Current Biology, Vol. 12, 307–312, February 19, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S0960-9822(02)00658-9

The Human Rho-GEF Trio and Its Target GTPase RhoG Are Involved in the NGF Pathway, Leading to Neurite Outgrowth

Soline Estrach,[2] Susanne Schmidt,[2] Sylvie Diriong, Aubin Penna, Anne Blangy, Philippe Fort, and Anne Debant[1]
CRBM-CNRS
UPR 1086 CNRS
1919 Route de Mende
34293 Montpellier Cédex 5
France

Summary

Rho-GTPases control a wide range of physiological processes by regulating actin cytoskeleton dynamics [1]. Numerous studies on neuronal cell lines have established that Rac, Cdc42, and RhoG activate neurite extension, while RhoA mediates neurite retraction [2–5]. Guanine nucleotide exchange factors (GEFs) activate Rho-GTPases by accelerating GDP/GTP exchange [6]. Trio displays two Rho-GEF domains, GEFD1, activating the Rac pathway via RhoG, and GEFD2, acting on RhoA, and contains numerous signaling motifs whose contribution to Trio function has not yet been investigated [7–9]. Genetic analyses in *Drosophila* and in *Caenorhabditis elegans* indicate that Trio is involved in axon guidance and cell motility via a GEFD1-dependent process, suggesting that the activity of its Rho-GEFs is strictly regulated [10–14]. Here, we show that human Trio induces neurite outgrowth in PC12 cells in a GEFD1-dependent manner. Interestingly, the spectrin repeats and the SH3-1 domain of Trio are essential for GEFD1-mediated neurite outgrowth, revealing an unexpected role for these motifs in Trio function. Moreover, we demonstrate that Trio-induced neurite outgrowth is mediated by the GEFD1-dependent activation of RhoG, previously shown to be part of the NGF (nerve growth factor) pathway [4]. The expression of different Trio mutants interferes with NGF-induced neurite outgrowth, suggesting that Trio may be an upstream regulator of RhoG in this pathway. In addition, we show that Trio protein accumulates under NGF stimulation. Thus, Trio is the first identified Rho-GEF involved in the NGF-differentiation signaling.

Results

Human Trio Induces Neurite Outgrowth in PC12 Cells

As Trio potentially activates antagonistic pathways in neuronal cells via its two Rho-GEF domains, we investigated the role of Trio in neurite outgrowth in PC12 cells. We first determined the effects on PC12 cell morphology of the two Trio Rho-GEFs when expressed individually. PC12 cells were transfected with different Trio cDNA constructs and were scored for the presence of neurites whose length was superior to at least two body lengths. Expression of GEFD1 promoted lamellipodia formation and cell spreading, but never induced neurite formation (Figure 1A). Expression of the constitutive form of RhoG (RhoGV12), whose wild-type counterpart is the direct target of GEFD1 in vitro and in fibroblasts [9], induced the same type of structures. In contrast, expression of the RhoA-specific GEFD2 induced cell retraction, as did the activated form of its GTPase target (Figure 1A).

Interestingly, PC12 cells expressing the full-length Trio protein (GFP-Trio) developed very long neurites in more than 50% of the transfected cells (Figures 1B and 1C). As previously observed [4], the wild-type form of RhoG also induced the extension of neurites (Figures 1B and 1C). All together, these observations suggest that the activity of the Trio Rho-GEF domains is highly regulated in the context of the full-length protein to allow Trio-induced neurite outgrowth.

Trio Is Localized in the Lamellipodia Structure of the Growth Cone

Analysis of phase-contrast time-lapse microscopy of GFP-Trio-expressing PC12 cells demonstrated that Trio-expressing growth cones possessed very dynamic structures such as filopodia and lamellipodia (Figure 2A). Moreover, as shown by fluorescence time-lapse microscopy of the same cell, GFP-Trio was enriched in these actin-rich structures, indicating that Trio was present in the motile growth cone (Figure 2A). To better visualize the localization of Trio, we used immunofluorescence images of GFP-Trio, actin, and tubulin, acquired at different Z planes for volume rendering of the image using Imaris software. The volume reconstruction of the growth cone clearly showed that Trio was present inside the F-actin-rich lamellipodia structure found at the tip of the growth cone (Figure 2B).

