Spy1 Protein Mediates Phosphorylation and Degradation of SCG10 Protein in Axonal Degeneration*

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Background: SCG10 is a novel axonal maintenance factor, and rapid SCG10 loss after injury requires JNK activity.

Results: Spy1 mediated SCG10 phosphorylation and degradation, partly in a JNK-dependent manner, and regulated injury-induced axonal degeneration.

Conclusion: Spy1 is an important regulator of SCG10 and axon degeneration.

Significance: Spy1 may be a novel axo-protective therapeutic target for axon loss.

Axon loss is a destructive consequence of a wide range of neurological diseases without a clearly defined mechanism. Recent data demonstrate that SCG10 is a novel axonal maintenance factor and that rapid SCG10 loss after injury requires JNK activity; how JNK acts to promote SCG10 loss is, however, unclear. Here we show that JNK phosphorylates the Speedy/RINGO family protein Spy1 in a JNK-dependent manner. SCG10 protein is rapidly degraded upon down-regulation of Spy1 in both cultured sympathetic neurons and mouse sciatic nerves. Our data demonstrate that Spy1 directly binds to SCG10 and modulates its phosphorylation and stability, delaying injury-induced axonal degeneration partly in a JNK-dependent manner. Therefore, Spy1 may be a novel axo-protective therapeutic target for axon loss.

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1 The abbreviations used are: CDK, cyclin-dependent kinase; DRG, dorsal root ganglion; Z, benzylxoycarbonyl.

2 The members have no homology to cyclin proteins (15, 16). Spy1 enhances cell proliferation, promotes the G1/S transition (17), and inhibits apoptosis in response to UV irradiation (18). Spy1 levels are up-regulated in various kinds of tumor tissues (19, 20); over-expression of Spy1 accelerates tumorigenesis (21), and knockdown of Spy1 can reduce breast cancer cell growth in vivo. In addition to its role in cell cycle regulation, Spy1 is implicated in mammary development (21). Our previous data showed that Spy1 was ubiquitously expressed in the lumbar spinal cord, including neurons and glial cells (22), and participated in the pathological process in response to sciatic nerve injury (23).

To tackle the possible role of Spy1 in the central nervous system, we performed a yeast two-hybrid screening using a human fetal brain complementary DNA library. Our data demonstrate that Spy1 directly binds to SCG10 and modulates its phosphorylation and stability, delaying injury-induced axonal degeneration partly in a JNK-dependent manner. Therefore,
Spy1 is a novel regulator of axon degeneration and as such positioning itself as a potential therapeutic target.

Materials and Methods

Expression Plasmid Preparation—SCG10 was isolated by PCR from a human fetal brain complementary DNA library and inserted into pcDNA3.1-Myc vector. The identical method was used for GST-SCG10, His-Spy1, HA-Spy1, and their mutants. SCG10 phosphorylation site mutants S50A, S62A, S73A, and S97A were created from wild-type SCG10. All constructs were verified by sequencing. GFP-tagged lentivirus sh-Spy1 was purchased from GeneChem (Shanghai, China).

Reagents and Antibodies—The following reagents and antibodies were used in this study: rabbit polyclonal anti-Spy1 (Abcam, ab86568), mouse monoclonal anti-SCG10 (Santa Cruz Biotechnology, sc-135620), mouse monoclonal anti-β3-tubulin (Millipore, MAB1637), rabbit polyclonal anti-GAPDH (Abcam, ab9484), mouse monoclonal anti-phosphoserine (Millipore, 05-1000), anti-His, -Myc, and -HA (GenScript Corp., Nanjing, China), JNK inhibitor VII, TAT-TI-JIP153-163 (Calbiochem, 420134), and Cdk2 inhibitor II (Santa Cruz Biotechnology, sc-221409) (24). Z-Leu-Leu-Leu-al (MG132) at 20 μM and SP600125 at 15 μM were purchased from Sigma-Aldrich.

