ErbB2 directly activates the exchange factor Dock7 to promote Schwann cell migration

Junji Yamauchi,1 Yuki Miyamoto,1 Jonah R. Chan,2 and Akito Tanoue1

1Department of Pharmacology, National Research Institute for Child Health and Development, Setagaya, Tokyo 157-8535, Japan
2Department of Biochemistry and Molecular Biology, Zilkha Neurogenetic Institute, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033

Introduction

During development, Schwann cells proliferate, migrate, sort, and ensheath individual axons, all of which contributes to the proper formation of myelin. Throughout these events, Schwann cells are in constant contact with axons, suggesting that glial-neuronal communication is essential for regulating these processes (Bunge, 1993). One such factor involved in Schwann cell proliferation and migration is neuregulin-1 (NRG1), which is expressed primarily by neurons and signals through the cognate ErbB family of receptors expressed by Schwann cells (Bunge, 1993; Mahanthappa et al., 1996; Garratt et al., 2000; Citri et al., 2003). Additionally, growing evidence clearly illustrates that NRG1 type III, a membrane-bound form, plays a critical role in the ensheathment and myelination of axons (Nave and Salzer, 2006). Although all of these processes are clearly distinct, they all require rapid and dynamic morphological changes in the Schwann cell, initiated by the activation of the ErbB2 and ErbB3 heterodimer of the receptor tyrosine kinase family (Garratt et al., 2000; Citri et al., 2003). How can the same ligand and receptor complex, namely NRG1 via ErbB2/3, regulate multiple cellular processes that are thought to be distinct and highly controlled in a temporal and spatial manner? How is specificity conferred to generate the proper number of Schwann cells to appropriately match the number of axons and then to determine which axons should be myelinated?

It is well established that the ability of cells to respond to extracellular signals to change cell morphology is controlled in part by the Rho family of small GTPases, including Rac1, Cdc42, and the downstream c-Jun N-terminal kinase. We show that the NRG1 receptor ErbB2 directly binds and activates Dock7 by phosphorylating Tyr-1118. Dock7 knockdown, or expression of Dock7 harboring the Tyr-1118–to–Phe mutation in Schwann cells, attenuates the effects of NRG1. Thus, Dock7 functions as an intracellular substrate for ErbB2 to promote Schwann cell migration. This provides an unanticipated mechanism through which ligand-dependent tyrosine phosphorylation can trigger the activation of Rho GTPase-GEFs of the Dock180 family.
The GEFs are largely divided into two major categories (Rossman et al., 2005). The first category is composed of ~80 genes related to the protooncogene Dbl. These gene products share a catalytic Dbl homology (DH) domain, which was first identified over a decade ago (Schmidt and Hall, 2002; Rossman et al., 2005). The second category consists of at least 11 GEFs, which contain a catalytic domain that is structurally distinct from the DH domain. This catalytic domain is named the Dock homology region (DHR)-2 (also called Caenorhabditis elegans Ced-5/mammalian Dock180/Drosophila melanogaster Mbc-zizimin homology domain) 2 or Dock; Rushton et al., 2005) and, until recently, little was known about the mechanisms that regulate the activities of the Dock180 (also called Dock1)-related GEFs. In this paper, we report the role of the atypical Dock180-related GEF Dock7 and the subsequent GTPase signaling cascade in NRG1-induced migration of primary Schwann cells. Furthermore, we identify Dock7 as the functional intracellular substrate of ErbB2, suggesting the importance of Dock7 in early peripheral nervous system development.

Results
NRG1 activation of ErbB2 and 3 promotes Schwann cell migration through Rho GTPases Rac1 and Cdc42 and the downstream JNK

Using Boyden chambers, we previously demonstrated that dorsal root ganglion (DRG) neurons secrete various growth factors, including neurotrophin-3 (NT3) and brain-derived neurotrophic factor, to regulate Schwann cell migration (Yamauchi et al., 2004). We placed primary Schwann cells onto filters of Boyden chambers coated with axonal membranes from DRG neurons and allowed them to migrate into the lower compartment along a concentration gradient of the neuronal conditioned medium for 6 h. When we added the NRG1 scavenger ErbB3-Fc to the DRG conditioned medium, migration was significantly diminished. Removal of the NRG1-like activity inhibited migration by ~80% (Fig. 1, A and B), implicating NRG1 as a positive regulator of Schwann cell migration. We next examined the effects of extracellular matrix proteins on Schwann cell migration induced by the conditioned medium. Filters in the Boyden chambers were coated with collagen (type I or IV), fibronectin, or laminin (Fig. 1, C and D). The conditioned medium stimulated migration on any of the extracellular matrices, although fibronectin and laminin enhanced Schwann cell migration in the absence of the conditioned medium. Therefore, it is possible that extracellular matrices act cooperatively with soluble factors to control migration. In contrast, the collagen-coated filters were similar to the filters coated with DRG axonal membranes. Because collagen modestly stimulates migration as compared with fibronectin and laminin, collagen (type I)-coated filters were used in the subsequent experiments. Next, we explored whether ErbB3-Fc is specific for the NRG1-like activity in the conditioned medium. As shown in Fig. 1 (E–G), ErbB3-Fc specifically inhibits migration induced by NRG1 but not by NT3 or insulin-like growth factor (IGF)-I, which stimulates Schwann cell migration (Cheng et al., 2000; Yamauchi et al., 2004). These results again suggest that the DRG conditioned medium contains NRG1-like activity, which can enhance Schwann cell migration. We further investigated the effect of the NRG1-like activity on migration of reaggregated Schwann cells on live DRG axons to mimic physiological conditions. The Schwann cell reaggregates initially spread out slowly and then begin to migrate out of the reaggregates along axons. Addition of ErbB3-Fc to the culture medium of the DRG neurons inhibits Schwann cell migration from the reaggregates (Fig. 1, H and I). This observation is consistent with the results from our Boyden chamber assay and, in fact, ErbB3-Fc has a greater initial inhibitory effect on migration from the reaggregates. It is important to note that after a longer time course the effects on migration distances in the presence or absence of ErbB3-Fc were diminished. Thus, it is likely that the effect of ErbB3-Fc delays migration rather than completely inhibiting it.

We examined whether migration of Schwann cells requires the tyrosine kinase activity of ErbB2. Pretreatment with AG825, an inhibitor of the ErbB2 tyrosine kinase (Tsai et al., 1996), reduced NRG1-induced migration by ~80% (Fig. 1, J and K). In addition, we transfected siRNA oligonucleotides for both ErbB2 and 3 in Schwann cells. Expression of ErbB2 and 3 was specifically down-regulated after transfection with the siRNA, whereas expression of control proteins was unaffected, as revealed by immunoblotting (Fig. 1 M). Knockdown of ErbB2 or ErbB3 attenuated migration induced by NRG1. Collectively, the ErbB2 and 3 heterodimer responds to NRG1, and the tyrosine kinase activity of ErbB2 is important for Schwann cell migration.

