GSK3 β Regulates Myelin-Dependent Axon Outgrowth Inhibition through CRMP4

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Myelin-associated inhibitors (MAIs) contribute to failed regeneration in the CNS. The intracellular signaling pathways through which MAIs block axonal repair remain largely unknown. Here, we report that the kinase GSK3 β is directly phosphorylated and inactivated by MAIs, consequently regulating protein–protein interactions that are critical for myelin-dependent inhibition. Inhibition of GSK3 β mimics the neurite outgrowth inhibitory effect of myelin. The inhibitory effects of GSK3 β inhibitors and myelin are not additive indicating that GSK3 β is a major effector of MAIs. Consistent with this, overexpression of GSK3 β attenuates myelin inhibition. MAI-dependent phosphorylation and inactivation of GSK3 β regulate phosphorylation of CRMP4, a cytosolic regulator of myelin inhibition, and its ability to complex with RhoA. Introduction of a CRMP4 antagonist attenuates the neurite outgrowth inhibitory properties of GSK3 β inhibitors. We describe the first example of GSK3 β inactivation in response to inhibitory ligands and link the neurite outgrowth inhibitory effects of GSK3 β inhibition directly to CRMP4. These findings raise the possibility that GSK3 β inhibition will not effectively promote long-distance CNS regeneration following trauma such as spinal cord injury.

Introduction

Inhibitory molecules at CNS lesion sites including myelin-associated inhibitors (MAIs) and chondroitin sulfate proteoglycans (CSPGs) activate RhoA in injured neurons to mediate neurite outgrowth inhibition (Liu et al., 2006). In a screen to identify proteins that functionally interact with RhoA in the context of neurite outgrowth inhibition, we previously identified the cytosolic phosphoprotein CRMP4 (Collapsin Response Mediator Protein 4) as a protein that functionally interacts with RhoA to mediate neurite outgrowth inhibition (Alabed et al., 2007). The CRMP family consists of five family members (CRMP1–5) in vertebrates (Goshima et al., 1995; Minturn et al., 1995; Byk et al., 1996; Gaetano et al., 1997; Inatome et al., 2000) that regulate aspects of axon pathfinding and neurite outgrowth (Hedgecock et al., 1985; Siddiqui and Culotti, 1991; Goshima et al., 1995; Minturn et al., 1995; Quinn et al., 1999, 2003; Yoshimura et al., 2005). Each CRMP allele produces two transcripts which differ in their N terminus, yielding long (L-CRMP) and short (S-CRMP) isoforms, which have alternatively been referred to as “a” and “b” isoforms (Quinn et al., 2003; Yuasa-Kawada et al., 2003; Alabed et al., 2007; Pan et al., 2010).

Treatment of neurons with the MAI Nogo specifically enhances the association between RhoA and L-CRMP4 (Alabed et al., 2007); however, the mechanism(s) regulating the formation of this complex is unknown and will add insight into the signaling mechanisms mediating neurite outgrowth inhibition. We find that the L-CRMP4–RhoA protein interaction is regulated by dephosphorylation of L-CRMP4 as a direct consequence of glycogen synthase kinase 3 β (GSK3 β) phosphorylation and inactivation. GSK3 α and β are serine/threonine kinases originally identified as regulatory kinases for glycogen synthase and subsequently implicated in signaling cascades downstream of Wnts, NGF (nerve growth factor), EGF (epidermal growth factor), semaphorins, and Hedgehog (Eickholt et al., 2002; Kockeritz et al., 2006). GSK3 has been widely studied as a potential therapeutic target for nerve regeneration and for a variety of diseases, including cancer and Alzheimer’s disease (Kockeritz et al., 2006).

Here, we show that MAIs phosphorylate and inactivate GSK3 β , leading to subsequent CRMP4 dephosphorylation. We confirm previous reports that inhibition of GSK3 β activity inhibits neurite outgrowth in cerebellar and dorsal root ganglion (DRG) neurons, mimicking the inhibitory effect of myelin, and demonstrate that the effects of GSK3 β inhibitors are markedly attenuated by antagonizing CRMP4. We also demonstrate that overexpression of GSK3 β attenuates myelin-dependent neurite outgrowth inhibition. We show that L-CRMP4 dephosphorylation enhances L-CRMP4 binding to RhoA and that a phospho-dependent change in L-CRMP4 conformation likely regulates this change in affinity. Together, these findings directly implicate GSK3 β in the MAI signaling cascade and link the neurite outgrowth inhibitory effects of GSK3 β inhibition to CRMP4.

Received Dec. 11, 2009; revised March 9, 2010; accepted March 12, 2010.

This study was supported by a grant to A.E.F. from the Canadian Institutes of Health Research (CIHR). A.E.F. is a Tier 2 Canada Research Chair. Y.Z.A. and S.O.T. are supported by fellowships from CIHR, and M.P. is supported by a fellowship from the Multiple Sclerosis Society of Canada. We thank Dr. Keith Burridge for RhoA constructs, Dr. Dennis Stacey for GSK3 β constructs, Isabel Rambaldi for technical assistance, Brigitte Ritter for assistance with lentivirus, Daniel Lee for assistance with the generation of the phospho-specific L-CRMP4 antibody, Dr. Craig Mandato for helpful discussions, and Drs. Phil Barker and Wayne Sossin for helpful comments on this manuscript.

