Control of axon elongation via an SDF-1α/Rho/mDia pathway in cultured cerebellar granule neurons

Yoshiki Arakawa, Haruhiko Bito, Tomoyuki Furuyashiki, Takahiro Tsuji, Sayaka Takemoto-Kimura, Kazuhiro Kimura, Kazuhiro Nozaki, Nobuo Hashimoto, and Shuh Narumiya

1Department of Pharmacology and 2Department of Neurosurgery, Kyoto University Faculty of Medicine, and 3PRESTO-Japan Science and Technology Corporation, Sakyo-ku, Kyoto 606-8315, Japan
4Department of Neurochemistry, University of Tokyo Graduate School of Medicine, Bunkyo-ku, Tokyo 113-0033, Japan

Rhō-GTPase has been implicated in axon outgrowth. However, not all of the critical steps controlled by Rhō have been well characterized. Using cultured cerebellar granule neurons, we show here that stromal cell–derived factor (SDF)-1α, a neural chemokine, is a physiological ligand that can turn on two distinct Rhō-dependent pathways with opposite consequences. A low concentration of the ligand stimulated a Rhō-dependent pathway that mediated facilitation of axon elongation. In contrast, Rhō/ROCK activation achieved by a higher concentration of SDF-1α caused repression of axon formation and induced no more increase in axon length. However, even at this higher concentration a Rhō-dependent axon elongating activity could be recovered upon removal of ROCK activity using Y-27632. SDF-1α–induced axon elongation activity under ROCK inhibition was replicated by the dominant-active form of the mammalian homologue of the Drosophila gene Diaphanous (mDia)1 and counteracted by its dominant-negative form. Furthermore, RNAi knockdown of mDia1 abolished SDF-1α–induced axon elongation. Together, our results support a critical role for an SDF-1α/Rhō/mDia1 pathway in mediating axon elongation.

Introduction

It has been widely accepted that rearrangement of actin and microtubule cytoskeleton lies at the heart of neuronal morphogenesis (Tanaka and Sabry, 1995). Drastic changes in neuronal shape occur immediately after the exit of neuronal cells from mitotic cycles, during embryonic and postnatal development. Process formation/extension and cell body migration must be spatially and temporally orchestrated in order to achieve patterned formation of neuronal cell layers and appropriate generation of synaptic circuits (Goodman and Shatz, 1995; Tessier-Lavigne and Goodman, 1996; Van Vactor and Flanagan, 1999).

A dynamic morphological alteration is initiated during the acquisition of neuronal polarity and must continue until the completion of synaptogenesis. Recent findings indicate a critical role for the antagonism between Rac– and Rhō–GTPases in these events (Narumiya et al., 1997; Hall, 1998; Luo, 2000; Dickson, 2001). A large body of work has now established that several soluble or transmembranous guidance molecule systems can exhibit either chemorepulsion or chemoattraction toward axonal growth cones, at least in part, via coupling to either Rhō or Rac, respectively. Such systems include EphrinA (Shamah et al., 2001), EphB reverse signaling (Lu et al., 2001), Semaphorin 4D/PlexinB (Driessens et al., 2001; Swierzcz et al., 2002), Semaphorin 3A/PlexinA-Neuropilin (Jin and Strittmatter, 1997; Liu and Strittmatter, 2001; Jurney et al., 2002), Netrin/DCC (Li et al., 2002), Slit/Robo (Wong et al., 2001), and neurotrophins/p75/trk (Yamashita et al., 1999; Huang and Reichardt, 2001; Ozdinler and Erzurumlu, 2001; Nusser et al., 2002). Thus, antagonistic interactions and hierarchical cascades between distinct small GTPases had been considered as most likely ways by which distinct gradient cues could be decoded into reliable maps of afferents projecting into one specific area of the central nervous system (CNS). ***

However, to date, a clear understanding regarding what specific effectors of the small GTPases contribute to each of these opposing signaling events is still missing. Furthermore, whether Rhō always antagonized with Rac remains controversial (Sebek et al., 1999; Bashaw et al., 2001).