Next, we tested whether the Rho GTPases Rac1 and Cdc42 are involved in the NRG1-induced migration of Schwann cells, as previously seen in NT3-induced migration (Yamauchi et al., 2004). Pretreatment of Clostridium difficile Toxin B, which glycosylates and blocks the functions of Rho GTPases such as RhoA, Rac1, and Cdc42 (Just et al., 1995), inhibited the NRG1 effect by ~70% (Fig. 2 A). In contrast, C3 exoenzyme, which ADP ribosylates RhoA and blocks its function (Hirose et al., 1998), did not have any obvious effect. Furthermore, we transfected a siRNA for Rac1 or Cdc42 into Schwann cells. Knockdown of Rac1 inhibited the NRG1-induced migration in Boyden chambers by ~25% (Fig. S1 D, available at http://www.jcb.org/cgi/content/full/jcb.200709033/DC1) as well as migration from reaggregates on DRG axons (compare videos 1–4 for cells transfected with control siRNA with videos 5 and 6 for cells transfected with Rac1 siRNA, available at http://www.jcb.org/cgi/content/full/jcb.200709033/DC1), which is consistent with recent studies (Benninger et al., 2007; Nodari et al., 2007). Transfection with nonoverlapping siRNA, Cdc42-1 or Cdc42-2, decreased the migration in Boyden chambers by ~15% and 25%, respectively (Fig. S1, E and F), as well as decreasing the migration from reaggregates on DRG axons (videos 7 and 8 for cells transfected with Cdc42-2 siRNA). However, because the effect after knockdown of Rac1 or Cdc42 is weaker than that of Toxin B, it is possible that Rac1 activity transduces an intracellular ...
Figure 1. NRG1 promotes Schwann cell migration through the ErbB2 and 3 heterodimer. (A and B) The migration of primary Schwann cells was measured by using Boyden chambers. Filters were coated with DRG axonal membranes. After incubation for 6 h with normal or conditioned medium from DRG neurons containing 5 μg/ml of control IgG or ErbB3-Fc, Schwann cells were stained with Giemsa solution and the number of migrating cells was counted (16 independent fields). Bar, 50 μm. (C and D) Filters in Boyden chambers were coated with DRG axonal membranes, collagen (type I or IV), fibronectin, or laminin. Schwann cell migration was measured in the presence of normal medium or conditioned medium (eight independent fields). (E–G) In the presence of control IgG or ErbB3-Fc, Schwann cells were incubated with or without 20 ng/ml of NRG1, NT3, or IGF-I in Boyden chambers (eight independent fields). Filters were coated with collagen (type I). (H and I) Schwann cell reaggregates were placed onto DRG neurons and control IgG or ErbB3-Fc was added. After 6 h, DRG axons were stained with an antineurofilament antibody (red), and Schwann cells were stained with an anti-S100β antibody (green). The distance of migration was measured (n = 16). Bar, 100 μm. (J and K) Schwann cells were pretreated in the presence or absence of 10 μM AG825 and then incubated with or without 20 ng/ml NRG1 in Boyden chambers. The number of migrating cells was counted (16 independent fields). Bar, 50 μm. (L and M) Schwann cells were transfected with control, ErbB2, or ErbB3 siRNA and incubated with or without NRG1 in Boyden chambers (16 independent fields). To confirm the effects of siRNAs, the lysates of transfected cells were immunoblotted with an anti-ErbB2, ErbB3, or actin antibody. Error bars show ± SD. Data were evaluated by using one-way ANOVA (*, P < 0.01; ***, P < 0.02).
2004) on NRG1-induced migration. SP600125 and JNK inhibitor I inhibited the NRG1 effect by 80 and 70%, respectively (Fig. 3 A). We next immunoblotted with an antiphosphorylated JNK antibody that recognizes the phosphorylated or active state of JNK. The levels of phosphorylated forms were normalized to the amount of total JNK (n = 5). Error bars show ± SD. Data were evaluated by using one-way ANOVA (*, P < 0.01).

signal from NRG1 together with Cdc42 and that Rac1 and Cdc42 may share a common downstream signaling pathway. To examine whether NRG1 directly activates Cdc42 and Rac1, we performed affinity precipitation using the Rac1-GTP and Cdc42-GTP binding domain of Pak1. The activities of Rac1 and Cdc42 reached maximum levels at 60–120 min after stimulation with NRG1 and remained activated for at least 360 min (Fig. 2, B–I). Therefore, NRG1 activation of ErbB2 and 3 can stimulate the increase of Rac1-GTP and Cdc42-GTP to enhance Schwann cell migration.

We previously reported that JNK acts downstream of Rac1 and Cdc42 in NT3-induced migration of Schwann cells (Yamauchi et al., 2005a, b). In addition, the JNK cascade is the direct target of Rho GTPases in many other types of cells (Schmidt and Hall, 2002). Therefore, we investigated the effect of structurally unrelated JNK inhibitors SP600125 and JNK inhibitor I (Heo et al., 2004) on NRG1-induced migration. SP600125 and JNK inhibitor I inhibited the NRG1 effect by ~80 and 70%, respectively (Fig. 3 A). We next immunoblotted with an antiphosphorylated JNK antibody that recognizes the phosphorylated or active state of JNK. The levels of phosphorylated forms were normalized to the amount of total JNK (n = 5). Error bars show ± SD. Data were evaluated by using one-way ANOVA (*, P < 0.01).

The atypical GEF Dock7 mediates NRG1-induced Schwann cell migration

To identify the specific GEFs involved in the NRG1 regulation of Rac1 and Cdc42, we designed siRNA against Tiam1 and Dbs, major GEFs of the Dbl family for Rac1 and Cdc42, respectively, in Schwann cells (Yamauchi et al., 2005a, b). Neither Tiam1 nor Dbs knockdown had any effect on the NRG1-induced migration of Schwann cells (Fig. S1, A and B). Therefore, we examined the involvement of Dock7, a Dock180-related GEF
expressed abundantly in Schwann cells (Fig. S1 C). Transfection of nonoverlapping siRNA, Dock7-1 or Dock7-2, into Schwann cells knocked down the expression of endogenous Dock7, as revealed by immunoblotting with an anti-Dock7 antibody (Fig. S2, A–C). Dock7-1 or -2 siRNA inhibited the NRG1-induced migration in Boyden chambers by ~70% and 50%, respectively (Fig. 4, A and B), as well as inhibiting migration from reaggregates on DRG axons (videos 9 and 10 for cells transfected with Dock7-1 siRNA, available at http://www.jcb.org/cgi/content/full/jcb.200709033/DC1). Similarly, knockdown of Dock7 by Dock7-1 siRNA reduced the NRG1 activation of Rac1 by ~80%, Cdc42 by 80%, and JNK phosphorylation by 60% (Fig. 4, C–E). Despite the potential involvement of other GEFs in these signaling pathways, our results hint at the possible role for Dock7 in Schwann cell migration and provide the rationale and impetus for the subsequent experiments.