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DOI:10.1523/JNEUROSCI.6154-09.2010

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Figure 1. L-CRMP4 dephosphorylation is stimulated by Nogo-P4 and regulates L-CRMP4–RhoA binding. **A–C**, PC12 cells (**A**) or HEK293T cells (**B**, **C**) were cotransfected with myc-RhoA constructs and L-CRMP4–V5 and subjected to immunoprecipitation with anti-myc antibody. **A**, PC12 cells were stimulated for 8 min with Nogo-P4 peptide. **B**, Cells were treated for 30 min with 200 nM calyculin A before lysis or cell lysates were treated for 30 min with 10 U SAP at 34°C before immunoprecipitation. **D**, **E**, PC12 cells (**D**) or cerebellar neurons infected with HSV-L-CRMP4–V5 (**E**) were stimulated for 1–8 min with Nogo-P4 peptide. Cell lysates were separated by SDS-PAGE and total and phosphorylated L-CRMP4 was detected by immunoblot. Quantifications in arbitrary units are from three experiments and are normalized for total L-CRMP4 loading. * $p < 0.05$ by paired t tests, two-tailed, compared with 0 min. a.u., Arbitrary units; IP, immunoprecipitation.

Figure 2. MAI-dependent phosphorylation of GSK3 β results in L-CRMP4 dephosphorylation. **A**, **B**, PC12 cells (**A**) or cerebellar neurons (**B**) were stimulated for 0.5–8 min with Nogo-P4 peptide or OMgp. Membrane fractions were separated by SDS-PAGE and total and phosphorylated GSK3 β was detected by immunoblot. Quantifications are from at least seven experiments and are normalized for total GSK3 β protein loading. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by Dunnett's post-test compared with 0 min following one-way RM ANOVA. ANOVA p values are indicated above the graphs. **C**, PC12 cells were transiently transfected with L-CRMP4–V5 and GSK3 β S9A for 24 h and then treated with Nogo-P4 peptide for 8 min. L-CRMP4–V5 was immunoprecipitated and separated by SDS-PAGE, and total and phosphorylated L-CRMP4 was detected by immunoblot. a.u., Arbitrary units; IP, immunoprecipitation.

Materials and Methods

Plasmids and antibodies. CRMP4, C4RIP, and RhoA constructs were described previously (Alabed et al., 2007). CRMP4AAA was generated using a site-directed mutagenesis kit (Stratagene). The S188ARhoA construct was provided by Dr. Keith Burrage (University of North Carolina–Chapel Hill, Chapel Hill, NC) and GSK3 β S9A by Dr. Dennis Stacey

(The Cleveland Clinic Foundation). L-CRMP4 antibody was generated by injecting rabbits with antigen RPTGTDQVPRQYK as per the study by Quinn et al. (2003). Antiserum was affinity purified on an antigen-Sepharose column. Phospho-specific antibody that recognizes CRMP4b phosphorylated at Thr622 was generated in rabbit with the phosphopeptide FDLTT (pT)PKGGTTPAGC (where pT is phosphothreonine). Anti-

Figure 3. Pharmacologic inhibition of GSK3 β enhances L-CRMP4–RhoA binding and inhibits neurite outgrowth. **A**, HSV-L-CRMP4–V5-infected cerebellar neurons were treated with 5 μ M SB216763, 7.5 μ M SB415286, 300 nM 6-bromoindirubin-3'-acetoxime, or 2 μ M CT99021 for 18 h. Cell lysates were subjected to immunoprecipitation with anti-V5 antibody and analyzed by immunoblot with anti-pT622 to detect L-CRMP4 phosphorylation and anti-V5 to detect total L-CRMP4. Densitometry from two experiments shows a statistically significant reduction in phospho-L-CRMP4 signal normalized to total CRMP4; the one-way repeated-measures ANOVA p value is 0.002 with Tukey's post-tests, indicating that values for all inhibitors are significantly different from those with DMSO ($p < 0.01$). **B**, myc-RhoA and CRMP4–V5 constructs were transiently coexpressed in PC12 cells. To inhibit GSK3 β , cells were treated overnight with 5 μ M SB216763. Cell lysates were subjected to immunoprecipitation (IP) with anti-myc antibody and analyzed by immunoblot with anti-V5 or anti-myc antibodies. L, Long; S, short. **C**, **D**, Dissociated rat cerebellar neurons (**C**) or DRG neurons (**D**) were treated overnight with 5 μ M SB216763, 7.5 μ M SB415286, 300 nM 6-bromoindirubin-3'-acetoxime, or 2 μ M CT99021, fixed, and stained with anti- β III tubulin antibody to visualize neurite outgrowth. Scale bar, 50 μ m. **E**, **F**, Quantification of cerebellar (**E**) or DRG (**F**) outgrowth/cell. Values are normalized to baseline outgrowth with DMSO vehicle control for each experiment. Determinations are from at least three experiments performed in duplicate. ** $p < 0.01$ and *** $p < 0.001$ by Dunnett's post-tests following one-way RM ANOVA. 6-bromo, 6-Bromoindirubin-3'-acetoxime.

serum was affinity purified by depleting antibodies that recognize unphosphorylated CRMP4 on a nonphosphorylated peptide column followed by selecting phospho-specific antibodies on a phosphopeptide antigen column. Other antibodies used were mouse and rabbit anti-V5 and

mouse anti-myc (Sigma-Aldrich), rabbit anti-phosphothreonine (Invitrogen), rabbit anti-phospho- and -total GSK3 β (Cell Signaling Technology), mouse anti- β III tubulin (Covance), and mouse anti-His (Qiagen).