***Abbreviations used in this paper: CRIB, Cdc42/Rac interactive binding; CNS, central nervous system; DA, dominant-active; DN, dominant-negative; EGFP, enhanced GFP; EGF, extracellular ligand; IGL, internal granule cell layer; mDia, mammalian homologue of the Drosophila gene Diaphanous; P, postnatal day; PAK, p21-associated kinase; RBD, Rhō-binding domain; RNAi, RNA interference; siRNA, short interfering double stranded RNA oligomer; SDF, stromal cell–derived factor.***
The rodent cerebellar granule cells represent a highly advantageous resource to study in detail the molecular sequence of events controlling axonogenesis, neuronal migration, dendritogenesis, and synaptogenesis. Indeed, each of these steps is critical for neuronal maturation and circuit formation occurs in an organized and sequential fashion in the cerebellum during the postnatal 3 wk after birth (Altman and Bayer, 1996; Hatten, 1999). Many of these steps involve dramatic changes in neuronal morphology, yet previous studies indicated that many of their features could be recapitulated, at least in part, in primary culture (Powell et al., 1997; Bito et al., 2000; Yamasaki et al., 2001). Thus, we considered the possibility that a factor supplied by the pia mater may be involved in the signaling pathway controlling axon initiation and elongation. One candidate factor we tested was stromal cell–derived factor (SDF)-1α, a chemokine heavily expressed in the pia mater and chemotaxant for migration of cerebellar granule cells (Lu et al., 2001; Klein et al., 2001; Tham et al., 2001; Zhu et al., 2002). Our interest was prompted by the striking phenotype of the knockout mice lacking either SDF-1α (Ma et al., 1998) or its cognate receptor CXCR4 (Ma et al., 1998; Zou et al., 1998). In both lines of mice, the formation of the cerebellar granule cell layers was abolished. This suggested that early events in cerebellar granule cell morphogenesis, such as axon initiation, axon elongation, or migration of the cell body, might be severely perturbed in the absence of SDF-1α signaling.

However, whether SDF-1α could stimulate axon formation via direct control of actin cytoskeletal signaling mechanisms in cerebellar granule cells has not been demonstrated. Here we show that SDF-1α activates Rho but not Rac small GTPase in cerebellar granule cells. A low concentration (100 ng/ml) of SDF-1α induced significant amount of axon outgrowth in a C3 exoenzyme–sensitive fashion. In contrast, at higher concentration SDF-1α rather repressed axon formation in a Rho-dependent manner. However, Y-27632 treatment was sufficient to uncover an axon elongating activity even under high dose of SDF-1α. Thus, SDF-1α stimulated two distinct opposing Rho effectors: a novel Rho effector, which promoted axon elongation, and ROCK to inhibit axonogenesis. The facilitatory effect of SDF-1α in the presence of Y-27632 could be mimicked by overexpressing a dominant-active (DA) form of the mammalian homologue of the Drosophila gene Diaphanos (mDia)1, a Rho effector involved in control of actin polymerization during cell polarization and directed cell growth. This dominant activation of mDia was dependent on intact Rac activity. Furthermore, the axon outgrowth induced by SDF-1α could be antagonized either by overexpression of a dominant interfering mutant or by RNAi knockdown of mDia1. Thus, we identify a novel function for mDia1 as a critical Rho effector–mediating SDF-1α–dependent axon elongation in concert with Rac.

Results

A pial chemokine SDF-1α triggers axon elongation via Rho early in culture in cerebellar granule cells