TrioGEFD1 Cooperates with the Spectrin Repeats and the SH3-1 Domain to Promote Efficient Neurite Outgrowth

In order to determine the contribution of the numerous signaling motifs of Trio in Trio-induced neurite outgrowth, we designed different deletion constructs (summarized in Figure 3A) and tested the effect of their expression on PC12 cell morphology. Their expression and their correct size were checked by Western blot analysis (data not shown). Interestingly, deletion of the kinase domain (GFP-Trio 1-2308) or deletion of the kinase and the GEFD2 domains (GFP-Trio 1-1813) did not affect the capacity of Trio to induce neurite outgrowth (compare Figures 3B, lanes 1 and 3, and 1C). Similarly, the deletion of the N-terminal part of Trio (GFP-Trio 250-2308) had little incidence on Trio-induced neurite outgrowth. We then tested the effect of a double point mutation in the DH1 domain Q1368A and L1376E (GFP-TrioAE), which drastically reduced GEFD1 in vitro exchange activity toward RhoG (data not shown). Indeed, the presence of this double mutation in the DH1 domain completely

[1]Correspondence: debant@crbm.cnrs-mop.fr
[2]These authors contributed equally to this work.

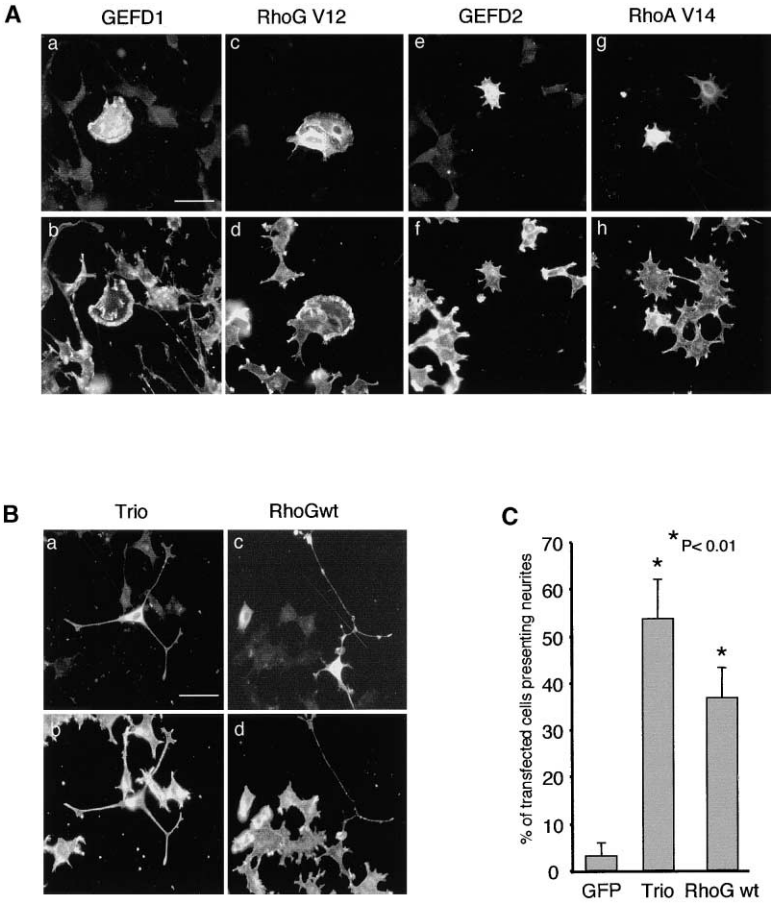


Figure 1. Full-Length Trio Induces Neurite Outgrowth in PC12 Cells

(A) PC12 cells were transfected with (A and B) HA-TrioGEFD1, (E and F) HA-TrioGEFD2, (C and D) GFP-RhoGV12, or (G and H) GFP-RhoAV14. After 48 hr, cells were fixed and permeabilized as described [18]. (C and G) Expression of the GFP-GTPases was detected directly, (A and E) whereas Trio constructs were detected using the anti-HA 12CA5 mAb, followed by incubation with FITC-conjugated anti-mouse IgG. Filamentous actin was stained with rhodamine-conjugated phalloidin (B, D, F, and H). The scale bar represents 5 μm.