Preparation of recombinant proteins. Stimulations to examine inhibitory responses were performed with Nogo-P4 peptide (Alpha Diagnostics), a 25 aa inhibitory peptide sequence (residues 31–55 of Nogo-66) sufficient to mediate the inhibitory properties of Nogo-66 (GrandPré et al., 2000), or His-tagged mouse OMgp (R&D Systems) preclustered for 30 min at room temperature with mouse anti-His antibody. Myelin extracts (Igarashi et al., 1993; Hsieh et al., 2006) and GST-Nogo-66 were prepared as described previously (GrandPré et al., 2000).

Preparation of recombinant viruses. For herpes simplex virus (HSV) production, pHSPVPr-PUC plasmids were transfected into 2–2 Vero cells that were superinfected with 5dl 1.2 helper virus 1 d later. Recombinant virus was amplified through three passages and stored at -80°C as described previously (Neve et al., 1997). Lentivirus particles were produced using a third-generation packaging system (Dull et al., 1998; Addgene) with GSK3 β SA-V5His cloned into the viral expression vector pRRLsinPPT. Recombinant viral particles were collected by high-speed centrifugation of supernatants from 293T cells transfected with the expression vector and packaging mix by using Lipofectamine 2000.

CRMP–RhoA coimmunoprecipitation assay. HEK293T cells were grown to subconfluence and transfected with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen), washed twice with ice-cold PBS, and lysed in lysis buffer A containing the following (in mM): 50 Tris, pH 7.4, 150 NaCl, 1 EDTA, 1% (v/v) Triton X-100, 1 Na_3VO_4 , 50 NaF, 1 PMSF, 100 nM calyculin A, and complete protease inhibitors (Roche Diagnostics). Lysates were precleared with protein A/G-agarose (Santa Cruz Biotechnology) and subjected to immunoprecipitation with myc-agarose or V5-agarose (Sigma). After washing three times with ice-cold lysis buffer, bound protein was eluted with SDS and immunoblotted with anti-Myc or anti-V5. For time course experiments, PC12 cells were transfected for 24 h using Lipofectamine 2000 (Invitrogen) and differentiated with 50 ng/ml NGF (Cedarlane Laboratories) for 24 h. Cells were treated with Nogo-P4 peptide for the indicated period of time at 37°C . Cells were then lysed, and proteins were immunoprecipitated as described above.

Assessment of protein phosphorylation. PC12 cells were differentiated in RPMI/1% BSA/50 ng/ml NGF for 24 h before treatment with recombinant proteins. P8 rat cerebellar neurons were prepared as previously described (Hsieh et al., 2006) and cultured in serum-free Sato's medium for 24 h before treatment. For L-CRMP4-V5-infected neurons, dissociated cerebellar neurons were cultured in the presence of virus for 4 h followed by serum starving for 20 h.

Cell lysates were prepared by washing cells twice with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer containing 20 mM

HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100 with a protease inhibitor mixture (Roche) and phosphatase inhibitors (1 mM Na₃VO₄, 50 mM NaF, 100 nM calyculin A). Membrane fractionation was performed as described previously (Alabed et al., 2006). Lysates and membrane fractions were analyzed by SDS-PAGE and immunoblotting with antibodies recognizing phospho- and total CRMP4 and/or GSK3 β . To quantify changes in protein phosphorylation, phospho-protein expression was assessed by densitometry and levels were normalized to the total level of the same protein in the lysate.

Neurite outgrowth assay. For outgrowth assays using pharmacologic inhibitors, SB216763, SB415286 (Sigma), 6-bromoindirubin-3'-acetoxime (EMD Biosciences), and CT99021 (generously provided by Dr. Rodolfo Marquez, School of Life Sciences, University of Dundee, Dundee, Scotland), were added to cultures after seeding. Dissociated embryonic day 13 (E13) chick and postnatal day 5 (P5) rat dorsal root ganglion (DRG) neurons were cultured in DRG medium (F-12 medium, 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 50 ng/ml NGF) in the presence of virus on poly-L-lysine- and laminin-coated substrates, fixed with 4% paraformaldehyde/20% sucrose in PBS, and double stained with anti- β III tubulin (Covance) and anti-V5 (Sigma) or anti-His (Qiagen) antibody. Dissociated cerebellar neurons were cultured in serum-free Sato's medium. Chick DRG neurite outgrowth lengths per cell were assessed using the NeuronJ plugin for ImageJ, a public domain JAVA image-processing program (<http://rsb.info.nih.gov/ij/>), as described previously (Fournier et al., 2003). Rat DRG and cerebellar neuron outgrowth was analyzed with the neurite outgrowth module of MetaXpress. For rat DRG cultures infected with lentiviruses, the neurons expressing the constructs were identified using the multiwavelength cell-scoring module of MetaXpress and the length of the neurites from only the expressing cells was measured using the NeuronJ plugin for ImageJ.