We tested whether SDF-1α was able to trigger any axon growth and whether this morphological change correlated, at least in part, with an alteration in either Rho or Rac activity in cultured cerebellar granule cells, where axonogenesis is well known to precede dendritogenesis. A 12-h exposure to SDF-1α at a concentration of 100 ng/ml induced a significant increase in the mean length of first appearing process compared with control (Fig. 1, A and B; Video 1, avail-
able at http://www.jcb.org/cgi/content/full/jcb.200210149/DC1), while having no effect on axon number (Fig. 1, A and B). This effect was abolished in the presence of C3 exoenzyme, a Rho inhibitor (Fig. 1, A and B). SDF-1α promoted a bell-shaped response in axon elongation with a peak effect at 100 ng/ml (Fig. 1, A and B). However, at a larger concentration this axon outgrowth effect was abolished and indistinguishable from the control (Fig. 1 B). In contrast, SDF-1α treatment significantly reduced axon number at concentrations over 250 ng/ml. Presence of C3 exoenzyme completely flattened either response (Fig. 1 B). Together, this indicated that SDF-1α may promote axon elongation via Rho, at least at lower concentrations, whereas it inhibited initiation of axon at higher concentrations. The reduction in SDF-1α–dependent axon growth with the maximal concentration of SDF-1α was unlikely to be caused by receptor desensitization or inactivation, since we confirmed that axon numbers were not negatively affected at the same dose. Furthermore, either 1- or 12-h exposure to either 100 or 500 ng/ml SDF-1α was accompanied with a similarly strong elevation in the amount of GTP-bound form of Rho (Fig. 1 C), whereas no apparent increase was discerned for GTP-bound Rac (unpublished data) as determined by pull-down assays using either a GST-fused Rho-binding domain (RBD) of Rhotekin or a GST-fused Cdc42/Rac interactive binding (CRIB) domain of p21-associated kinase (PAK) were performed (Ren et al., 1999; Tsuji et al., 2002). These results indicate the possibility that stimulation of Rho pathway with a physiological ligand such as SDF-1α may mediate axon elongation.

**Antagonism between a Rho-activated axon elongating pathway and ROCK-mediated control of axonogenesis**

To test whether the biphasic responsiveness could be accounted for by the existence of distinct thresholds of activation of two separate Rho effectors, we carefully reexamined the dose–response relationship between C3 concentration and resulting axon length. The sampling number was increased substantially in order to detect even small differences that might have been overlooked before. At 10 μg/ml of C3, a significant increase in axon length and number was achieved (Fig. 2). When the dose was augmented to 30–50 μg/ml, this facilitation in axon length was substantially diminished compared with 10 μg/ml treatment, though a small but significant net increase was still detectable; however, no additional effect was seen on axon number (Fig. 2). Therefore, different concentrations of active Rho were likely to gear two distinct pathways, repression and facilitation of axon extension, via two distinct Rho effectors. In contrast, only one Rho effector was likely to contribute to axon number control (Fig. 2), consistent with our previous work (Bito et al., 2000). A similar C3 dose–response curve was also reported in PC-12 cells (Winton et al., 2002).

What are these two Rho effectors? The finding that SDF-1α seemed to antagonize axonogenesis at higher doses in a Rho-dependent manner (Fig. 1 B) was in keeping with our previous finding that Rho/ROCK signaling critically regulates axon numbers (Bito et al., 2000). To test whether ROCK, a Rho-associated kinase (Narumiya et al., 1997; Bito et al., 2000; Ishizaki et al., 2000), was indeed mediating the SDF-1α effect, we bath-applied 50 μM Y-27632, a potent and selective ROCK inhibitor (Uehata et al., 1997; Ishizaki et al., 2000). As expected, treatment with Y-27632 reversed the negative SDF-1α effect on axon numbers, indicating that SDF-1α is indeed able to activate ROCK (Fig. 3, A and B). Surprisingly, however, exposure to SDF-1α in the presence of the ROCK inhibitor now resulted in a significant net increase in axon extension (Fig. 3, A and B). Thus, SDF-1α activated a novel ROCK-independent, yet C3-sensitive, effector pathway that was coupled to axon elongation.
The Rho effector mDia1, a regulator of actin polymerization, is heavily expressed in the external granule cell layer during early postnatal development and positively regulates axon outgrowth

Recently, mDia was found to be a critical Rho effector that may predominantly act as an inducer/regulator of actin polymerization in HeLa cells and may be required for establishment of cell polarity and directed growth (Watanabe et al., 1997; Ishizaki et al., 2001; Ozaki-Kuroda et al., 2001; Tsuji et al., 2002). Indeed, we found that Swiss3T3 cells can elongate prolonged neurite-like processes best when higher mDia activity was achieved by overexpression a DA form of mDia1 (mDia1-ΔN3; Fig. 4 A; Ishizaki et al., 2001) and was coupled with lower ROCK activity in the presence of 30 μM Y-27632 (Fig. 4 B), consistent with previous work from our laboratory (Tsuji et al., 2002).