(B) PC12 cells were transfected as indicated and treated as described in (A). Expression of (A) GFP-Trio and of (C) GFP-RhoGwt was detected directly, and (B and D) filamentous actin was stained with rhodamine-conjugated phalloidin. The scale bar represents 5 μm.

(C) Quantification of neurite outgrowth. Transfected cells were scored for the presence of neurites whose length was superior to at least two body lengths. Results are expressed as the percentage of cells with neurites versus the total number of transfected cells. At least 100 cells were counted in each experiment, and data are the means ± standard errors of at least 3 independent experiments.

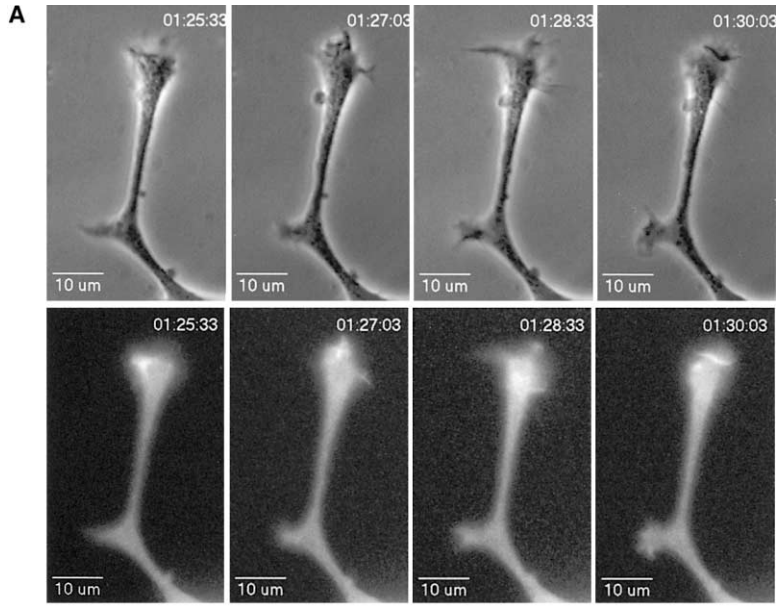
abolished Trio function (Figure 3B, lane 7). Furthermore, deletion of the SH3-1 domain (GFP-Trio 1-1579) or progressive deletions of the spectrin repeats (GFP-Trio 696-1813, GFP-Trio 926-1813) significantly abrogated Trio effect on neuronal morphology (Figure 3B, lanes 4–6). Consistently, mutation in the TrioSH3-1 domain of a conserved tryptophane residue that has been shown elsewhere to be crucial for binding SH3 ligands [15] strongly affected Trio-induced neurite outgrowth (W1636P, GFP-TrioP, Figure 3B, lane 8). Furthermore, cells expressing the triple Trio mutant (GFP-TrioAEP) were unable to produce any neurites, as expected (Figure 3B, lane 9). All together, these data point to an unexpected role of TrioSH3-1 and the spectrin repeats in modulating the GEFD1-dependent effect of Trio on neuronal morphology.