Densitometry and statistical analysis. Densitometry was performed using Adobe Photoshop and all quantifications were normalized for total protein loading. Statistical analysis was performed using GraphPad Prism and the specific tests used are indicated in the text or in the figure legends.

Results

L-CRMP4–RhoA binding is regulated by MAI-dependent dephosphorylation

As reported previously, the association between RhoA and L-CRMP4 is enhanced by stimulation with Nogo-P4 peptide, an inhibitory fragment of Nogo-A (GrandPré et al., 2000), in transfected PC12 cells (Fig. 1A) and cerebellar neurons (Alabed et al., 2007). The rapid enhancement of this protein–protein interaction led us to investigate the potential regulatory role of protein phosphorylation on this process. In 293T cells transfected with myc-wild-type (wt)-RhoA and L-CRMP4-V5, myc immunoprecipitates contain L-CRMP4-V5 (Fig. 1B). Treatment of transfected 293T cells with the serine/threonine phosphatase inhibitor calyculin A causes an upward mobility shift of L-CRMP4-V5 indicative of L-CRMP4 phosphorylation. While there is no apparent mobility shift in wt-RhoA following calyculin A treatment, this does not exclude the possibility that RhoA is also phosphorylated. Calyculin A treatment diminishes the L-CRMP4–wt-RhoA coimmunoprecipitation, demonstrating that phosphorylation of L-CRMP4 and/or RhoA disrupts their binding. When 293T cell lysates are treated with shrimp alkaline phosphatase (SAP) to stimulate protein dephosphorylation, the association between L-CRMP4-V5 and myc-wt-RhoA is enhanced, similar to the effect of Nogo treatment. We then asked whether dephosphorylation of RhoA and/or L-CRMP4 is capable of enhancing RhoA–L-CRMP4 binding. The binding properties of a RhoA mutant with the phospho-residue serine 188 mutated to alanine (nonphosphorylatable S188ARhoA) and of an L-CRMP4 triple alanine substitution mutant (L-CRMP4-AAA) for the three carboxy terminal phospho-residues targeted by GSK3 β (Thr622, Thr627, Ser631) were assessed. RhoAS188A binds more weakly than wt-RhoA to

Figure 4. Effects of myelin and GSK3 β inhibitors on neurite outgrowth inhibition are not additive. **A–D**, Quantification of DRG neurite outgrowth/cell from neurons plated on myelin and treated with vehicle control (DMSO) and increasing doses of SB216763 (**A**), SB415286 (**B**), 6-bromoindirubin-3'-acetoxime (**C**), or CT99021 (**D**). Values are normalized to outgrowth with no myelin. Determinations are from at least four experiments performed in duplicate. For DMSO-treated neurons, $p < 0.001$ for all doses of myelin compared with no myelin by Dunnett's post-tests following one-way RM ANOVA. Two-way ANOVA shows no significant differences in outgrowth on myelin between cultures treated with DMSO and those treated with any GSK inhibitor.

wt-L-CRMP4 (Fig. 1C). However, L-CRMP4-AAA binds more strongly than wt-L-CRMP4 to wt-RhoA (Fig. 1C). Together, these findings indicate that dephosphorylation of L-CRMP4 favors L-CRMP4–RhoA binding as does Nogo stimulation.

Figure 5. Pharmacologic inhibition of GSK3 β inhibits neurite outgrowth in an L-CRMP4-dependent manner. **A**, HSV-GFP- or HSV-C4RIP-infected dissociated DRG neurons were grown overnight in the presence of 5 μ M SB216763, fixed, and stained with anti- β -III tubulin antibody. Scale bar, 100 μ m. **B**, Quantification of DRG neurite outgrowth/cell from HSV-GFP- or HSV-C4RIP-infected neurons treated overnight with 5 μ M SB216763 or 300 nM 6-bromoindirubin-3'-acetoxime (6-bromo). Values are normalized to baseline outgrowth with DMSO vehicle control. Determinations are from three experiments performed in duplicate. ** p < 0.01 by Student's t test compared with DMSO.

To evaluate the effect of Nogo stimulation on L-CRMP4 phosphorylation, PC12 cells or L-CRMP4-V5-infected cerebellar neurons were treated with Nogo-P4 peptide and L-CRMP4 phosphorylation was assessed by Western blotting with a phospho-specific antibody recognizing pThr622 of L-CRMP4 [corresponding to Thr 509 of S-CRMP4 (Cole et al., 2004b)]. Nogo-P4 stimulation diminishes L-CRMP4 phosphorylation in both PC12 cells (Fig. 1D) and cerebellar neurons (Fig. 1E).