We also found that expression of mDia1 coincided with early axonogenesis in postnatal cerebellum in mice (Fig. 5, A and B). Immunohistochemical analysis showed that mDia1 was especially abundant in postnatal day 1 (P1) cerebellum at and beneath the external granule cell layer where the earliest events in axonogenesis occurred (Fig. 5 B). In round cerebellar granule cells, mDia1 protein was already colocalized with F-actin and tubulin at spots where an axon was likely to initiate (Fig. 5 C). After axon outgrowth started, mDia1 was heavily enriched at the base of early initiating process and within its growth cones (Fig. 5 C, arrowheads) in close spatial vicinity with actin filaments and microtubules (Figs. 5 C).
To ask directly whether mDia1 activity facilitated, at least in part, axon formation and elongation in cerebellar granule neurons, we tested whether expression of the DA-mDia1 mutant was sufficient to replicate the SDF-1α–stimulated axon elongation activity in the absence of ROCK pathway which was blocked with Y-27632. Transfection was performed immediately after trituration and during initial plating of the neurons so that expression of exogenous protein was initiated ~6 h after plating (Bito et al., 2000, and this study). Neurons were fixed using PFA at 12 h after transfection to examine the effect of mDia1 activity on axon initiation and elongation. Indeed, mDia1-ΔN3–expressing cerebellar granule cells revealed a similar number of axons as GFP-expressing control cells; however, the axons were significantly longer compared with the mock-transfected neurons in the presence of Y-27632 (Fig. 6, A–D), consistent with the idea that mDia1 may work downstream of Rho to facilitate axon growth. During transfection of mDia1-ΔN3, we noticed that the newly formed processes were abnormal in neurons untreated with Y-27632. Axon outgrowth seemed to be prematurely aborted as processes were filled with an exuberant amount of filamentous actin and β-tubulin (Fig. 6 B, mDia1-ΔN3), and thus exhibited an abnormal width (Fig. 6 B and unpublished data). Basal ROCK activity, in the context of excessive mDia1 activity, might account for a prominent increase in actin polymerization (Watanabe et al., 1999), while also sustaining a tonic level of actomyosin contractility, thereby negatively acting on axon elongation.

Rho/mDia pathway mediates SDF-1α–stimulated axon elongation in cerebellar granule neurons in part via a coordination with a Rac-dependent pathway

We next examined the contribution of endogenous mDia1 on SDF-1α–stimulated axon elongation by use of a dominant-negative (DN) form of mDia1, mDia1-ΔN3(Hind-III) (Fig. 4 A; Tsuji et al., 2002). This mutant was able to abolish the effect of DA-mDia1 on axon length (Fig. 7 A). DN-mDia1 also inhibited axon numbers back to baseline levels (Fig. 7 A); however, since DA-mDia1 had little effect per se (Fig. 6 D), we currently favor the simplest view that rather than acting on the axonogenesis itself, mDia’s axon elongating activity may be required in order to visualize even the smallest process. Consistently, when this DN mutant was transfected in neurons stimulated with SDF-1α in the presence of Y-27632, both axon number and axon length were significantly diminished (Fig. 7 B and C). Together, these results support the notion that axon elongation induced by SDF-1α may be regulated in an mDia-dependent manner.

To obtain an independent confirmation of these results, we applied the RNA interference techniques using short interfering double stranded RNA oligomers (siRNAs) (Elbashir et al., 2001). mDia1–specific siRNA was designed and its efficiency in knocking down mDia1 protein was tested in NIH3T3 cells by Western blot analysis (Fig. 8 A). Loss of mDia1 immunoreactivity was specifically obtained in cells expressing a cotransfection marker enhanced GFP (EGFP; unpublished data). Using identical transfection procedures, EGFP-positive cerebellar granule neurons were screened to identify the neurons, which have taken up the mDia1 siRNA, and axon lengths were measured in these neurons. A significant reduction was observed both in axon numbers and in axon length (Fig. 8, B and C), compared with scramble siRNA-treated neurons, in keeping with the DN approach.

We finally wanted to examine the relevance of mDia1 activity vis-à-vis of Rac, a small GTPase classically found to mediate axon outgrowth. A DN form of Rac, RacN17, was introduced into cerebellar granule cells along with a DA-mDia1 mutant mDia1-ΔN3. Axon elongation facilitated by DA-mDia1 in the presence of Y-27632 was repressed, in the presence of RacN17, back to baseline levels (Fig. 9 A). These findings are most consistent with the idea that Rac may significantly contribute to the mDia effect on axon outgrowth. Thus, mDia may directly regulate Rac or one of its upstream regulators.