**ErbB2 directly binds and activates Dock7 by phosphorylating Tyr-1118 to regulate Schwann cell migration**

Because the DHR-2 domain of the Dock180-related GEFs shows catalytic activity (Brugnera et al., 2002; Coté and Vuori, 2002; Meller et al., 2002), we tested whether Rho GTPases could be activated by the DHR-2 domain of Dock7. The purified DHR-2 domain (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200709033/DC1) promoted the exchange, binding, and release of the guanine nucleotide for Rac1 and Cdc42 in a time-dependent manner (Fig. 5, A, B, D, and E), whereas no effect was observed for RhoA (Fig. 5, C and F). Catalytically active GEFs preferentially interact with guanine nucleotide–free forms of the small GTPases (Arthur et al., 2002; Schmidt and Hall, 2002; Rossman et al., 2005). A Gly-to-Ala mutation in the P loop of the small GTPases decreases their guanine nucleotide binding activities (Arthur et al., 2002). We performed an affinity precipitation of the DHR-2 domain of Dock7 with guanine nucleotide–free Rac1G15A, Cdc42G15A, or RhoAG17A as well as wild-type Rac1, Cdc42, or RhoA. DHR-2 specifically coprecipitated with Rac1G15A (Fig. 5 G) and Cdc42G15A (Fig. 5 I) but not with RhoAG17A (Fig. 5 K), which is consistent with the results from the guanine nucleotide exchange assays. In contrast, the DH and pleckstrin homology (PH) domains of Dbs affinity precipitated with Rac1G15A (Fig. 5 H) or Cdc42G15A (Fig. 5 J) as well as with RhoAG17A (Fig. 5 L). Similarly, the affinity precipitation with wild-type Rac1 or Cdc42 also showed binding to DHR-2 but was slightly weaker than the precipitation with each GTPase harboring the Gly-to-Ala mutation. To investigate whether NRG1 activation of the ErbB2 and 3 heterodimer stimulates the GEF activity of Dock7, we cotransfected the plasmids encoding wild-type Dock7, ErbB2, and ErbB3 into 293T cells and measured the exchange of the guanine nucleotide from immunoprecipitated Dock7 for Rac1 and Cdc42. The activity of wild-type Dock7 was significantly increased after stimulation with NRG1 (Fig. 5, M and O). Similarly, NRG1 promoted the affinity-precipitation of Dock7 with Rac1G15A or Cdc42G15A (Fig. 5, N and P).

We asked if ErbB2 could directly phosphorylate Dock7 because Dock7 is stimulated after the activation of the ErbB2 and 3 heterodimer and apparently possesses various tyrosine phosphorylation sites. The recombinant intracellular kinase domain of ErbB2 (HTScan ErbB2 kinase) phosphorylated the purified wild-type Dock7 protein (Fig. S3 B) and coprecipitated with Dock7 in vitro (Fig. 6 A). To investigate the potential sites on Dock7 that could be tyrosine phosphorylated by ErbB2, we transfected a plasmid encoding the N-terminal region containing DHR-1 (aa 1–691), middle region 1 (aa 692–1110), middle region 2 (aa 1111–1431), or DHR-2 (aa 1432–1992) into 293T cells (Fig. 6 B). Because the amino acid sequence positioned between DHR-1 and -2 is quite extensive, it was divided into two regions. The ErbB2 kinase coprecipitated with both middle regions...
and phosphorylated the middle region 2 (Fig. 6 C). These results are also supported by findings that the middle region 2, acting as a specific substrate for ErbB2, has a dominant-negative effect on NRG1-induced migration (Fig. S4, A–D), and phosphorylation of Tyr-1118 by the ErbB2 kinase is associated with activation of Rac1 (Fig. S4, E, F, and I) and Cdc42 (Fig. S4, G, H, and J). Because the middle region 2 of Dock7 contains six tyrosine residues that may be phosphorylated by ErbB2 (Fig. 6 D), we made a series of constructs harboring Tyr-to-Phe mutations in the middle region 2. The ErbB2 kinase had the ability to phosphorylate the Y1138F, Y1225F, Y1233F, Y1375F, and Y1429F mutants of the full-length Dock7 and analyzed the phosphorylation by the ErbB2 kinase. In Fig. 6 F, the Y1118F mutation greatly reduced the phosphorylation of Dock7 by the ErbB2 kinase, whereas the Y1138F, Y1225F, Y1233F, Y1375F, and Y1429F mutations did not possess inhibitory effects on phosphorylation. Similarly, the purified Y1118F Dock7 (Fig. 6 G) reduced phosphorylation by the ErbB2 kinase (Fig. 6 E). Amino acid sequences surrounding Tyr-1118 in Dock7 are uniquely conserved among mammalian Dock7 proteins but not in the Dock180-related GEFs homologous to Dock7 and those from other species (Fig. 6 H).

To clarify whether ErbB2 directly activates Dock7 and whether Tyr-1118 is critical for ErbB2-dependent GEF activity, we incubated the purified wild-type or Y1118F Dock7 with the ErbB2 kinase and performed guanine nucleotide release assays in vitro. ErbB2 stimulated guanine nucleotide release.
Figure 6. **ErbB2 directly binds and activates Dock7 by phosphorylating Tyr-1118.** (A) 250 ng of immobilized full-length FLAG-Dock7 protein was incubated in 30 μl of reaction buffer containing 20 μM of cold ATP in the presence or absence of 100 ng ErbB2 kinase for 30 min, washed, and immunoblotted with an anti-phospho-tyr or ErbB2 antibody. Immobilized FLAG-Dock7 was also stained with Coomassie brilliant blue. (B) The schematic structures of Dock7 and the domains are illustrated. Red rectangle, Tyr in the middle region 2. (C) 293T cells were transfected with the plasmid encoding DHR-1, middle region 1, middle region 2, or DHR-2 of Dock7. The lysates of transfected cells were immunoprecipitated with an anti-FLAG antibody, incubated with ErbB2 kinase and ATP, and immunoblotted with an anti-phospho-tyr or ErbB2 antibody. A shift in the mobility of the bands for the tyrosine-phosphorylated protein was observed. The cell lysates were also immunoblotted with an anti-FLAG antibody. (D) The amino acid sequences containing six tyrosine residues in the middle region 2 are shown. (E) Cells were transfected with the plasmid encoding each middle region 2 containing one Tyr-to-Phe mutation. The samples, immunoprecipitated with the anti-FLAG antibody, were incubated with ErbB2 kinase and ATP. A shift in the mobility was observed in bands of the tyrosine-phosphorylated protein. The tyrosine phosphorylation of the constructs and their expression are also shown. (F) Cells were transfected with each full-length Dock7 harboring one Tyr-to-Phe mutation in the middle region 2, immunoprecipitated with anti-FLAG antibody, and incubated with ErbB2 kinase and ATP. The tyrosine phosphorylation of the constructs and their expression are also shown. (G) 250 ng of immobilized full-length FLAG-Dock7 or FLAG-Dock7Y1118F was incubated with ErbB2 kinase and ATP. (H) A comparison of the amino acid sequences surrounding the ErbB2 phosphorylation sites (red squares) of mammalian Dock7 and other homologous proteins is shown. Black, conserved amino acids; grey, nonconserved amino acids. (I and J) Immobilized FLAG-Dock7 or the Y1118F mutant was incubated in 30 μl of reaction buffer containing 20 μM of cold ATP in the presence or absence of ErbB2 kinase and washed. The release of [3H]GDP from GST-Rac1-[3H]GDP or Cdc42-[3H]GDP by immobilized proteins was measured (n = 3). Error bars show ±SD. Data were evaluated by using one-way ANOVA (*, P < 0.01).
of Dock7 for Rac1 and Cdc42, whereas the Y1118F mutation in Dock7 abolished the release (Fig. 6, I and J). These results provide evidence that ErbB2 directly binds to Dock7 and phosphorylates the Tyr-1118 position to activate Rac1 and Cdc42 in vitro.