L-CRMP4 is dephosphorylated in a GSK3 β -dependent manner in response to MAIs

Dephosphorylation of L-CRMP4 suggests engagement of a CRMP4-directed phosphatase and/or inactivation of an L-CRMP4-directed kinase in response to MAIs. L-CRMP4 phosphorylation is sequentially regulated by GSK3 β on residues Ser631, Thr627, and Thr622 following a priming phosphorylation event that may be mediated by DYRK2 (Cole et al., 2004b). Inactivation of GSK3 β by phosphorylation on Ser9 leads to a rapid decrease in phospho content of its substrates. GSK3 β phosphorylation and inactivation are an important regulatory step in response to many factors including NGF and Wnt (Cohen and Frame, 2001; Zhou et al., 2004); therefore, we assessed the role of GSK3 β in Nogo signaling. We find that GSK3 β is phosphorylated in membrane fractions from Nogo-P4- or OMgp-stimulated PC12 cells (Fig. 2A) and cerebellar neurons (Fig. 2B). To examine the subcellular distribution of inactive GSK, we performed immunostaining and observed an increase in phospho-GSK in the central domain of growth cones undergoing collapse in response to both Nogo-P4 and OMgp (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). To test whether GSK3 β phosphorylation and inactivation lead to L-CRMP4 dephosphorylation, we overexpressed a constitutively active form of GSK3 β (GSK3 β S9A) and examined the effect of Nogo on L-CRMP4 phosphorylation. Overexpression of GSK3 β S9A blocks the Nogo-P4-dependent decrease in L-CRMP4 dephosphorylation, indicating that L-CRMP4 dephosphorylation is GSK3 β dependent (Fig. 2C).

Inactivation of GSK3 β inhibits neurite outgrowth in an L-CRMP4-dependent manner

Our data support a model whereby Nogo induces GSK3 β inactivation, resulting in L-CRMP4 dephosphorylation and enhanced

L-CRMP4–RhoA complex formation. If this is the case, then GSK3 β inactivation should diminish CRMP4 phosphorylation, increase L-CRMP4 association with RhoA, and inhibit neurite outgrowth. To test this, levels of L-CRMP4 phosphorylation were assessed in PC12 cells treated with GSK3 inhibitors. As expected for a known GSK3 β substrate (Cole et al., 2004b), phospho-L-CRMP4 levels were dramatically reduced in cells treated with the GSK3 inhibitors SB216763, SB415286, 6-bromoindirubin-3'-acetoxime, and CT99021 (Fig. 3A) [reductions in phospho-L-CRMP4 of 94 $\pm$ 4, 76 $\pm$ 9, 78 $\pm$ 15, and 90 $\pm$ 6% ($\pm$ SEM), respectively, normalized to total L-CRMP4 loading, n = 2]. Overnight stimulation of PC12 cells with SB216763 (Fig. 3B) or 6-bromoindirubin-3'-acetoxime (data not shown) increases the association of RhoA with L-CRMP4 but not S-CRMP4. The specific effect of the pharmacologic

GSK3 β inhibitors on the long isoform of CRMP4 mimics that of Nogo treatment (Alabed et al., 2007). Previous reports have shown that strong GSK3 inhibition reduces neurite outgrowth (Kim et al., 2006). Consistent with this, we find that treatment of rat cerebellar neurons (Fig. 3C,E) or DRG neurons (Fig. 3D,F) with several GSK3 inhibitors diminishes neurite outgrowth. Weak GSK3 inhibition has previously been shown to promote branching of immature hippocampal and DRG neurons (Kim et al., 2006) but to have no significant effect on branching in later stage neurons (Dill et al., 2008). Consistent with this, we observed no increase in the number of primary processes or branches with low doses of GSK inhibitors in postnatal rat DRGs (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). The decrease in branching observed at high doses of GSK inhibitors is likely due to the decrease in the overall growth of these neurons (Fig. 3F). In our hands, every GSK3 inhibitor tested inhibits neurite outgrowth except for SB415286 (Fig. 3F), raising the possibility that the known SB415286 effects on additional kinases (Bain et al., 2007) may neutralize the neurite outgrowth inhibitory effect of GSK3 β inhibition.

To test whether myelin and GSK3 inhibition has an additive effect on neurite outgrowth inhibition, we examined neurite outgrowth from rat DRG neurons with combined exposure to myelin and GSK3 inhibitors. SB216763, 6-bromoindirubin-3'-acetoxime, and CT99021 inhibit neurite outgrowth in a dose-dependent fashion (Fig. 3C–F) but do not enhance myelin-dependent inhibition (Fig. 4A–D) or inhibition by a purified GST-Nogo66 substrate (supplemental Fig. 3, available at www.jneurosci.org as supplemental material) further supporting our data that GSK3 is part of the myelin signaling pathway leading to neurite outgrowth inhibition.

To determine whether the reduced neurite outgrowth that accompanies GSK3 inhibition requires L-CRMP4, we assessed the effects of C4RIP (CRMP4–RhoA Interfering Peptide), an antagonist of L-CRMP4–RhoA binding (Alabed et al., 2007). Remarkably, the neurite outgrowth inhibitory effect induced by GSK3 inhibition is dramatically attenuated by infecting neurons with HSV-C4RIP (Fig. 5). This indicates that the neurite outgrowth inhibition induced by GSK3 inhibitors requires L-CRMP4.