The GTPase RhoG Mediates Trio-Induced Neurite Formation

We then investigated which GTPase mediated Trio-induced neurite outgrowth in PC12 cells. Since RhoGwt induced neurite outgrowth in those cells, by acting upstream of Rac and Cdc42 [4], and since RhoG is a target of GEFD1 [9], we investigated whether it could mediate Trio effect on neuronal morphology. We coexpressed Trio with two dominant-negative forms of RhoG, RhoGN17 and RhoGA37 (Figure 3C). The latter mutant is impaired in its binding to known effectors of the GTPase and efficiently inhibits GEFD1 activity on actin cytoskeleton remodeling in fibroblasts [9]. Both RhoG mutants very efficiently inhibited Trio-induced neurite outgrowth (Figure 3C), suggesting that Trio may stimulate neurite outgrowth through the activation of RhoG. However, since TrioGEFD1 activates both RhoG and Rac in vitro, we cannot rule out the possibility that Trio also acts directly through the GTPase Rac to promote neurite outgrowth. Expression of the dominant-negative form of Rac (RacN17) partially inhibited Trio effect on neuronal morphology, while expression of Cdc42N17 only mildly affected Trio effect (Figure 3C). This observation is reminiscent of RhoG-induced neurite outgrowth, which had been previously shown to be more affected by RacN17 than by Cdc42N17 [4]. In addition, Trio effect on neuronal morphology was completely blocked by dominant-negative forms of the RhoG-specific effectors kinectin and RhoGIP122 (Figure 3C), which do not bind to Rac or Cdc42 GTPases and thus specifically interfere with the RhoG pathway [9, 16]. Thus, Trio likely stimulates neurite outgrowth through the activation of RhoG, and not through the direct activation of Rac.

Trio Protein Accumulates in Response to NGF (Nerve Growth Factor) Stimulation of PC12 Cells

Since RhoG mRNA has been shown to be induced by NGF [4], we checked whether Trio expression was also



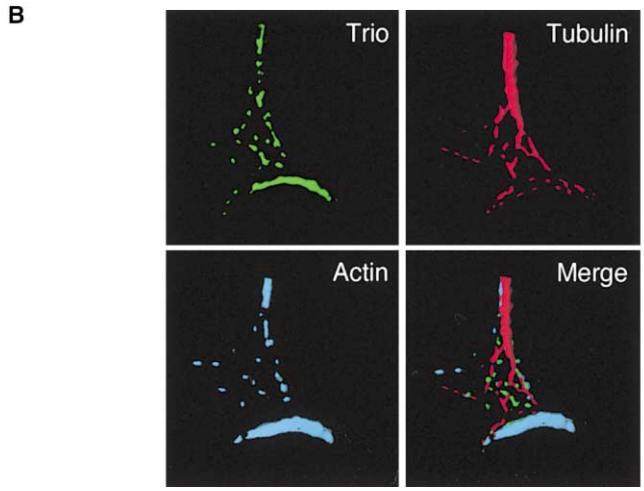


Figure 2. Trio Is Localized in the Lamellipodia Structure of the Growth Cone

(A) Growth cone morphology of GFP-Trio-expressing PC12 cells was monitored by video microscopy. Cell images were captured both in epifluorescence and phase contrast every 90 s. A representative selection of the images is shown here. See the Supplementary Material available with this article online for movies.

(B) Volume rendering of growth cones from Trio-expressing PC12 cells. After 48 hr of expression, cells were fixed using the DSP fixation protocol as described [19]. A stack of 19 optical sections (0.2 μm) was restored using the Huygens system. Restored images were processed with Imaris 2.7 for volume rendering. See the Supplementary Material for details regarding image acquisition.

modulated in response to NGF. The endogenous Trio protein was present at a weak level in the soluble fraction of serum-starved and proliferative PC12 cells (Figure 4A and data not shown). The amount of Trio increased several fold after 7 hr of NGF stimulation (Figure 4A). However, no significant induction was observed at the mRNA level, as analyzed by quantitative PCR (data not shown), suggesting that NGF likely regulates Trio expression at a posttranscriptional level.

Trio Mutants Interfere with the NGF-Differentiation Pathway

RhoG has been recently proposed to be a mediator of NGF-induced neurite outgrowth [4]. Indeed, RhoGN17, RhoGA37, and the deletion mutant of the RhoG-specific effector kinectin efficiently blocked NGF-induced neurite outgrowth (Figure 4B). We thus investigated whether Trio could also contribute to the NGF pathway. For that purpose, we tested the capacity of different Trio mutants to interfere with the NGF pathway. Expression of the GFP-TrioAE or GFP-TrioP mutants partially inhibited NGF-induced neurite outgrowth. Interestingly, GFP-TrioAEP and the N-terminal part of Trio containing the spectrin repeats (GFP-Trio 1-1203) very efficiently blocked NGF-induced neurite formation (Figure 4B). All together, these data suggest that Trio participates in NGF-differentiation signaling as an upstream regulator of RhoG.