Could Rho/mDia pathway regulate Rac? We recently proposed a candidate mechanism in HeLa and Swiss3T3 cells: as ROCK is known to down-regulate Rac activity, ROCK inhibition, under some circumstances, may suffice to up-regulate Rac via mDia1 (Tsuji et al., 2002). We tested whether such a possibility may be true in cerebellar granule
Figure 6.  **DA mDia1 facilitates axon elongation.** Morphology of cerebellar granule cells overexpressing GFP (A), GFP-mDia1-ΔN3 alone (B), or GFP-mDia1-ΔN3 in the presence of Y-27632 (C). When ROCK activity was reduced, expression of GFP-mDia1-ΔN3 resulted in a significantly enhanced elongation (D, left) of axons (n = 65–157) compared with EGFP-expressing controls (A). Overexpression of GFP-mDia1-ΔN3 alone successfully induced an axon, which, however, exhibited a significantly altered shape (enlarged width, premature stop), presumably due to an increased actin stability in the presence of intact ROCK activity (B). Basal ROCK activity, in the context of excessive mDia1 activity, might cause a prominent increase in actin polymerization, while also sustaining a tonic level of actomyosin contractility, thereby negatively acting on axon elongation. *P < 0.05; ***P < 0.001. Bars, 5 μm.

Figure 7.  **A DN mDia1 mutant interferes with SDF-1α-dependent axon elongation.** (A) Coexpression of the GFP-mDia1-ΔN3(HindIII) mutant abolished the effect of FLAG-mDia1-ΔN3 expression on axon length (left), n = 33–80. (B and C) The effect of GFP-mDia1-ΔN3(HindIII) overexpression was examined on SDF-1α-facilitated axon elongation in the presence of Y-27632. A potent inhibition on both SDF-1α-dependent axon elongation (B and C, left) and axon initiation (B and C, right) was detected. n = 35–145. Bars, 5 μm.
cells. Consistent with previous work, Y-27632 treatment in SDF-1α–stimulated neurons was sufficient to increase the GTP-bound form of Rac (Fig. 9 B).

Together, our data establish a critical role for the Rho/mDia1 pathway in mediating axon elongation in SDF-1α–stimulated cerebellar granule neurons, presumably in concert with Rac activity (Fig. 10).

Discussion
Discovery of a Rho/mDia-dependent signaling pathway crucial for axon elongation

Previous reports have shown an antagonism between Rac and Rho-mediated pathways in neurite extension and control of axonal growth cones (for review see Narumiya et al., 1997; Gallo and Letourneau, 1998; Hall, 1998; Luo, 2000; Dickson, 2001; Nikolic, 2002). In the mammalian CNS, we showed that enhanced Rho/ROCK activity led to inhibition of axonogenesis, whereas inhibition of Rho/ROCK pathway induced precocious outgrowth of neurites (Bito et al., 2000). In spite of a few reports suggesting a distinct, rather facilitatory effect for Rho during neuritogenesis (Threadgill et al., 1997; Sebok et al., 1999; Bashaw et al., 2001), it has remained unclear whether these experimental results truly reflected a genuine primary effect mediated by Rho.

In this study, we have formally demonstrated that Rho may in fact mediate both stimulation and inhibition of axon outgrowth downstream of the same ligand SDF-1α in the context of early postnatal cerebellar granule cells. Although a classically recognized Rho/ROCK pathway repressed axon formation, a Rho/mDia pathway was identified which rather potently facilitated axon elongation. How ROCK can domi-
intrinsically antagonize the expression of the axon elongation machinery, while another Rho effector mDia is facilitating this event, is currently being investigated. One possibility that is consistent with our finding is that ROCK and mDia may locally respond to different levels of GTP-bound Rho. Our finding of a multiplicity in Rho-mediated sites of action is reminiscent of the recent findings suggesting that Rac also acts on multiple steps of axon outgrowth during Drosophila development (Ng et al., 2002).