Overexpression of GSK3 β attenuates myelin-dependent inhibition

A second prediction from our biochemical data is that overexpression of GSK3 β would overcome myelin inhibition by diminishing binding between RhoA and phosphorylated L-CRMP4. Overexpression of GSK3 β enhances the phosphorylation of both S-CRMP4 and L-CRMP4 (Fig. 6A) but specifically reduces binding between RhoA and the long isoform of CRMP4 (Fig. 6B). Further, DRG neurons infected with a GSK3 β S9A lentivirus (Fig. 6C) grow significantly better on an inhibitory myelin or GST-Nogo66 substrate (Fig. 6C,D), demonstrating that GSK3 β inactivation is necessary for myelin inhibition.

A phospho-dependent conformation of L-CRMP4 affects its binding properties

Regulation of GSK3 β affects phosphorylation of S-CRMP4 and L-CRMP4, yet only the L-CRMP4 isoform demonstrates GSK3 β - or Nogo-regulated RhoA binding (Alabed et al., 2007) (Figs. 3, 6). Further, RhoA binds more robustly to L-CRMP4 than S-CRMP4 (Alabed et al., 2007). We therefore considered the possibility that RhoA binds to L-CRMP4 on two distinct binding sites, one in the carboxy terminal region that is shared by S-CRMP4 and L-CRMP4 and one in the unique N-terminal portion of L-CRMP4. We assessed the ability of individual L-CRMP4 domains to interact with RhoA by coimmunoprecipitation from transfected 293T cells (Fig. 7A,B). We detected a binding site for RhoA in the common dihydropyrimidinase (DHP) region of CRMP4 but failed to detect binding between RhoA and the unique N-terminal domain of L-CRMP4, C4RIP (Fig. 7B).

We next considered the possibility that L-CRMP4 may exist in a phospho-dependent conformation that regulates binding to RhoA. To test this possibility, we examined binding between RhoA and the triple alanine substitution mutant of L-CRMP4 or S-CRMP4. L-CRMP4 AAA binds to RhoA more strongly than wt-L-CRMP4; however, binding between RhoA and S-CRMP4 AAA is indistinguishable from RhoA binding to wt-S-CRMP4 (Fig. 7C). This suggests that the phosphorylation status of the carboxy terminus of L-CRMP4 affects a RhoA binding site that is dependent on the unique N terminus of L-CRMP4. This could be ascribed to a folded conformation that stabilizes a single RhoA binding site or to a generation of a new conformation-dependent RhoA binding site in the unique N terminus of L-CRMP4 (Fig. 7D). Importantly, we find that in PC12 cells, stimulation with Nogo-P4 fails to further enhance binding between L-CRMP4 AAA and RhoA (Fig. 7E) demonstrating

Figure 6. GSK3 β overexpression attenuates myelin inhibition. **A**, Lysates from 293T cells transfected with GSK3 β and CRMP4–V5 constructs were subjected to immunoprecipitation with an anti-V5 antibody and probed with anti-phosphothreonine or anti-V5 antibody. **B**, Lysates from 293T cells transfected with myc-RhoA, GSK3 β , and CRMP4–V5 constructs were subjected to immunoprecipitation with an anti-myc antibody and probed with anti-V5, anti-myc, and anti-GSK3 β antibodies. **C**, HSV-GFP- or GSK3 β S9A-V5-infected dissociated DRGs were grown overnight on myelin, fixed, and stained with anti- β -III tubulin antibody (red) and Hoechst nuclear stain (blue). The green image reflects GFP signal from the control virus or anti-V5 staining detecting GSK3 β S9A overexpression. Skeletonized output images of HSV-GFP- or GSK3 β S9A-V5-positive neuronal cell bodies (green) and their associated neurites (red) used for quantification are shown in the bottom row. Scale bar, 50 μ m. **D**, Quantification of DRG neurite outgrowth/cell from HSV-GFP- or GSK3 β S9A-infected DRG neurons grown on myelin or GST-Nogo-66 substrates. Determinations are from three experiments. * p < 0.05 and *** p < 0.001 by Bonferroni's post-tests following two-way RM ANOVA. IP, Immunoprecipitation; L, long; S, short.

that Nogo-dependent dephosphorylation of L-CRMP4 is responsible for enhancing L-CRMP4–RhoA binding. Finally, we infected DRG neurons with recombinant HSV-L-CRMP4 AAA and assessed neurite outgrowth. We find that overexpression of L-CRMP4 AAA alone modestly but significantly inhibits neurite outgrowth ($15.1 \pm 3.48\%$ reduction $\pm$ SEM, $p = 0.049$ by two-tailed paired t test, $n = 3$) indicating that dephosphorylation of CRMP4 alone is sufficient to mediate some neurite outgrowth inhibition; however, dephosphoryla-

Figure 7. L-CRMP4 conformation is phospho dependent and regulates RhoA recruitment. **A**, Schematic representation of full-length and mutant CRMP4 constructs. **B**, **C**, HEK293T cells were transiently cotransfected with myc-RhoA and CRMP4-V5 constructs. Lysates were subjected to immunoprecipitation with anti-myc antibody and immunoblotted with anti-myc or anti-V5 antibodies. **D**, A model of how L-CRMP4 binding to RhoA may be regulated by a phosphorylation-dependent conformational change in L-CRMP4. Dephosphorylation of the carboxy terminal region of L-CRMP4 may enhance the folding interaction between the amino and carboxy termini of the protein and modify the protein conformation to favor binding to RhoA or may change the conformation of the N terminus of L-CRMP4, exposing a second RhoA binding site. **E**, PC12 cells were transiently cotransfected with myc-RhoA and CRMP4 AAA-V5. Cells were stimulated with Nogo-P4 peptide and lysates were subjected to immunoprecipitation with anti-myc antibody and immunoblotted with anti-myc or anti-V5 antibodies. IP, Immunoprecipitation; L, long; S, short.

tion of L-CRMP4 in combination with RhoA activation mediates more robust inhibitory effects.

