Molecular and Cellular Neuroscience 47 (2011) 265–273

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

The adaptor protein Nck2 mediates Slit1-induced changes in cortical neuron morphology

Jennifer E. Round *, Hui Sun

Department of Molecular, Cellular and Developmental Biology, Yale University, 266 Whitney Avenue, New Haven, Connecticut 06520, United States

ARTICLE INFO

Article history:
Received 16 December 2010
Revised 18 April 2011
Accepted 28 April 2011
Available online 6 May 2011

Keywords:
Slit
Robo
Nck
SH2/SH3 adaptor
Cortical neuron
Dendrite

ABSTRACT

Slits are multifunctional guidance cues, capable of triggering neurite repulsion, extension, or branching, depending on cell type and developmental context. While the Robo family of Slit receptors is a well-established mediator of axon repulsion, a role for Robos in Slit-mediated neurite growth and branching is not well defined, and the signaling molecules that link Robo to the cytoskeletal changes that drive neurite outgrowth are not well characterized in vertebrates. We show that Slit stimulates cortical dendrite branching, and we report that Slit also triggers a robust increase in the length of cortical axons in vitro. Moreover, neurons derived from *Robo1; Robo2* deficient mice do not display an increase in neurite length, indicating that endogenous Robos mediate Slit's growth-promoting effects on both axons and dendrites. We also demonstrate that the SH2/SH3 adaptor proteins Nck1 and Nck2 bind to Robo via an atypical SH3-mediated mechanism. Furthermore, we show that only Nck2 is required for the Slit-induced changes in cortical neuron morphology in vitro. These findings indicate a specific role for Nck2 in linking Robo activation to the cytoskeleton rearrangements that shape cortical neuron morphology.

Introduction

During nervous system development, the extension of axons and dendrites is influenced by extracellular cues, which are essential for axon guidance, synaptogenesis, and the construction of precise neuronal circuits in the developing nervous system. Many of the extracellular cues that direct axon pathfinding also influence the size and shape of dendrite arbors (Moreno-Flores et al., 2002; Suli et al., 2006; Whitford et al., 2002). Significant progress has been made in identifying the membrane receptors and intracellular signaling molecules that mediate the effect of guidance cues on axon pathfinding (reviewed in Huber et al., 2003; Round and Stein, 2007; Ypsilanti et al., 2010). However, whether these cues utilize the same receptors and signaling pathways to shape dendrite morphology is not known.

Slits are multifunctional guidance cues, capable of triggering neurite extension, branching, and repulsion, depending on the cell type and developmental context (Brose et al., 1999; Wang et al., 1999; Whitford et al., 2002). The three vertebrate Slits, Slit1, Slit2, and Slit3, cooperate to repel commissural axons of the developing spinal cord (Long et al., 2004; Zou et al., 2000) and guide the trajectory of major axon tracts in the developing forebrain, olfactory bulb, and optic system (Bagri et al., 2002; Nguyen-Ba-Charvet et al., 2002; Plump et al., 2002). Slits are also capable of stimulating neurite outgrowth, as evidenced by their ability to stimulate axon extension in mouse sensory and *Xenopus* spinal neurons (Stein and Tessier-Lavigne, 2001; Wang et al., 1999) and their ability to stimulate dendrite extension and branching in cortical neurons (Whitford et al., 2002).

It is well established that the Robo family of membrane receptors mediates Slit-induced axon repulsion in vertebrates (Andrews et al., 2006; Fouquet et al., 2007; Long et al., 2004; Lopez-Bendito et al., 2007). However, a role for Robos in the growth-stimulating effect of Slit on axons and dendrites is not well characterized. *Robo1*$^{-/-}$; *Robo2*$^{-/-}$ mice exhibit reduced trigeminal sensory axon branching, suggesting that Robos mediate Slit-induced axon branching *in vivo* (Ma and Tessier-Lavigne, 2007). Chimeric receptor studies utilizing a Met receptor ectodomain indicate that the intracellular domain of Robo is capable of mediating changes in cortical dendrite morphology (Whitford et al., 2002), but the role of endogenous Robos in cortical dendrite growth and branching has not been tested directly. All three Slits and two Robo family members, Robo1 and Robo2, exhibit overlapping expression patterns in the developing cortex (Andrews et al., 2008; Marillat et al., 2002; Whitford et al., 2002), highlighting the possibility that Slit/Robo interactions shape dendrite morphology *in vivo*.