To explore whether ErbB2 phosphorylates endogenous Dock7 in Schwann cells, we made an antibody specific to phosphorylated Tyr-1118 of Dock7 (Fig. S2, D and E). Stimulation with NRG1 dramatically enhanced the phosphorylation of Tyr-1118, and this phosphorylation was reduced by AG825 or ErbB3-Fc (Fig. 7 A). NT3 or IGF-1 did not enhance the phosphorylation of Dock7, indicating that the Tyr-1118 position is specific for NRG1. In addition, Dock7 was immunoprecipitated with ErbB2, and this interaction was enhanced by stimulation with NRG1 (Fig. 7 B). Stimulation with NRG1 also increased the colocalization of Dock7 with ErbB2 (Fig. 7 C, middle) and phosphorylated Dock7 at Tyr-1118 with ErbB2 (Fig. 7 C, bottom) and phosphorylated Dock7 at Tyr-1118 in cell bodies and in processes. In addition, after stimulation with NRG1, colocalization of phosphorylated Dock7 with phosphorylated ErbB2 was observed in punctate structures (Fig. 7 C, top). Furthermore, NRG1 induced an affinity precipitation of Dock7 with Rac1G15A or Cdc42G15A (Fig. 7 D, D and E). Thus, stimulation with NRG1 can promote the binding of Dock7 with ErbB2, phosphorylate Dock7 at the Tyr-1118 position, and regulate the Dock7 activity in Schwann cells.

Next, we investigated the role of the phosphorylation of Dock7 at the Tyr-1118 position in Schwann cell migration. We co-transfected a plasmid encoding Dock7-1 siRNA-resistant wild-type or Y1118F Dock7 together with a control or Dock7-1 siRNA into Schwann cells. Expression of siRNA-resistant wild-type Dock7 reversed the Dock7-1 siRNA-mediated inhibition of NRG1-induced migration in Boyden chambers, whereas Y1118F Dock7 failed to rescue Dock7-1 siRNA-mediated inhibition of migration (Fig. 8, A and B). Because there is the possibility that the Y1118F mutation has an effect on the protein conformation of Dock7, we tested the effects of the other mutants, Y1138F, Y1225F, Y1233F, Y1375F, and Y1429F, on migration. The Y1138F, Y1225F, Y1233F, or Y1375F mutant rescued the phosphorylation of Dock7 at Tyr-1118 with ErbB2, and this interaction was enhanced by stimulation with NRG1 (Fig. 7 C, bottom), indicating that the Tyr-1118 position is required for migration. (Fig. 8, D and E), indicating that the Y1118F mutation mimics the nonphosphorylated form and that the phosphorylation at the Tyr-1118 position is required for migration. The Y1429F mutant could rescue siRNA-mediated inhibition of migration but did not completely. The reason may be that Tyr-1429 interacts functionally with the catalytic DHR-2 because it is adjacent to DHR-2. Alternatively, because the Tyr-1429 position is contained in the canonical phosphatidylinositol-3-kinase binding motif Tyr-X-X-Met (Fig. 5 D; Ponzetto et al., 1993), the binding may partially affect Dock7 activation (Côté et al., 2005). Expression of Dock7-1 siRNA-resistant constructs was not down-regulated by cotransfection with Dock7-1 siRNA, which specifically reduced expression of native siRNA-sensitive nucleotide sequence of Dock7 (Fig. 8 C).

Consistent with the results in the previous paragraph, expression of Dock7-1 siRNA-resistant wild-type Dock7 reversed Dock7-1 siRNA-mediated inhibition of NRG1-induced migration from reaggregates on DRG axons (Fig. 9, A and B). In contrast, expression of siRNA-resistant Y1118F Dock7 did not rescue siRNA-mediated inhibition of migration. These results indicate again that the phosphorylation of Dock7 at the Tyr-1118 position by ErbB2 plays a key role in promoting Schwann cell migration.

Discussion

Each stage in Schwann cell development involves characteristic morphological changes regulated by reciprocal and complex glial-neuronal interactions. Membrane-bound NRG1, expressed primarily on axons, represents an essential determinant in controlling myelination by Schwann cells (Nave and Salzer, 2006). In this paper, we demonstrate that NRG1 binding to the ErbB2 and 3 heterodimer promotes migration of premyelinating Schwann cells and that this effect is mediated by the direct activation of the Dock180-related GEF Dock7 and the subsequent Rho GTPase cascade. This conclusion is supported by the findings that blocking the signaling molecules coupling the ErbB receptor to the Rho GTPase cascade results in the attenuation of migration. Importantly, we identify Dock7 as the functional intracellular substrate for the ErbB2 receptor. ErbB2 directly binds to Dock7 and promotes the GEF activities for Rho GTPases by phosphorylating Tyr-1118 in vitro. Stimulation with NRG1 in Schwann cells leads to the phosphorylation of Dock7 at Tyr-1118 and activation. Transfection of Dock7 harboring the Tyr-1118-to–Phe mutation inhibits the NRG1-induced migration. These results demonstrate that NRG1 activation of the ErbB2 and ErbB3 heterodimer induces Schwann cell migration through an unexplored mechanism, namely that a receptor-mediated tyrosine phosphorylation event triggers the activation of Dock7. Dock7 has a GEF activity that is preferential for Cdc42 rather than Rac1; however, Schwann cell migration by NRG1 requires both Rac1 and Cdc42. Because Schwann cells modestly express Dock3, 4, and 5 of the Rac1-specific Dock180-related GEFs, they may cooperatively support the remaining NRG1-dependent Rac1 activity.

Possible alternative regulation of Dock7 in Schwann cell migration

It is clear that ErbB2 phosphorylates and activates Dock7 in vitro; however, the question of whether Dock7 can be activated by an alternative mechanism remains. Besides the catalytic DHR-2 domain, Dock180-related GEFs contain another conserved domain, termed DHR-1 (also called City-zizim homology domain 1). The putative phospholipid-binding C2 domain is found in the DHR-1 domain of Dock180. Dock180 binds to phosphatidylinositol-3,4,5-triphosphate, the product of phosphatidylinositol-3-kinase (Côté et al., 2005). Because ErbB2 can activate phosphatidylinositol-3-kinase (Garratt et al., 2000; Citri et al., 2003), it is possible that phosphatidylinositol-3,4,5-triphosphate modulates cellular functions of Dock7. Dock180 also has some protein-protein interactive domains. Dock180 interacts with the proline-rich region of engulfment and cell motility (ELMO) family proteins and the Src homology (SH) 3 domain of CrkII through the N-terminal SH3 domain and the C-terminal proline-rich sequence, respectively (Hasegawa et al., 1996; Brugnera et al., 2002).
The ELMOs–CrkII–Dock180 complex is required for activating Rac1 along the periphery of a cell, leading to lamellipodial formation. The activation of Rac1 through Dock180 has an alternative mechanism when dealing with the Rho GTPase RhoG. Once activated, RhoG forms a ternary complex with ELMOs–CrkII–Dock180 (Katoh and Negishi, 2003). The RhoG–GTP–ELMOs–CrkII–Dock180 complex induces morphological changes at the cell periphery. However, Dock7 does not interact with ELMO1 and CrkII (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200709033/DC1) because it is unlikely that Dock7 contains either an SH3 domain or a proline-rich region. It will be of interest to examine the binding partners of Dock7.