Cell Culture and Transfection—HEK-293 cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco). Medium was supplemented with 10% fetal bovine serum and cultured at 37 °C in a humidified incubator at 5% CO2. Cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Dorsal root ganglions (DRGs) from embryonic day 17.5–18.5 rat (Charles River) were cultured in poly-d-lysine-(Sigma) and laminin- (Sigma) coated 24-well plates (Corning). Collected DRGs were trypsinized for 20 min at 37 °C, triturated in medium, and plated as a spot at a density of approximately two DRGs per well in 2 μl of medium. The plates were incubated for 20 min at 37 °C to attach cells to the plastic followed by the addition of Neurobasal medium (Invitrogen) supplemented with 2% (v/v) B27 (Invitrogen), 25 ng/ml nerve growth factor (Promega), and 1 μM 5-fluoro-2’-deoxyuridine and 1 μM uridine (Sigma) to block cell division of nonneuronal cells. Cultures were maintained for 8–9 days before axotomy or drug treatment. We axotomized cultures of DRG neurons with a FIGURE 1. SCG10 is a binding partner of Spy1. A, Spy1 interacts with SCG10 in vitro. The GST pulldown assay was performed by incubating in vitro-translated His-tagged Spy1 with purified GST-SCG10 immobilized on glutathione-Sepharose beads. GST-SCG10 but not GST was shown to pull down Spy1. Arrows indicate GST and GST-SCG10 bands, and Coomassie Blue staining indicates the loading amounts. WB, Western blot. B, HA-tagged Spy and Myc-tagged SCG10 were co-expressed in HEK-293 cells for immunoprecipitation assays. C and D, the stathmin-like domain (SLD) of SCG10 was required for binding to Spy1. E and F, the Speedy/RINGO domain (S/R) of Spy1 was required for binding to SCG10.

### Table 1

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FIGURE 2. Spy1 expression inversely correlates with SCG10 during the early stage of axonal injury. A, immunoblot analysis of endogenous SCG10 and Spy1 in cultured DRG axons with or without axotomy. Immunoblot against neuron-specific β3-tubulin confirms comparable amounts of protein loaded. B, the ratio of Spy1 or SCG10 protein relative to β3-tubulin in the uncut and cut axons by densitometry. The data are mean ± S.E. (n = 3, *, #, p < 0.01, significantly different from the uncut group). C, immunoblot analysis of endogenous SCG10 and Spy1 in sciatic nerve sections with or without axotomy. Immunoblot against neuron-specific β3-tubulin confirms comparable amounts of protein loaded. D, the ratio of Spy1 or SCG10 protein relative to β3-tubulin in the uncut and cut axons by densitometry. The data are mean ± S.E. (n = 3, *, #, p < 0.01, significantly different from the uncut group).

Results

SCG10 Is a Binding Partner of Spy1—To identify novel Spy1-binding partners, we performed yeast two-hybrid screening using a human fetal brain complementary DNA library with Spy1 as bait and identified SCG10 as a putative binding protein (Table 1). To verify the potential interaction obtained with the yeast two-hybrid screening, we performed an in vitro GST binding assay and showed that in vitro-translated Spy1 interacted with GST-SCG10 but not with GST alone (Fig. 1A). Immunoprecipitation assays were then undertaken to test the intracellular interaction between Spy1 and SCG10. HA-tagged Spy and Myc-tagged SCG10 were co-expressed in HEK-293 cells, and they could pull down each other (Fig. 1B). Importantly, immunoprecipitation of endogenous SCG10 from DRG neurons using a SCG10-specific antibody also pulled down Spy1 protein (Fig. 1C).

To determine the protein domains required for the interaction between Spy1 and SCG10, Myc-tagged SCG10 mutants and HA-tagged Spy mutants were co-expressed in HEK-293 cells for immunoprecipitation assays. The data suggested that the stathmin-like domain of SCG10 was required for binding to...
Spy1 (Fig. 1, D and E) and that the Speedy/RINGO domain of Spy1 was required for binding to SCG10 (Fig. 1, F and G).

Spy1 Expression Inversely Correlates with SCG10 during the Early Stage of Axonal Injury—Previous data showed that SCG10 was a novel axonal maintenance factor (14). Given that our recent data showed that Spy1 participated in the pathological process response to sciatic nerve injury (23), we hypothesized that Spy1 might modulate SCG10 activity and participate in axon regeneration/degeneration. We first examined the expression of both proteins in an in vitro model of axonal injury.

In DRG neurons, SCG10 expression was lost rapidly from injured distal axons during the first 3 h after axotomy (Fig. 2, A and B), which was in agreement with previous studies (14, 25). Interestingly, Spy1 level was increased in injured distal axons during the same period (Fig. 2, A and B). To further confirm these results, we performed an in vivo model of axonal injury of sciatic nerve. Indeed, SCG10 level decreased rapidly in injured distal sciatic nerves at the early stage after transection (Fig. 2, C and D), whereas Spy1 level was increased in parallel (Fig. 2, C and D). Therefore, our data suggested that Spy1 expression was inversely correlated with SCG10 during the early stage of axonal injury.

