). Activated Cdk5 phosphorylates Tau and causes microtubule reorganization induced by Sema3A (62). Recently, our group and several other groups have reported that CRMP-2 is phosphorylated by Cdk5, dual tyrosine-regulated kinase (DYRK), and GSK-3β (10, 14, 73). Cdk5 and DYRK phosphorylate CRMP-2 at Ser-522, and this phosphorylation site acts as a priming site for subsequent GSK-3β phosphorylation at Ser-518 and Thr-514. Here, we found that the phosphorylation of CRMP-2 by Cdk5 and GSK-3β cancels the binding activity to tubulin and Numb. Thus, both signaling pathways downstream of Sema3A and ephrin-A5 as well as LPA seem to terminate in the same molecule, CRMP-2, through activation by different kinases, followed by microtubule reorganization (Fig. 7). On the other hand, we reported that neurotropic factors BDNF and NT-3, but not NGF, inhibit the phosphorylation of CRMP-2 via PI 3-kinase and Akt in hippocampal neurons (73). These growth factors are known to prevent growth cone collapse in response to the repulsive guidance cues (20, 67). Thus, it appears that attractive and repulsive extracellular cues regulate the balance of phosphorylation and dephosphorylation in CRMP-2, thereby managing microtubule formation and endocytosis. The function of CRMP-2 would be dramatically regulated by several kinds of kinases in response to extracellular stimulation.

Rho kinase has multiple substrates regulating the dynamics of the actin filament (35, 42). Rho kinase regulates the phosphorylation of MLC, resulting in actomyosin contractility, which is observed during ephrin-A5-induced growth cone collapse (71). We recently found that Rho kinase phosphorylates the microtubule-associated proteins (MAPs) Tau and MAP-2 (3, 4). This phosphorylation dissociates MAPs from microtubule filaments in neurons. In fact, in this study the mutant that mimicked CRMP-2 phosphorylated by Rho kinase could not inhibit the ephrin-A5-induced growth cone collapse completely, suggesting that Rho kinase is likely to phosphorylate multiple substrates, including MLC and MAPs, to achieve growth cone collapse. Because nonphosphorylated CRMP-2 can enhance the assembly of microtubules, we speculate that phosphorylated CRMP-2 may lose the ability to drive the microtubule formation, thus causing the destabilization of microtubule formation, in association with phosphorylated MAPs.

We found actin to be a CRMP-2-interacting protein. Both phosphorylated and nonphosphorylated forms of CRMP-2 bound to actin in vitro, and CRMP-2 was detected on actin filaments in DRG neuron growth cones. We examined whether CRMP-2 associates with actin monomer in vitro by use of purified actin monomers and recombinant CRMP-2 proteins, but a direct association was not observed. In another study, we have recently found that CRMP-2 interacts with Specifically Rac1-Associated protein (Sra-1/CYFIP1) (43a), which directly interacts with actin filaments (45). Thus, CRMP-2 may associate with actin filaments through Sra-1 in growth cones. In this study, Rho kinase-induced phosphorylation of CRMP-2 had no effect on the actin binding ability of CRMP-2. CRMP-2 is a highly conserved phosphoprotein, and its phosphorylation states alter upon NGF-induced neuronal differentiation or in the formation of degenerating neurites in the brains of patients with Alzheimer’s disease (12, 33). These findings raise the possibility that other kinases up- or down-regulate CRMP-2 activity and mediate actin reorganization in the Rho family GTPase-mediated signal cascade. Further studies characterizing the protein kinases may shed some light on other functions of CRMP-2.
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REFERENCES