Figure 7. NRG1, acting through the phosphorylation of Dock7 at Tyr-1118, regulates Schwann cell migration. (A) After stimulation with vehicle or 20 ng/ml of NRG1, NT3, or IGF-I for 30 min, Schwann cells were lysed, immunoprecipitated with an anti-Dock7 antibody, and immunoblotted with an anti-(pTyr1118)Dock7 antibody. In some experiments, cells were treated with or without AG825 or ErbB3-Fc. The cell lysates were also immunoblotted with an anti-Dock7 antibody. (B) After stimulation with vehicle or NRG1, immunoprecipitated Dock7 was immunoblotted with an anti-ErbB2 antibody. Immunoblots for ErbB2, ErbB3, and Dock7 are shown. (C) After stimulation with vehicle or NRG1, Schwann cells were costained with the following antibodies: anti-(pTyr1118)Dock7 (green; top) and anti-(pTyr1118)Dock7 (red; top), anti-(pTyr1118)Dock7 (green; middle) and anti-ErbB2 (red; middle), and anti-Dock7 (green; top) and anti-ErbB2 (red; bottom). After stimulation with vehicle or NRG1, increased colocalization (indicated by arrows) was observed (Bar, 25 μm). a–d are magnifications of the boxed areas as indicated (Bar, 10 μm). Dotted lines indicate the outlines of cells. (D and E) Affinity precipitation with GST-Rac1G15A or Cdc42G15A was performed and immunoblotted with an anti-Dock7 antibody. Immunoblots for Dock7 are shown.
using yeast two-hybrid or affinity chromatography techniques and to analyze various regulatory mechanisms of Dock7 in Schwann cells. In addition, elucidation of the 3D structures of Dock7 and the other Dock180-related GEFs should provide valuable information concerning how the phosphorylation by ErbB2 activates Dock7 in Schwann cells.

The regulation of downstream signaling pathways involved in Schwann cell morphology and function

Rac1 and Cdc42 control the formation of membrane protrusions, including lamellipodia and filopodia, which are essential for the migration of many types of cells including neurons. Rac1 and
Cdc42 regulate actin polymerization by activating the Arp2/3 complex through their effectors, the Wiskott-Aldrich syndrome protein (WASP) and the WASP family verprolin homologous protein families (Takenawa and Miki, 2001). In addition, the Rac1 and Cdc42 effector Pak family controls actin filament dynamics by phosphorylating myosin light chain kinases or LIM domain kinases (Zhao and Manser, 2005). The JNK signal is a key downstream effector of the Dock7-activated Rho GTPases in Schwann cell migration, but it is conceivable that effector proteins such as WASP, WASP family verprolin homologous protein, and Pak families can also influence migration by altering the actin cytoskeleton. Further studies may explain how signals through Rho GTPases are coordinately transduced with JNK to induce cell migration.

JNK has been originally identified as the kinase that phosphorylates the transcription factor c-Jun. Indeed, fibroblast migration likely requires c-Jun phosphorylation, but JNK has some key substrates that include cytoskeletal components (Huang et al., 2004). One particular JNK substrate candidate implicated in cell migration is the focal adhesion adaptor protein paxillin because JNK phosphorylates paxillin to regulate migration of bladder tumor epithelial NBT-II cells (Huang et al., 2003). Additionally, another candidate molecule may be the microtubule-associated proteins (MAPs). Mice deficient in JNK1, as well as pharmacological inhibition of JNK activity, exhibit a progressive morphological alteration associated with defective neuronal migration (Chang et al., 2003; Kawauchi et al., 2003). Hypophosphorylation of MAP2 and MAP1B is also observed with an increase in microtubule stability, although it is unclear whether JNK regulates microtubule dynamics by phosphorylating MAP2 and MAP1B. Because paxillin, MAP2, and MAP1B are widely expressed and control various cellular functions, they may act cooperatively as targets of JNK to assist migration of Schwann cells.

In the present study, we identify Dock7 as a downstream effector of ErbB2. This interaction mediates NRG1-induced migration of premyelinating Schwann cells. It is noteworthy to add that NRG1, acting through the ErbB2 and 3 heterodimer, enhances myelination by Schwann cells (Bunge, 1993; Garratt et al., 2000; Citri et al., 2003) as well as migration. Because migration precedes myelination, certain mechanisms may be preserved in both processes. Myelination by Schwann cells is mediated by the polarity protein Par-3, whose complex generally involves the Rac1 and Cdc42 effector Par-6 (Chan et al., 2006). Recently, Watabe-Uchida et al. (2006) reported that Dock7 regulates the polarity formation of axons and dendrites through Rac1 in hippocampal neurons. It is possible that the Dock7-mediated Rho GTPase activation may lead to the formation of a polarity complex that will ultimately trigger myelination. The chemical compound NSC23766 is a first generation Rac1-specific inhibitor identified by a structure-based in silico screening (Gao et al., 2004). It fits into a small GTPase binding groove on the Rac1-specific GEFs Tiam1 and Trio of the Dbl family. The development of chemical inhibitors specific for Dock7 and the in vivo application of siRNA oligonucleotides will help to elucidate the role of Dock7 in the myelination process both in vitro and in vivo, as well as in various pathological states originating from aberrant regulation of the ErbB receptors (Tsai et al., 1996; Citri et al., 2003).
Materials and methods

Antibodies and inhibitors

The following antibodies were purchased: anti-ErbB2, anti-ErbB3, anti-JNK1, anti-Tiam1, anti-Dsb, anti-HA, and anti-MBP (Santa Cruz Biotechnology, Inc.); anti-autophosphorylated (Tyr1182) ErbB2 (Invitrogen); anti-phosphorylated active (pThr185/pTyr186) JNK (Cell Signaling Technology); anti-phosphorylated Tyr (pY) and anti-NGF (Millipore); anti-Rac1, anti-Cdc42, and anti-actin (BD Biosciences); anti-S100β (Dako); antineurofilament (Covance); anti-FLAG (Sigma-Aldrich); and anti-GFP (Medical & Biological Laboratories).

The rabbit antisera for Dock7 was generated against a KEAFLHP-SPDEEE peptide. The polyclonal anti-Dock7 antibody was affinity purified using a peptide-conjugated resin. The rabbit antisera for phosphorylated (pY1182) Dock7 was generated against a phosphorylated peptide ETVPQpYDFTET. The polyclonal anti-(pY1182)Dock7 antibody was affinity purified using a phosphorylated peptide conjugated resin from nonadsorbed fractions of a nonphosphorylated peptide ETPQYLpYDFTET-conjugated resin. Peroxidase and fluorescence-labeled secondary antibodies were purchased from GE Healthcare and Invitrogen, respectively. The following antibodies were purchased: ErbB3-Fc, which possesses the extracellular domain of ErbB3 fused to the Fc region of an IgG (R&D Systems); and C. difficile toxin B, Clavium dulotum C3 esterase, AG825, SP600125/JNK inhibitor II, and JNK inhibitor I (EMD).