Spy1 Mediates SCG10 Phosphorylation and Degradation Partly in a JNK-dependent Manner—As Spy1 levels showed an inverse correlation with SCG10 after axonal injury, we speculated whether Spy1 could regulate SCG10 level. Forced expression of ectopic Spy1 resulted in a decrease of SCG10 in a dose-dependent manner (Fig. 3, A and B). In contrast, treatment with
the proteasome inhibitor MG132 blocked the loss of SCG10 (Fig. 3, C and D), indicating that Spy1 might impact SCG10 stability rather than its de novo synthesis.

Previous data showed that JNK phosphorylation of SCG10 targeted SCG10 for degradation (14). We then set out to determine whether Spy1 might affect SCG10 phosphorylation in a JNK-dependent manner. Consistent with the observation that Spy1 promoted SCG10 degradation, it also enhanced SCG10 phosphorylation (Fig. 3E). To further identify the specific phosphorylation site(s) of SCG10 by Spy1, four candidate phosphor-
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Axon loss is a destructive consequence of a wide range of neurological diseases. Therapies targeting the axon loss process itself are notably absent. Clarifying the mechanism of axon loss may help develop such therapies. Regulated protein degradation promotes the degeneration of injured axons (3), potentially via the degradation of vulnerable axonal maintenance factors. Recent data have demonstrated that SCG10, being similar to NAMAT2 (29), is a novel axonal maintenance factor (14); rapid SCG10 loss after injury requires JNK activity. However, pharmacologically inhibiting JNK activity further slows the degradation of the mutant SCG10 in which two JNK phosphorylation sites (serines 62 and 73) were replaced by alanines, demonstrating that JNK may promote the degradation of SCG10 through other mechanisms, in addition to the phosphorylation of SCG10 (14). Therefore, understanding the mechanisms regulating SCG10 stability may be helpful in finding ways to attenuate axonal destruction, for example, by inhibiting specific degradation machinery targeting SCG10.

Spy1, as a member of the MAPK family, is implicated in mammalian development, cell growth, and cell cycle regulation. In addition, our previous data showed that Spy1 expression could be detected in a human fetal brain complementary DNA library, including neurons and glial cells (22), and that Spy1 may participate in the pathological mechanisms of neural repair and injury (23). To identify the possible function of Spy1 in the nervous system, we performed yeast two-hybrid studies and found that SCG10 was a binding partner of Spy1. Deletion and point-mutation analysis showed that Spy1 affects axonal injury in a SCG10-dependent manner. Our work further showed Spy1-mediated SCG10 phosphorylation and injury-induced axonal degeneration, which confirmed our hypothesis.

Previous data showed that JNK phosphorylation of SCG10 targeted SCG10 for degradation (14), and our data showed that Spy1 increased JNK kinase activity. These data are in line with previous reports that Spy1 was an activator of CDKs (15, 16), suggesting that Spy1 may exert its pathobiological functions via modulating kinase activities. On the other hand, recent data showed that dual leucine zipper kinase (DLK) regulated stress-induced JNK activity in axons via interaction of dual leucine zipper kinase with the scaffolding protein JIP3 (30, 31). Whether Spy1 has an identical role like dual leucine zipper kinase needs further studies. Furthermore, treatment with JNK kinase inhibitor SP600125 only partially abolished Spy1-mediated phosphorylation of SCG10, suggesting that other mechanisms may take part in the Spy1-mediated phosphorylation of SCG10.
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SCG10. Clearly, more work is warranted to address this lingering issue.

Most studies show that Spy1 is a cell cycle regulator and that it promotes tumorigenesis (15–21). Here, we demonstrate a cell cycle-independent role for Spy1. Two pieces of evidence may support our data. First, SCG10 binds to the Speedy/RINGO domain of Spy1, but not the CDK-binding domain on the N terminus. Second, the functional interplay between Spy1 and SCG10 is staged in the axons instead of the nucleus where cell cycle regulation takes place. Further investigation is needed to clarify whether SCG10 might modulate Spy1 reciprocally in the nucleus.

In conclusion, our data indicate that Spy1 is an important regulator of axonal maintenance factor SCG10. As such, understanding its regulatory mechanisms may help to find new methods for attenuating axonal degeneration. Spy1 may be a novel axo-protective therapeutic target for axon loss.

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References
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