Discussion

The Rac and RhoA GTPases have antagonistic effects on neuronal morphology. Rac activation leads to neuritogenesis, while RhoA activation promotes cell retrac-

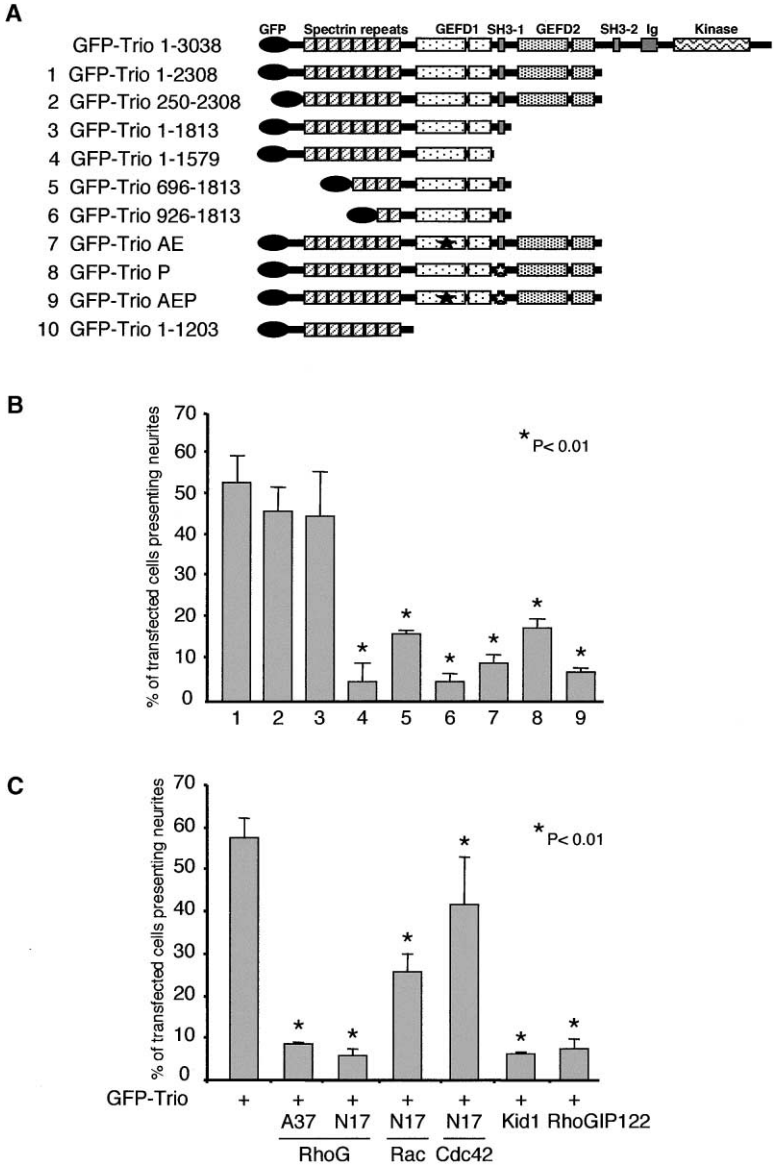


Figure 3. TrioGEFD1 Cooperates with the Spectrin Repeats and the SH3-1 Domain to Promote an Efficient Neurite Outgrowth Mediated by the GTPase RhoG

(A) A schematic representation of the different Trio constructs used in this study.

(B) Effect of the different Trio deletion or point mutants on the morphology of PC12 cells. PC12 cells were transfected with the different constructs as indicated (the numbering refers to [A]) and were treated as described in Figure 1. Quantification of transfected cells displaying neurites whose length was superior to at least two body lengths was done as in Figure 1C.