Plasmids

The coding regions of three alternative splicing variants of Dock7 (available from GenBank/EMBL/DDBJ under accession nos. DQ118679, DQ118680, and DQ309763) were isolated by the method of 3′ and 3′ rapid amplification of cDNA from human brain (Marathon-Ready cDNA, Clontech Laboratories, Inc.), according to the manufacturer’s protocol. The major variant DQ118679 was ligated into the mammalian FLAG- and GFP-tagged expression vectors pCMVFLAG and pEGFP-C1. The cDNA fragments encoding DHR-1 (aa 1 – 691), middle region 1 (aa 692 – 1110), middle region 2 (aa 1111 – 1431), and DHR-2 (aa 1432 – 1992) of Dock7 were also inserted into pCMVFLAG. The constructs of the full-length Dock7 harboring the Tyr1118-to-Ph, Tyr1138-to-Ph, Tyr1225-to-Ph, Tyr1233-to-Ph, Tyr1375-to-Ph, or Tyr1429-to-Ph mutation and the Y1118F, Y1138F, Y1225F, Y1233F, Y1375F, and Y1429F mutants of the full-length Dock7 were also inserted into pEGFP-C1. The wild-type and Y1118F, Y1138F, Y1225F, Y1375F, and Y1429F mutants of the full-length Dock7 were produced by the overlapping PCR method and ligated into pCMV-FLAG. The wild-type and Y1118F, Y1375F, and Y1429F mutants of the full-length Dock7 were subcloned into pcDNA3.1(+) (Invitrogen) containing 10% FBS and 100 ng/ml NGF on collagen-coated dishes (Yamauchi et al., 2004). After 2–3 wk, DRG neurons were cultured in DME-Glutamax containing 10% FBS and 20 ng/ml NGF. 293T and Cos-7 cells (Human Science Research Resource Bank) were cultured in tissue culture dishes in DME containing 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin, and Cos-7 cells were plated for experiments on collagen-coated dishes. Before the experiments, 293T and Cos-7 cells were cultured in DME containing 1% FBS and 1 mg/ml BSA for 24 h. Unless otherwise indicated, Schwann cells and Cos-7 cells were pretreated with or without 2 ng/ml C. difficile Toxin B for 24 h, 2 μg/ml C3 exoenzyme for 24 h, 10 μM AG825 for 45 min, 10 μM SP600125 for 45 min, or 20 μM JNK inhibitor I for 24 h before stimulation with 20 ng/ml NT3 (R&D Systems), NT3 (PeproTech), or IGF-I (Invitrogen) for 0–360 min. To confirm cell localization under these experimental conditions, Schwann cells and Cos-7 cells were stained with 0.4% trypan blue. Trypan blue–incorporating cells were <1% in each experiment.

Boyden chamber migration assay

Cell migration was routinely measured using a 24-well Boyden chamber, as previously described (Yamauchi et al., 2004). In brief, in the case of assessing the effect of ErbB3-Fc on DRG neurons’ conditioned medium, polyethylene terephthalate (8-μm pore size) filters were coated with axonal membranes from DRG neurons (Grimes et al., 1996). In other experiments, filters were essentially coated with collagen (type I), except for the comparison of extracellular matrix proteins collagen (type IV), fibronectin, and laminin. Cells (0.5 × 10^6 cells for Schwann cells or 5 × 10^5 cells for Cos-7 cells) in 500 μl of normal medium per well were loaded into the upper chamber, which was inserted into the tissue culture dish in 750 μl of conditioned medium containing 5 μg/ml anti-NGF antibody in the presence of 5 μg/ml of control IgG or ErbB3-Fc per well or in normal medium containing 50 ng/ml NGF, NT3, or IGF-I per well. After incubation at 37°C for 6 h, the filters were stained with Giemsa solution or fixed with PFA to detect cells expressing GFP. No difference in cell number was observed at 6 h in the presence or absence of NGF1, NT3, or IGF-I. The number of stained or GFP-fluorescent migrating cells at the bottom surface of the filters was counted at four fields per filter in two to four independent experiments. In the presence of 5 μg/ml of control IgG or ErbB3-Fc for 8 h, trypan blue–incorporating cells were <0.5%.

Migration assay using reaggregated Schwann cells

To mimic physiological conditions, Schwann cell migration was also measured using DRG neurons and reaggregated Schwann cells as described previously (Yamauchi et al., 2004). In brief, DRG neurons were plated onto the center of a collagen-coated dish and allowed to extend axons outwardly. Schwann cell reaggregates were formed by plating Schwann cells on Ultra Low Attachment dishes (Corning) for 4 h and on Petri dishes (Boraworld Scientific) for 20 h with gentle agitation every 3–4 h. In the case of analyzing the effect of 5 μg/ml ErbB3-Fc, 5 μg/ml anti-NGF antibody was added into culture medium of DRG neurons. For the other experiments, the medium was changed into normal medium containing vehicle or 20 ng/ml NGF. Individual Schwann cells were allowed to migrate out of the reaggregates along the axons. After incubation at 37°C for 6 h, cells were fixed with PFA, blocked, and immunostained. The distance of migration was calculated by measuring the size of the reaggregates over time, subtracting the mean initial size of the reaggregates, and dividing the remaining distance in half. Experiments were performed by measuring eight reaggregates per dish in two independent experiments.

Fluorescence images

Cells on collagen-coated glass coverslips or filters of Boyden chamber and reaggregated Schwann cells were air-dried, fixed with 4% PFA, blocked with Immuno-Block (Dainippon Sumitomo Pharma) in phosphate-buffered saline–0.1% Tween-20, incubated with primary antibodies, and treated with fluorescence-labeled secondary antibodies. The coverslips and filters were mounted with Vectashield (Vector Laboratories) onto slides for observation by confocal and fluorescence microscopy. The confocal images were collected with a microscope (IX81; Olympus) with a laser-scanning FV500 confocal (Olympus) equipped with 1 μm filter set. The primary antibodies used for confocal images were anti-Dock7, anti- (pY1182)Dock7, anti-ErbB2, and anti- (pY1182)ErbB2. The fluorescence images were captured with a microscope system (TE-300; Nikkon) and analyzed with AxioVision software (Carl Zeiss, Inc.). The primary antibodies used for the fluorescence images were anti-S100β to identify Schwann cells when GFP constructs were not transfected and anti-neurofilament to identify DRG axons. The live imaging was performed using a microscope.
system (DM4000B, Leica) equipped with an INKJ2-ZILCS stage top incubator (Tokai Hit) and AF6000 software (Leica). The time frame was 60–300 min after putting Schwann cell reaggregates on DRG neurons, which were replaced with a fresh medium in the presence or absence of 20 ng/ml NRG1. To avoid fading of the GFP fluorescence, the intensity levels were fixed at less than position 2. Captured images were thus adjusted using the brightness switch on AF6000 software. Image sequence was recorded at one frame per 5 s and played at three frames per second.