Much of what is known about Robo signaling is derived from genetic and biochemical studies in *Drosophila melanogaster*. A number of cytoskeletal regulators bind to Robo's intracellular domain in the fly, including Enabled, Abl, and Dreadlocks (Dock), where they cooperate with Robo to guide commissural axons at the ventral

* Corresponding author at: Department of Biology, Box 7118, Davidson College, Davidson, North, Carolina 28036, United States. Fax: +1 704 894 2512.
E-mail address: jeround@davidson.edu (J.E. Round).

1044-7431/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.mcn.2011.04.009

midline (Bashaw et al., 2000; Fan et al., 2003; Wills et al., 2002). While the mammalian orthologs of Enabled and Abl have been confirmed to interact with Robo (Park et al., 2003; Rhee et al., 2007), a role for Nck, the mammalian ortholog of Dock, in Robo signaling has not been established.

The two vertebrate Nck genes, Nck1 and Nck2, are non-enzymatic adaptor proteins composed of three Src homology (SH) 3 domains and a C′-terminal SH2 domain. Both Nck1 and Nck2 are expressed in the developing nervous system, including the embryonic and early postnatal forebrain (Fawcett et al., 2007; Guan et al., 2007). Nck1 has been shown to bind to the Netrin receptor DCC to mediate Netrin-induced neurite outgrowth (Li et al., 2002; Shekarabi et al., 2005), while Nck2 binds to the guidance receptor EphB1, coupling receptor activation to the activation of c-jun N′-terminal kinase (JNK) (Stein et al., 1998). Whether the Nck adaptors bind to Robo and mediate Slit's effects on neuron outgrowth in vertebrates is not known.

We employed loss-of-function genetic approaches and soluble peptide inhibition in cultured cortical neurons to explore the role of endogenous Robos and Ncks in the neuronal responses to Slit1. We show that, in addition to influencing dendrite morphology, Slit1 stimulates elongation of cortical axons, and that endogenous Robos mediate Slit's effects on axons and dendrites. We show that both Nck1 and Nck2 bind to Robos via an atypical SH3 domain-dependent mechanism, yet only Nck2 mediates Slit-induced cortical neurite outgrowth. These results highlight the central role played by the Nck2 adaptor in mediating Slit's diverse effects on cortical neuron morphology.

Results

Slit signals via endogenous Robos to stimulate the growth of cortical axons and dendrites

Previous in vitro experiments in mouse and *Xenopus* spinal neurons demonstrate that Slit increases the length of axons in addition to its repellent effect (Wang et al., 1999; Stein and Tessier-Lavigne, 2001), and experiments in cortical neurons indicate that Slit stimulates the growth and branching of cortical dendrites (Whitford et al., 2002). However, the ability of Slit1 to stimulate the growth of cortical axons has not been reported. To determine if Slit influences cortical axon outgrowth, we established primary cultures of dissociated cortical neurons derived from mouse E15.5 embryos. We then stimulated the neurons with control or Slit1-enriched medium at 1 day *in vitro* (DIV) and subjected the cultures to a low-efficiency calcium phosphate transfection of eYFP at 2 DIV to allow for the morphological analysis of individual cells by fluorescent microscopy at 3 DIV. In agreement with Whitford et al. (2002), we found that Slit1-enriched medium induces a significant increase in dendrite outgrowth and branching ($P<0.0001$, Fig. 1B, D). In addition, we observed that Slit1 triggered a robust increase in cortical axon length ($p<0.0001$, Fig. 1A, D). The addition of soluble Robo1-Fc blocked the Slit1-induced increase in axon length, dendrite length, and dendrite branching, whereas addition of Fc alone or DCC-Fc did not ($p<0.0001$, Fig. 1D), confirming that the effect of enriched medium on cortical axons and dendrites is specific to Slit1.