Discussion

We find that MAIs induce phosphorylation and inactivation of GSK3 β , which regulate CRMP4 phosphorylation and binding to RhoA (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). GSK3 β inhibition mimics the effect of myelin on neurite outgrowth and this requires CRMP4. We also demonstrate that GSK3 β inactivation is necessary for MAI signaling because overexpression of active GSK3 β attenuates MAI-dependent neurite outgrowth inhibition. Together, these findings provide novel insights into the neuronal mechanism of action of GSK3 β and suggest additional molecular targets to promote neuronal repair following CNS injury.

MAI-dependent regulation of GSK3 β

The molecular links between cell surface MAI receptors and RhoA regulation of the cytoskeleton have not been fully elucidated. We have previously implicated an L-CRMP4–RhoA interaction in this pathway and have now demonstrated that this interaction is negatively regulated through L-CRMP4 phosphorylation by GSK3 β . The kinase responsible for GSK3 β phosphorylation in response to MAI stimulation remains to be determined. PKC is an intriguing candidate because it is activated by MAIs and blockade of PKC attenuates myelin-dependent inhibition (Sivasankaran et al., 2004). GSK3 β -mediated phosphorylation of the C terminus of L-CRMP4 is also dependent on priming phosphorylation at Ser635. Although both CDK5 and DYRK2 prime CRMP4 *in vitro*, the *in vivo* priming kinase is undetermined (Cole et al., 2006). Whether the priming kinases are directly regulated in response to MAI stimulation remains unknown.

Neurite outgrowth inhibition and GSK3 β inactivation

We provide the first example of a neurite outgrowth inhibitory ligand that stimulates phosphorylation and inactivation of GSK3 β . Our findings are consistent with several reports demonstrating that pharmacologic inhibition of GSK3 inhibits neurite outgrowth, but differ from those reporting promotion of axon branching with GSK3 inhibition (Owen and Gordon-Weeks, 2003; Shi et al., 2004; Kim et al., 2006). In an elegant study to examine why GSK3 inhibition can both enhance branching and inhibit outgrowth, Kim et al. (2006) have described a correlation between GSK3 activity toward primed or nonprimed substrates and neuronal phenotypes. Specifically, introduction of a GSK3 β mutant that selectively phosphorylates nonprimed substrates (GSK3 β R96A) results in reduced axon branching. Further, low

concentrations of GSK3 inhibitors that increase axon branching mainly diminish the phosphorylation of primed GSK3 substrates. GSK3 β regulates L-CRMP4 phosphorylation on priming-dependent and -independent residues (Y. Z. Alabed and A. E. Fournier, unpublished data) and these sites may be differentially affected by various concentrations of GSK3 β inhibitors. MAI-dependent inactivation of GSK3 β may impact additional priming-independent substrates, leading to neurite outgrowth inhibition; however, this is difficult to reconcile with the ability of C4RIP to reverse myelin- and SB216763-dependent outgrowth inhibition.

Spatial targeting of GSK3 β

MAI effects on GSK3 β phosphorylation were variable in whole-cell lysates but consistent in membrane fractions. This suggests that a specific pool of GSK3 β may be regulated in response to MAIs. A commonly accepted view is that GSK3 may be regulated

at discrete sites within the axon and growth cone to target specific substrates. The engagement of distinct spatially segregated pools of target substrates could explain how inhibitory MAIs and growth-promoting neurotrophins both phosphorylate and inactivate GSK3 β (Zhou et al., 2004).

GSK3 β as a therapeutic target

Based on findings indicating growth-promoting and neuroprotective effects of GSK3 β inhibition, clinical studies in spinal cord injury using stem cells and the GSK3 β inhibitor lithium are being pursued (Cyranoski, 2007). Our findings demonstrate that robust GSK3 β inhibition impedes axon extension, raising concerns regarding the efficacy of such a treatment. A recent study has demonstrated that lithium and SB415286 increase neurite outgrowth on myelin and CSPG substrates and stimulate growth of corticospinal tract fibers around the site of a spinal cord injury (Dill et al., 2008). We do not detect enhanced outgrowth of SB415286-treated DRG neurons on myelin substrates (Fig. 4) and this drug does not inhibit neurite outgrowth on a laminin substrate while other GSK3 β inhibitors do (Fig. 3F). The off-target effects of lithium and SB415286 (Bain et al., 2003, 2007) raise the possibility that the effects of these drugs on neurite outgrowth are not through GSK3 β . We are confident that the neurite outgrowth inhibitory effects described here are attributable to GSK3 β , since CT99021 is a very specific GSK3 β inhibitor. The only other identified substrate for CT99021 is CDK2-CyclinA, but this substrate is strongly targeted by SB415286, which does not inhibit neurite outgrowth (Bain et al., 2007). The *in vitro* inhibition of outgrowth does not, however, preclude the possibility that the doses used *in vivo* elicit an axonal sprouting phenotype.