How then does mDia, an adaptor protein containing multiple formin homology domains, exert its effect on axon elongation? A clear picture is still missing, since only a few binding partners of mDia such as profilin (Watanabe et al., 1997), IRSp53 (Fujiwara et al., 2000), or mDia-interacting protein (Satoh and Tominaga, 2002) have been reported. Interestingly, in Saccharomyces cerevisiae the Diaphanous homologue Bni1p was shown to be critically involved in controlling assembly of actin cables required for establishment of cell polarity and directed growth (Ozaki-Kuroda et al., 2001; Pryyne et al., 2002; Sagot et al., 2002). Furthermore, recently a DA mutant of mDia1 was also shown to affect orientation of microtubules in HeLa cells, thus implying that mDia may perhaps spatially coordinate actin polymerization and the stability/ rate of assembly of microtubule polymers (Ishizaki et al., 2001; Palazzo et al., 2001).

Intriguingly, in our study Rac activity was shown to be required to support an mDia-based change in neuronal morphology. Indeed, a high amount of basal Rac activity is found in the early postnatal cerebellum (unpublished data). In vivo and in vitro studies examining the consequence of defects in Rac/Pak/Cdk5/p35 signaling have provided numerous lines of evidence in support for a role for Rac-mediated control of cytoskeletal dynamics during axon outgrowth (Luo et al., 1994, 1996; Nikolic et al., 1996, 1998; Kaufmann et al., 1998; Hing et al., 1999; Newsome et al., 2000; Zukerberg et al., 2000; Ng et al., 2002). Since we detected an increase in Rho but not Rac activity upon SDF-1α stimulation, we presume that Rac may play a rather permissive role in mediating the Rho/mDia-dependent events, although we cannot exclude the possibility that mDia may also directly regulate Rac or one of its upstream regulators (Fig. 9; Tsuji et al., 2002).

A critical role for a pial chemokine SDF-1α as a guidance molecule regulating axon outgrowth

Recently, SDF-1α (Tashiro et al., 1993; Nagasawa et al., 1994) has attracted a lot of attention as a prototypical chemokine that acts as a mitogen and participates in positional control of a large variety of highly differentiated cell types. Gene targeting of either the ligand molecule SDF-1α or its receptor CXCR4 resulted in a strikingly similar phenotype in the respective knockout mice, thereby demonstrating a crucial role for SDF-1α/CXCR4 signaling in the development of appropriate cell layer formation in the cerebellum, the hippocampal dentate gyrus, the thymus, the spleen, vascular endothelium, and the intestinal epithelium (Nagasawa et al., 1996; Ma et al., 1998; Tachibana et al., 1998; Zou et al., 1998; Lu et al., 2002). In the cerebellum, absence of SDF-1α/CXCR4 signaling resulted in premature invasion of many proliferating granule cells into the cerebellar anlage, and aberrant onset of migration from the external granule cell layer (EGL) to the internal granule cell layer (IGL) (Ma et al., 1998; Zou et al., 1998). Complementary experiments demonstrated that SDF-1α acted as a chemoattractant for migration of both EGL- and IGL-derived cerebellar granule cells in culture (Klein et al., 2001; Lu et al., 2001; Zhu et al., 2002). Together, these lines of evidence are consistent with the idea that neuronal migration and patterning may heavily involve chemokine and G protein–coupled receptor signaling.

However, it was not known which exact step of neuronal morphogenesis was directly targeted by SDF-1α, nor was it clear what intracellular signaling it activated in order to fulfill its role as a neural guidance molecule. Here we found that a large part of the initial morphogenetic and cytoskeletal action of SDF-1α might be specifically mediated by the Rho signaling pathway, independent of its potency as a mitogen, in a pertussis toxin-insensitive manner (unpublished data). Previously, it was suspected that SDF-1α might exhibit its growth promoting or chemotactic effect on neurons via pertussis-sensitive Gi/o heterotrimeric G proteins (Klein et al., 2001) and/or via PLC-mediated activation of intracellular calcium stores (Klein et al., 2001; Lu et al., 2002). The possible contribution of these signaling pathways to the neurite elongation still needs to be properly addressed in our cultured cerebellar granule neurons.
Control of actin dynamics via multiple effectors of small GTPases: potential antagonism and coordination between distinct signaling pathways