The finding that Slit1 stimulates cortical axon growth, combined with the previous identification of Slit1 as a factor that promotes dendrite growth and branching, prompted us to examine if the Robo family of Slit receptors mediates the effects of Slit1. We generated polyclonal antibodies against both Robo1 and Robo2 (Supp. Fig. 1), and used these antibodies to confirm that both Robo1 and Robo2 are expressed by cortical neurons in culture (Supp. Fig. 1D). We then established primary cultures of cortical neurons derived from *Robo1; Robo2* double mutant mice, which have been described previously (Long et al., 2004). We found that the addition of Slit1-enriched medium failed to stimulate axon growth, dendrite growth, or branching in the absence of both receptors (Fig. 1C, 1E). In contrast, Slit1 induced a robust increase in axon length, dendrite length and branching in neurons derived from age-matched wild-type embryos (Fig. 1E). This finding indicates that endogenous Robos mediate all three effects of Slit1 on cortical neuron morphology.

The adaptor protein Nck binds to Robo1 and Robo2

The immediate downstream signaling molecules that mediate Slit's diverse effects on cortical neuron morphology are not well characterized. The intracellular domains of mouse Robo1 and Robo2 contain a number of consensus binding motifs, including two putative tyrosine phosphorylation sites, termed CC0 and CC1, and two EVH1 binding sites, designated CC2 and CC3 (Fig. 3B). Robos also contain a number of PxxP motifs between CC2 and CC3, which serve as potential binding sites for proteins that contain SH3 domains.

Previous biochemical studies in *Drosophila* indicate that the SH2/SH3 adaptor protein Dock binds directly to the intracellular domain of Robo (Fan et al., 2003). The two mammalian orthologs of Dock, Nck1 and Nck2, share 68% amino acid identity and are widely expressed in the developing nervous system (Bladt et al., 2003). In order to determine if Nck1 and/or Nck2 bind to Robo in vertebrates, we performed a GST pull-down assay. We found that full-length radiolabeled Robo1 and Robo2 bind to both GST-Nck1 and GST-Nck2, while versions of Robo1 and Robo2 that lack the intracellular domain do not bind Nck (Fig. 2A). These results indicate that both Nck1 and Nck2 are capable of binding to the intracellular domain of Robo1 or Robo2 in vitro. To quantify the affinity of the interaction, we performed an equilibrium binding assay to determine the dissociation constant for Nck2 and Robo1 (Fig. 2B). By subtracting non-specific binding of Robo1 to GST from that of Robo1 to GST-Nck2, we estimate the dissociation constant to be approximately 30 nM.

Nck binds via its SH3 domains to the PxxP-rich region between CC2 and CC3 of Robo

Nck has been shown to bind to guidance cue receptors via both SH2-mediated and SH3-mediated mechanisms (Li et al., 2002; Stein et al., 1998). Therefore, we performed mapping experiments to determine if the binding of Nck to Robo1 and Robo2 is SH2-mediated, SH3-mediated or a combination of the two. We generated recombinant versions of GST-Nck2 that lacked either the C′-terminal SH2 domain or all three of the N′-terminal SH3 domains (Fig. 3A). We found that deletion of the SH2 domain had no effect on the ability of GST-Nck to bind to radiolabeled Robo1 or Robo2 (Fig. 3C, top). In contrast, the deletion of all three SH3 domains completely abolished the interaction of Nck with Robo1 and Robo2, indicating that the SH3 domains of Nck are necessary and sufficient for binding to the Robo family of Slit receptors *in vitro*.

In order to determine which SH3 domain(s) of Nck participate in the interaction with Robo, we generated a single point mutation in each of the three SH3 domains, which renders the SH3 domain unable to bind to its target PxxP motif (Tanaka et al., 1995). We found that a single point mutation in any one of the SH3 domains failed to disrupt the binding of Nck to Robo *in vitro* (Fig. 3C, bottom). We also found that mutating any two SH3 domains of Nck in combination reduced but failed to disrupt the interaction completely. Only when we introduced a point mutation in all three of the SH3 domains did we observe a severe reduction in the ability of Nck to bind Robo. These data suggest that multiple SH3 domains contribute to the association between Nck and Robo, or that the three SH3 domains function interchangeably and redundantly *in vitro*.

In order to identify the region of the Robo intracellular domain that is bound by Nck, we generated recombinant versions of Robo1 that are truncated at various points in the intracellular domain (Fig. 3B). A truncation that removes the CC3 domain of Robo1 and the remainder of the C′-terminus (T1) did not disrupt the interaction between Robo1 and