L-CRMP4 and neurite outgrowth inhibition

Our data suggest that overexpression of GSK3 β inhibits formation of an L-CRMP4–RhoA complex and may be protective in the context of myelin inhibition. The partial nature of the rescue is likely explained by exposure of the neurons to the inhibitory substrate during the delay between lentiviral transduction and expression of GSK3 β S9A, but it is also possible that alternative parallel pathways are involved in myelin inhibition of outgrowth. The previously reported proapoptotic function of GSK3 β (Zhou et al., 2004) makes its overexpression an unlikely route for therapeutics, highlighting the importance of understanding its targets for promoting outgrowth on myelin. GSK3 regulates the phosphorylation and activation of many microtubule-associated proteins, including APC, CRMP2, CRMP4, MAP1b, MAP2, NF, Tau, and kinesin light chain, which would be affected in an overexpression paradigm (Cole et al., 2004a; Zhou and Snider, 2005). CRMP2 is phosphorylated in a ROCK (Rho-associated kinase)-dependent manner during Nogo or MAG signaling and may contribute to neurite outgrowth inhibition via dysregulated microtubule dynamics (Mimura et al., 2006). While CRMP4 is capable of binding to microtubules (Fukata et al., 2002), it is not a known ROCK substrate and its *in vivo* function likely differs from CRMP2 for several reasons. First, overexpression of S-CRMP4 in hippocampal neurons or SHSY5Y cells has a modest effect on axon outgrowth when compared with the robust elongation effect of S-CRMP2 (Cole et al., 2004a). Second, L-CRMP4 colocalizes with SV2-positive vesicles and binds to the endocytic adaptor protein intersectin, suggesting a role in endocytosis (Quinn et al., 2003). Third, L-CRMP4 overexpression promotes an actin-based phenotype in DRG neurons promoting the extension of filopodia and neurite branches (Alabed et al., 2007). This actin-based phe-

notype is consistent with the ability of CRMP4 to bundle F-actin (Rosslenbroich et al., 2005) and to bind to RhoA (Alabed et al., 2007). Overexpression of a splice variant of CRMP1 together with CRMP2 antagonizes Rho signaling (Leung et al., 2002) and overexpression of CRMP2 can switch RhoA- and Rac-1-dependent morphological changes in N1E-115 cells (Hall et al., 2001). However, CRMP4 siRNA treatment does not affect levels of phospho-LIMK or phospho-cofilin (M. Pool and A. E. Fournier, unpublished data), nor does it affect neurite outgrowth on laminin substrates (Alabed et al., 2007), indicating that CRMP4 does not directly regulate signaling downstream of RhoA. Further, the modest inhibitory effect of L-CRMP4 AAA expression on neurite outgrowth suggests that dephosphorylated CRMP4 and active RhoA cooperate to mediate neurite outgrowth inhibition, perhaps by regulating the localized formation of a signaling complex. How RhoA phosphorylation may be regulated to modulate MAI signaling and binding to CRMP4 is also an open question, since RhoAS188A binds more weakly to CRMP4 (Fig. 1C). Finally, the long isoforms of CRMPs can serve different functions from the short isoforms, perhaps even serving as short-isoform antagonists (Yuasa-Kawada et al., 2003). The ability of C4RIP to inhibit L-CRMP4–RhoA binding and to attenuate Nogo- and SB216763-dependent outgrowth inhibition suggests that the role of dephosphorylated L-CRMP4 in mediating neurite outgrowth inhibition may be linked to its ability to bind to RhoA and is suggestive of an actin-dependent phenotype.

CRMP4 structure

The crystal structures of murine CRMP1 (Deo et al., 2004) and human CRMP2 (Stenmark et al., 2007) have been solved, but the structures do not include the N-terminal extension of the long isoforms or the carboxy terminal region containing the GSK3 β target residues. The lack of structural data for the carboxy terminus is a function of proteolytic susceptibility of this region (Deo et al., 2004). Our findings suggest that full-length L-CRMP isoforms may undergo a fold resulting in a phospho-dependent conformation that regulates additional protein–protein interactions. For simplicity, our model is presented with a single CRMP molecule; however, it is known that CRMPs form heterotetramers (Wang and Strittmatter, 1997). It is possible that intermolecular binding of RhoA to the N terminus of one L-CRMP4 molecule and the DHP region of a second molecule may occur. Further, it is possible that phosphorylation of L-CRMP4 in the carboxy terminus may affect the oligomerization properties of L-CRMP4 and that RhoA may favor binding to L-CRMP4 monomers or oligomers. Additional interactions conferred by phospho-dependent conformational changes in L-CRMP4 could play a key role in CRMP function by regulating binding affinities to upstream regulators such as GSK3 and/or to potential effectors such as RhoA. A better understanding of the impact of phosphorylation on L-CRMP4 binding interactions will likely yield additional insights into L-CRMP4 function and into intracellular mechanisms regulating neurite outgrowth inhibition.

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