Immunoprecipitation and immunoblotting
Cells were lysed in lysis buffer B (50 mM Hepes-NaOH, pH 7.5, 200 mM MgCl₂, 150 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethane sulfonylflouride, 1 μg/ml leupeptin, 1 mM EDTA, 1 mM NaVO₄, 10 mM NaF, and 0.5% NP-40) and the lysates were centrifuged. The supernatants were mixed with protein G resin that was preadsorbed with various antibodies. The immunoprecipitates or the proteins in the cell lysates were denatured and then subjected to SDS-PAGE. The electrophotothoretically separated proteins were transferred to PVDF membranes, blocked, and immunoblotted. The bound antibodies were detected using the ECL or ECL-Plus system (GE Healthcare). The band images were captured with a GT-7000U scanner (Epson) and analyzed with ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/).

In vitro tyrosine-phosphorylation reaction
250 ng of purified immobilized FLAG-Dock7 proteins were incubated with 20 μM of cold ATP in the presence or absence of 100 ng ErbB2 kinase in 30 μl of reaction buffer (20 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethane sulfonylfluoride, 1 μg/ml leupeptin, and 1 mM EDTA) at 30°C for 30 min and then chilled on ice. Tyrosine-phosphorylated FLAG-Dock7 proteins were washed with reaction buffer and used for guanine nucleotide releasing assays for Rac1 and Cdc42.

Guanine nucleotide exchange assays
Guanine nucleotide exchange assays were performed as previously described (Yamauchi et al., 2005a). In brief, for the guanine nucleotide binding assay, 125 ng of immobilized FLAG–Dock7–DHR-2 or the immunoprecipitates were incubated in 30 μl of reaction buffer containing 16 ng/μl each of GST–Rho GTPase, 33 ng/μl BSA, and 3 μM [32P]GDP (0.3 cpm/μl) at 30°C for 0–30 min. The reactions were stopped by adding 1 μl of ice-cold wash buffer (20 mM Hepes–NaOH, pH 7.5, and 10 mM MgCl₂) and filtered through 0.45-μm nitrocellulose membranes. The membranes were immediately washed with ice-cold wash buffer and air dried. The radioactivity remaining on each membrane was measured using a LSC-6100 liquid scintillation counter (Alkata). For the guanine nucleotide–releasing assay, [32P]GDP-bound GST–Rho GTPases were obtained by incubation with reaction buffer containing 125 ng/μl each of Rho GTPase, 250 ng/μl BSA, 1 μM GDP, and 0.3 μM [32P]GDP (0.3 cpm/μl) at 30°C for 90 min. The reaction was stopped by adding 5 mM MgCl₂, and mixtures were immediately cooled on ice. 125 ng of immobilized FLAG–Dock7–DHR-2, 250 ng FLAG–Dock7 proteins, or the immunoprecipitates were incubated in 30 μl of reaction buffer containing 16 ng/μl GST–Rho GTPase–[32P]GDP, 33 ng/μl BSA, and 3 μM of cold GDP at 30°C for 0–30 min. The reaction was stopped and filtered. The radioactivity remaining on each membrane was measured. 3–10 separate experiments were performed.

RNA preparation and RT-PCR analysis
Total RNA was isolated by Trizol reagent (Invitrogen). The cDNA were prepared from 1 μg of total RNA with Superscript II (Invitrogen), according to the manufacturer’s instructions. PCR amplification (Takara Bio, Inc.) was performed at 25 cycles, each cycle consisting of denaturation at 94°C for 1 min, annealing at 56.5°C for 1 min, and extension at 72°C for 1 min. The primers used were the following: 5′-GGACCTGGAGTGCATCTTCTTC-3′ (sense) for Dock7; 5′-ACCTTCTAATCGACAAGCTGC-3′ (sense) and 5′-TCACTCTGCGACTCACTTAAGCT-3′ (antisense) for Dock8; 5′-ATCTTGAAGTGATGTGTCCAC-3′ (sense) and 5′-TACCCAGCAAGTCTGATGAGGAG-3′ (antisense) for Dock9; 5′-GACTTACAACATTTGGCCTCATTAGTATG-3′ (sense) and 5′-GAGATTTGGGAGTTCTTGCATCTTTGG-3′ (antisense) for Dock10; 5′-GAGAGAACGTCACTCAGATGACTAC-3′ (sense) and 5′-CATAAGAAGCTCCAGCTGTCGCT-3′ (antisense) for Dock11; and 5′-ATGGAGATATCGCTGCGCTC-3′ (sense) and 5′-GACGATATCGCTGCGCTC-3′ (antisense) for Dock12.

Recombinant proteins
Unless otherwise indicated, all steps were performed at 4°C as previously described (Yamauchi et al., 2005a,b; Chan et al., 2006). FLAG-tagged DHFR, wild-type, and Y1118F proteins of Dock7 were purified from serum-starved 293T cells transiently transfected with pcMV–FLAG–Dock7–DHR-2, pCMV–FLAG–Dock7 Y1118F, respectively, using the CalPhos transfection reagent (Takara Bio Inc.) according to the manufacturer’s protocol. In brief, cells were lysed in lysis buffer A (50 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 3 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethane sulfonylfluoride, 1 μg/ml leupeptin, 1 mM EDTA, and 0.5% NP-40) and centrifuged. The supernatants were mixed with protein G resin (GE Healthcare) that was preadsorbed with an anti-FLAG antibody. Bound Flag–Dock7 proteins were extensively washed with lysis buffer A containing 500 mM NaCl and subsequently with lysis buffer A containing 500 mM NaCl and 50 mM EDTA and eluted with lysis buffer containing 20 mM FLAG peptide (Sigma-Aldrich), according to the manufacturer’s protocol. The buffer contained in elution fractions was exchanged with reaction buffer (20 mM Hepes–NaOH, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethane sulfonylfluoride, 1 μg/ml leupeptin, and 1 mM EDTA). The aliquots were stored at −80°C until use. The intracellular kinase domain of ErbB2 (HTScan ErbB2 kinase) was purchased from Cell Signaling Technology. GST-tagged Pak1-CRIB, wild-type Rho GTases, and guanine nucleotide–free Rho GTases were purified from E. coli BL21 (DE3) plus5 cells transformed with pET42a-Pak1-CRIB, pET42o–wild-type Rho GTases, and pET42a–guanine nucleotide–free Rho GTases, respectively. In brief, cells were treated with 0.4 mM isopropyl-1-thio-β-galactopyranoside at 37°C for 1.5 h, then centrifuged at 5000 g. A cell-free extract was made by the addition of 500 μg/ml lysozyme and 100 μg/ml DNase I in extraction buffer (50 mM Tris–HCl, pH 7.5, 15 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethane sulfonylfluoride, 1 μg/ml leupeptin, 1 mM EDTA, and 0.5% NP-40). The lysates were centrifuged, and the supernatants were mixed with glutathione resin (GE Healthcare). Bound proteins were washed with reaction buffer and eluted with extraction buffer containing 20 mM glutathione. The buffer contained in elution fractions was dialyzed against reaction buffer GST-Pak1-CRIB or GST-GTPases reaction buffer containing 0.1 μM GDP for GST-Pak1-CRIB or GST-GTPases. The aliquots were stored at −80°C until use. The Coomassie brilliant blue staining was performed by using the Rapid Coomassie or One Step Coomassie kit (Nakalai), according to the manufacturer’s protocol.

siRNA transfection
The siRNAs were transfected into primary Schwann cells using the OligoFectamine or Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. The medium was replaced at 24 h after transfection.