(C) PC12 cells were transfected with GFP-Trio 1-2308 alone or in combination with different HA-tagged cDNA constructs as indicated. Cells were then treated as described in Figure 1. Quantification of transfected cells displaying neurites whose length was superior to at least two body lengths was done as in Figure 1C.

tion and prevents NGF-differentiation signaling [5]. We show here that the human Rho-GEF Trio efficiently induces neurite outgrowth in PC12 cells by a process that is dependent only on GEFD1 activity and not on GEFD2, which is consistent with the data obtained with Trio orthologs [10]. These observations indicate that the activity of the Trio Rho-GEFs is strictly regulated. In the case of GEFD2, the expression of this RhoA-specific GEF alone induces cell retraction in PC12 cells, showing that it is functional in intact cells. Thus, it is likely that the GEFD2 activity is negatively regulated in the context of the full-length protein, and that it may have no major function in neurite outgrowth of PC12 cells. However, mutations in *Drosophila* Trio lead to overextension of the dendrites in the mushroom bodies (MBs), and, interestingly, this phenotype is also observed in RhoA mutants [11, 17]. Thus, Trio may regulate dendritic growth via its RhoA-specific GEF domain.

Trio is a complex protein displaying numerous signaling motifs that may participate in the regulation of Trio Rho-GEF activity. We show here that the C-terminal kinase domain of Trio is not required for neurite outgrowth, consistent with the finding that Trio orthologs involved in axon guidance do not contain a similar kinase domain. While GEFD1 activity is required for Trio-induced neurite outgrowth, expression of GEFD1 alone does not lead to neuronal differentiation, but rather promotes the formation of a large lamellipodia. This observation indicates that the activity of GEFD1 must be strictly regulated in the context of the full-length protein. Using deletion and point mutants of Trio, we show that the GEFD1-dependent neurite outgrowth induced by Trio requires the presence of the spectrin repeats and the SH3-1 domain, revealing an unexpected role for these motifs in Trio function. These domains could, for example, target Trio to an appropriate specific location within the cell. However, their exact role in the regulation of Trio activity is still unclear.

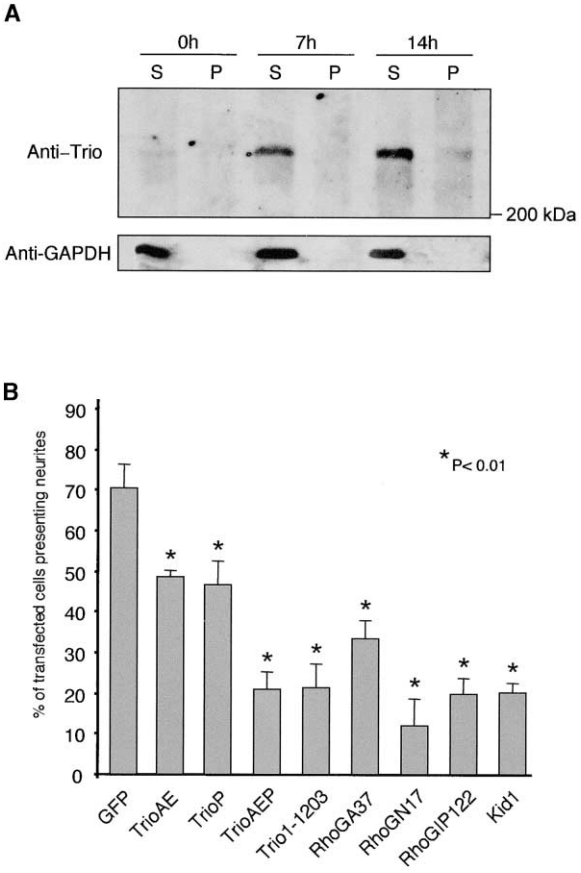


Figure 4. Trio Is Involved in the NGF-Differentiation Pathway

(A) Serum-starved PC12 cells were differentiated with NGF (50 ng/ml) and subjected to subcellular fractionation at the indicated times after NGF addition. Soluble (S) and particulate (P) fractions were obtained as described in the Experimental Procedures section provided in the Supplementary Material. The presence of the endogenous Trio protein was revealed by Western blot analysis using a polyclonal anti-Trio antibody directed against amino acids 2627–3038. The equivalent amount of protein in each fraction was assessed by Western blot analysis using a polyclonal anti-GAPDH antibody.