Our study demonstrates that the Rho-dependent signaling cascade implicated in the development control of neural hardwiring may consist of two apparently opposing streams of signals: an inhibitory pathway mediated by ROCK (Bito et al., 2000) and a facilitatory pathway mediated by mDia1 (this study). Coordination of these two pathways may be suited to sequentially express the unique property of SDF-1α either as a repellent or as an attractant. These findings shed light on the new possibility that Rho- and Rac-dependent pathways may interact not just in an antagonistic but also in a cooperative way in the regulation of the timing and the extent of axon elongation in cultured cerebellar granule cells (Fig. 10). Our study raises the possibility that the stimulation of Rho/mDia-mediated pathway by SDF-1α might provide a modulatory link between the Rho- and Rac-based modes.

Together, these findings underscore the significance of ROCK/mDia antagonism during early CNS development. An appropriate balance and coordination between these two signaling systems may be key to controlling the initial timing and extent of axon outgrowth as a function of the strength of stimuli exhibited by the gradient of external guidance cues such as the pial chemokine SDF-1α.

Materials and methods

Primary culture of mouse cerebellar granule cells

Mouse cerebellar granule cell cultures were as described in Bito et al. (2000). A detailed protocol can be obtained from the authors upon request.

Materials and reagents

Y-27632, a ROCK inhibitor, was supplied by Dr. Masayoshi Uehata (Mitsubishi Pharma Co., Osaka, Japan). Botulinum C3 exoenzyme was purified as described (Morii and Narumiya, 1995). SDF-1α was purchased from PeproTech EC. GST-Rhotekin RBD and GST-PAK CRIB proteins were prepared as described (Ren et al., 1999; Tsuji et al., 2002). pEGFP-C1 was purchased from CLONTECH Laboratories, Inc. pEGFP-mDia1 from N3 (HindIII), and pEGFP-N17Rac were as described (Watanabe et al., 1999). The shorter first process. Since an axon was equal or larger than 3 μm in length, its contour could be unambiguously traced from the cell soma up to the very tip of the processes; all turning points within the longest process was defined and the sum of the cumulated distances between these points was considered as axon length. The calculation was performed off-line using the software plug-in of the LSM 510 system. Measurements in transfected neurons were performed identically except that transfected neurons were first identified using an antitag immunostaining (with an anti-GFP or anti-p140mDia1 antibody (Watanabe et al., 1997), anti-FLAG mAb (M2; Eastman Kodak Co.), rat antitubulin mAb (Chemicon), anti–β-tubulin mAb (TUB.2.1) (Sigma-Aldrich); anti-GFP monoclonal and polyclonal (Molecular Probes); and Alexa Fluor 488–, 594–, and 633–conjugated goat anti–rabbit, anti–rat, and anti–mouse secondary (Molecular Probes). Alexa Fluor 594–, and 633–phalloidin (1:1,000; Molecular Probes) were used to stain the F-actin. In all image analyses, no background subtraction was performed, and all pseudocolor representations were assembled using Adobe Photoshop® version 6.0 for illustrative purpose only.

Quantitation of axon length and number was performed on cerebellar granule neurons cultured in the presence of 10% FCS using parameters similar to those described previously (Bito et al., 2000). Axons were distinguished from the filopodia by the presence of microtubules as verified by β-tubulin staining. As established previously (Bito et al., 2000), β-tubulin immunoreactivity was missing in virtually all processes with less than 3 μm in length. Because the average axon number was below two (i.e., exact number was usually one or zero) during the experimental time window in most of our assays in this study, we restricted our measurement of axon length to the first process. Since an axon was equal or larger than 3 μm in length, its contour could be unambiguously traced from the cell soma up to the very tip of the processes; all turning points within the longest process was defined and the sum of the cumulated distances between these points was considered as axon length. The calculation was performed off-line using the software plug-in of the LSM 510 system. Measurements in transfected neurons were performed identically except that transfected neurons were first identified using an antitag immunostaining (with an anti-GFP or an anti-FLAG antibody) to maximize the probability of detection; when directly compared, FLAG- and GFP-tagged constructs yielded similar results and phenotypes. Statistical analyses were performed using Prism 3.02 (Graphpad Software). All data are indicated as means ± SEM. Unpaired t test with Welch's correction was employed to determine statistical significance and p values below 0.05 was considered as significant.