(B) PC12 cells were transfected with different GFP-tagged cDNA constructs as indicated and were treated as described in Figure 1, except that, before fixation, NGF (50 ng/ml) was added to the cells for 24 hr. Quantification of transfected cells displaying neurites whose length was superior to at least two body lengths was done as in Figure 1C.

By video microscopy, we show that Trio-expressing cells possess growth cones with highly dynamic filopodia and lamellipodia, indicative of a concurrent Rac and Cdc42 activation. We propose that Trio-induced neurite outgrowth is likely to be mediated by the direct activation of RhoG by GEFD1, since RhoG has been shown to be a much better substrate than Rac for TrioGEFD1 in vitro and has been shown to be the in vivo direct target of GEFD1 in fibroblasts; expression of RhoGwt in PC12 cells stimulates neurite outgrowth; two dominant-negative forms of RhoG, which acts upstream of Rac and Cdc42, fully block Trio-induced neurite outgrowth (this work, Figure 3C); RhoGA37 is impaired in RhoG effector binding and does not bind TrioGEFD1 (data not shown), suggesting that the inhibition of Trio signaling by RhoGA37 does not result from Trio titration; and, more importantly, fragments of RhoG-specific effectors, which interfere only with the RhoG pathway, efficiently inhibit Trio effect, strongly suggesting that Rac is not the direct target of Trio. The fact that RacN17 partially inhibits Trio-induced neurite outgrowth can be explained by the fact that RhoG is an upstream activator of Rac [4, 18].

RhoG has been proposed to participate in NGF-differentiation signaling, and its mRNA expression is induced by NGF treatment of PC12 cells. The Rho-GEF responsible for the activation of RhoG in this system has not been identified. Here, we present evidence that Trio may be one mediator of RhoG activation in response to the NGF-differentiation signal. We show that Trio protein accumulates under NGF treatment, providing a strong link between Trio and the NGF-signaling pathway. Moreover, expression of different dominant-negative mutants of Trio significantly blocks NGF neurite outgrowth. These observations together with the fact that RhoG is likely to be the direct target of Trio strongly suggest that Trio acts as an upstream regulator of RhoG in the NGF-differentiation pathway. Finally, Trio is the first identified Rho-GEF regulated by the NGF-triggered differentiation signal.

Supplementary Material

Supplementary Material including the Experimental Procedures and the movies corresponding to the selection presented in Figure 2A is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

We thank E. Portales-Casamar for helpful discussions, P. Travo, head of the CRBM Integrated Imaging facility, for interest and support, and Naomi Taylor for helpful comments on the manuscript. S.S. was supported by a European Molecular Biology Organisation long-term fellowship. This work was funded by the Centre National de la Recherche Scientifique institutional grants and a grant from the "Association pour la Recherche contre le Cancer" (n°5850).

Received: July 4, 2001
Revised: November 21, 2001
Accepted: December 7, 2001
Published: February 19, 2002

References

1. Hall, A. (1998). Rho GTPases and the actin cytoskeleton. Science *279*, 509–514.
2. Kozma, R., Sarner, S., Ahmed, S., and Lim, L. (1997). Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. Mol. Cell. Biol. *17*, 1201–1211.
3. Jalink, K., van Corven, E.J., Hengeveld, T., Morii, N., Narumiya, S., and Moolenaar, W.H. (1994). Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. J. Cell Biol. *126*, 801–810.
4. Katoh, H., Yasui, H., Yamaguchi, Y., Aoki, J., Fujita, H., Mori, K., and Negishi, M. (2000). Small GTPase RhoG is a key regulator for neurite outgrowth in PC12 cells. Mol. Cell. Biol. *20*, 7378–7387.
5. Gallo, G., and Letourneau, P.C. (1998). Axon guidance: GTPases help axons reach their targets. Curr. Biol. *8*, R80–82.

6. Stam, J.C., and Collard, J.G. (1999). The DH protein family, exchange factors for Rho-like GTPases. Prog. Mol. Subcell. Biol. *22*, 51–83.
7. Debant, A., Serra-Pages, C., Seipel, K., O'Brien, S., Tang, M., Park, S.H., and Streuli, M. (1996). The multidomain protein Trio binds the LAR transmembrane tyrosine phosphatase, contains a protein kinase domain, and has separate rac-specific and rho-specific guanine nucleotide exchange factor domains. Proc. Natl. Acad. Sci. USA *93*, 5466–5471.
8. Bellanger, J.M., Lazaro, J.B., Diriong, S., Fernandez, A., Lamb, N., and Debant, A. (1998). The two guanine nucleotide exchange factor domains of Trio link the Rac1 and the RhoA pathways in vivo. Oncogene *16*, 147–152.
9. Blangy, A., Vignal, E., Schmidt, S., Debant, A., Gauthier-Rouviere, C., and Fort, P. (2000). TrioGEF1 controls Rac- and Cdc42-dependent cell structures through the direct activation of rhoG. J. Cell Sci. *113*, 729–739.
10. Newsome, T.P., Schmidt, S., Dietzl, G., Keleman, K., Asling, B., Debant, A., and Dickson, B.J. (2000). Trio combines with dock to regulate Pak activity during photoreceptor axon pathfinding in *Drosophila*. Cell *101*, 283–294.
11. Awasaki, T., Saito, M., Sone, M., Suzuki, E., Sakai, R., Ito, K., and Hama, C. (2000). The *Drosophila* trio plays an essential role in patterning of axons by regulating their directional extension. Neuron *26*, 119–131.
12. Bateman, J., Shu, H., and Van Vactor, D. (2000). The guanine nucleotide exchange factor trio mediates axonal development in the *Drosophila* embryo. Neuron *26*, 93–106.
13. Liebl, E.C., Forsthoefel, D.J., Franco, L.S., Sample, S.H., Hess, J.E., Cowger, J.A., Chandler, M.P., Shupert, A.M., and Seeger, M.A. (2000). Dosage-sensitive, reciprocal genetic interactions between the Abl tyrosine kinase and the putative GEF trio reveal trio's role in axon pathfinding. Neuron *26*, 107–118.
14. Steven, R., Kubiseski, T.J., Zheng, H., Kulkarni, S., Mancillas, J., Ruiz Morales, A., Hogue, C.W., Pawson, T., and Culotti, J. (1998). UNC-73 activates the Rac GTPase and is required for cell and growth cone migrations in *C. elegans*. Cell *92*, 785–795.
15. Hing, H., Xiao, J., Harden, N., Lim, L., and Zipursky, S.L. (1999). Pak functions downstream of Dock to regulate photoreceptor axon guidance in *Drosophila*. Cell *97*, 853–863.
16. Vignal, E., Blangy, A., Martin, M., Gauthier-Rouviere, C., and Fort, P. (2001). Kinectin is a key effector of RhoG microtubule-dependent cellular activity. Mol. Cell. Biol. *21*, 8022–8034.
17. Lee, T., Winter, C., Marticke, S.S., Lee, A., and Luo, L. (2000). Essential roles of *Drosophila* RhoA in the regulation of neuroblast proliferation and dendritic but not axonal morphogenesis. Neuron *25*, 307–316.
18. Gauthier-Rouviere, C., Vignal, E., Meriane, M., Roux, P., Montcourier, P., and Fort, P. (1998). RhoG GTPase controls a pathway that independently activates Rac1 and Cdc42Hs. Mol. Biol. Cell *9*, 1379–1394.
19. Bell, P.B., Jr., and Safiejko-Mroczka, B. (1995). Improved methods for preserving macromolecular structures and visualizing them by fluorescence and scanning electron microscopy. Scanning Microsc. *9*, 843–857; discussion 858–860.