Videomicroscopy

Cerebellar granule cells labeled with PKH-26 (Sigma–Aldrich) were seeded at a density of 104 per dish in a 35-mm glass-bottom dish (Matsunami Glass) and cultured for 3 h. The dish was transferred to a temperature-controlled CO2 incubator (Carl Zeiss MicroImaging, Inc.) attached to the microscope stage. SDF-1α (100 ng/ml, a final concentration) was then added, and the cell movement was monitored at 37°C in 5% CO2 for 60 min using a confocal laser scanning unit (LSM 510-V2;5; Carl Zeiss MicroImaging, Inc.). One optical section was acquired every 3 min, and the video image were constructed from these sequential images.

Immunohistochemistry

P1 or adult brain was taken out and immediately frozen in TissueTek O.C.T. Compound (Sakura) on dry ice. Cryostat sections of 20-μm width were obtained and subjected to indirect immunofluorescence staining using an anti-p140mDia1 primary (AP50; Watanabe et al., 1997) and Alexa
Fluor 488–conjugated goat anti–rabbit secondary antibodies. TO-PRO-3 iodide (Molecular Probes) was used for nuclear staining. Specimens were examined on an Axiosplan or LSM 510 confocal imaging system (Carl Zeiss MicroImaging, Inc.). Stacked optical sections were merged using Maximum Projection Software (Carl Zeiss MicroImaging, Inc.).

**Western blot analysis**

Total brain or cerebellar tissues were collected from ICR mice of indicated ages. Thin slices were cut in parasagittal directions in ice-cold HANKS and lysed in the lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 1× Complete (Protease Inhibitor Cocktail Tablets) (Roche Diagnostics). The samples were centrifuged at 100,000 g for 30 min, and the supernatant was collected as the cell lysates. Protein concentration of the lysates was determined by the Lowry method. For Western blot analysis using the total cell lysates, one fifth volume of the 5× Laemmli sample buffer was added to the lysates. The mixtures were boiled for 5 min and subjected to SDS-PAGE and Western blot analysis using anti-p140mDia1 antibody (AP50).

**Protein knockdown of mDia1 by RNAi using siRNA**
siRNA corresponding to mDia1 mRNA sequences were designed as recommended with 5′-phosphate, 3′-hydroxy, and two-base overhangs on each strand; it was chemically synthesized and annealed for duplex siRNA formation by Dharmacon Research, Inc. The following gene-specific sequences were used successfully: si-mDia1 (K2) sense 5′-GCCGUGUCA-GAGCCAU-UGAU-3′ and antisense 5′-GCAGCUGUCGGUACCUC-3′. Transfection of si-mDia1 siRNA for NIH-3T3 cells (10^4 cells/ml) was performed with Lipofectamine 2000 in 6-well plates. Per well, 2 μl lipofectamine 2000 diluted in 100 μl Opti-MEM was applied to a premix consisting of siRNA (20 μM, 4 μl Opti-MEM (100 μl) and incubated for 30 min. The whole mixture was added to the medium, which then was changed to DME 3 h after transfection. Cells were incubated for 24–48 h before analysis of knock-down mDia1 by Western blot as described above. Cotransfection of pEGFP-C1 and siRNA to cerebellar granule cells (5 × 10^3 cells/ml) were performed with Lipofectamine 2000. Briefly, for 10^6 cells 2 μl Lipofectamine 2000 diluted in 100 μl Opti-MEM was applied to a pEGFP-C1 (1 μg/siRNA (20 μM, 8 μl Opti-MEM (200 μl) mixture and incubated for 30 min. The entire mixture was then added to the cell-containing medium, which was refed with the original culture medium 3 h after transfection. Cells were incubated for 12 h in solution, then finally plated onto 12-mm round Matrigel-coated coverslips placed in a 24-well plate. After another 12 h of culture on the coverslips, measurements of axon parameters were performed as described above.

**Online supplemental material**

Video 1, showing facilitation of axon elongation by a 100 ng/ml SDF-1α treatment in cultured cerebellar granule neurons, is available online at http://www.jcb.org/cgi/content/full/jcb.200210149/DC1).

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