The efficiencies of protein depletion were 95 ± 3.1% for Dock7-1 siRNA, 87 ± 6.7% for Dock7-2 siRNA, 93 ± 5.5% for ErbB3 siRNA, 92 ± 5.7% for Tiam1 siRNA, 91 ± 7.9% for Dbs siRNA, 98 ± 0.33% for Rac1 siRNA, 81 ± 2.9% for Cdc42-1 siRNA, and 98 ± 1.8% for Cdc42-2 siRNA at 48 h after transfection.

siRNA preparation
The 21-nt siRNA duplexes were synthesized by Nippon Eptg. The target nucleotide sequences for the first Dock7 (Dock7-1) siRNA (5′-AACAGGGTCCTGATCATAAAG-3′), the second, nonoverlapping Dock7 (Dock7-2) siRNA (5′-AAGGTTAATAGGTCGACGAAGG-3′), the third, nonoverlapping Dock7 (Dock7-3) siRNA (5′-TGCAACATATTATGCAACTG-3′), the fourth, nonoverlapping Dock7 (Dock7-4) siRNA (5′-TGCAACATATTATGCAACTG-3′), and the fifth, nonoverlapping Dock7 (Dock7-5) siRNA (5′-TGCAACATATTATGCAACTG-3′) were designed according to an online software, siRNA Sequence Selector (Clontech Laboratories, Inc.; http://bioinfo. clontech.com/maidesigner/). The target sequences of the cDNA of Phthius pyralis luciferase siRNA was 5′-AAGCGGACTTACATCTGAG-3′, which
does not have significant homology to any mammalian gene sequences. To confirm cell viability under these experimental conditions, Schwann cells were stained with trypan blue. Trypan blue–positive cells in tissue culture dishes numbered <1% at 48 h after siRNA transfection (<0.5% for control luciferase siRNA, 0.7 ± 0.02% for Dock7-1 siRNA, 0.5 ± 0.03% for Dock7-2 siRNA, 0.5 ± 0.03% for ErbB2 siRNA, 0.7 ± 0.00% for ErbB3 siRNA, 0.5 ± 0.09% for Tiam1 siRNA, 0.7 ± 0.07% for Dbs siRNA, 0.7 ± 0.05% for Rac1 siRNA, 0.5 ± 0.02% for Cdc42-1 siRNA, and 0.8 ± 0.03% for Cdc42-2 siRNA).

Plasmid transfection
For primary Schwann cells, pEGFP, pEGFP-Dock7-1 siRNA-resistant wild-type, Y1118F, Y1138F, Y1225F, Y1375F, or Y1429F Dock7 was cotransfected with control, Dock7-1, Rac1, or Cdc42-2 siRNA by using the lipofectamine 2000 reagent or Nucleofector II (Amazxa Biosystems) with the Basic Neonucleofector Transfection kit (Amazxa Biosystems), according to the manufacturer’s protocol. Transfection efficiency was 15–20% using GFP-expressing plasmid as the control. The medium was replaced at 24 h after transfection. To perform the Boyden chamber migration assay, Schwann cells were cultured in Sato medium containing 1 mg/ml BSA for another 24 h. To assay the migration of reaggregated Schwann cells, cells were allowed to form reaggregates in DME containing 10% FBS for another 24 h. For 293T and Cos-7 cells, pCMV-FLAG-Dock7, pCMV-FLAG-Dock7Y1118F, pCMV-FLAG-Dock7Y1138F, pCMV-FLAG-Dock7Y1225F, pCMV-FLAG-Dock7Y1375F, pCMV-FLAG-Dock7Y1429F, pCMV-FLAG-Dock7-DHR1, pCMV-FLAG-Dock7-middle region 1, pCMV-FLAG-Dock7-middle region 2 or the tyrosine mutant, or pCMV-FLAG-Dock7-DHR2 was transfected with or without pCMV-ErbB2 and pCMV-ErbB3 using the CalPhos transfection reagent. Transfection efficiency typically exceeded 95% using GFP-expressing plasmid as the control.

Affinity precipitation of GEFs
Dock7 proteins or Dbs-DVIPH in the cell lysates was affinity precipitated with 20 μg GST-Rac1G15A, GST-Cdc42G15A, or GST-RhoAG17A, which are guanine nucleotide–free Rho GTPases. A Glide-Alo mutation of residue 15 in Rac1 and Cdc42 or residue 17 in RhoA decreases their nucleotide binding (Arthur et al., 2002). Active GEFs preferentially interact with guanine nucleotide-free forms of the small GTPases (Arthur et al., 2002; Schmid and Hall, 2002; Rossman et al., 2005). The affinity precipitation was also performed using 20 μg GST wild-type Rho GTPase (Rac1, Cdc42, or RhoA). Affinity-precipitated GEFs were detected by immunoblotting (Yamauchi et al., 2005a,b).

Detection of active Rho GTPases
To detect active GTP-bound Rac1 and Cdc42 in the cell lysates, we performed affinity precipitation by using 20 μg GST-Fox1-C relie, which binds to their GTP-bound forms. To compare the total amount of GTPase, immunoblotting was also performed with an anti-Rac1 or Cdc42 antibody. Two to five separate experiments were performed. The band intensity in the immunoblot was quantified, and the levels of Rac1-GTP and Cdc42-GTP were normalized to the amount of each total GTPase (Yamauchi et al., 2005a,b).

JNK assay
The cell lysates were immunoblotted with an anti-(pThr183/pTyr185)JNK antibody that recognizes the active form. To compare the total amount of JNK, immunoblotting was also performed with an anti-JNK antibody. Three to five separate experiments were performed. The band intensity in the immunoblot was quantified, and the levels of the phosphorylated forms were normalized to the amount of total kinase.

Statistical analysis
Values shown represent the mean ± SD from separate experiments. Analysis of variance (ANOVA) was followed by Fisher’s protected least significant difference post hoc comparisons (*, P < 0.01; **, P < 0.001; ***, P < 0.002).

Online supplemental material
Fig. S1 demonstrates that Schwann cell migration requires the activation of the Rhox family of small GTPases but is not dependent on Tiam1 or Dbs of the Dbl family GEFs. Fig. S2 characterizes the anti-Dock7 and anti-(pTyr11)-Dock7 antibodies. Fig. S3 shows the purification of the DHR2–wildtype, and Y1118F mutant proteins of Dock7. Fig. S4 illustrates that the middle region 2 of Dock7 inhibits NR31-induced migration and activation of Rac1 and Cdc42 in Cos-7 cells cotransfected with ErbB2 and ErbB3. Fig. S5 demonstrates that Dock7 does not interact with EIM01 and Crikil. Videos 1 and 2 illustrate time-lapse imaging of vehicle-stimulated migration from reaggregates of control siRNA-transfected Schwann cells. Videos 3 and 4 demonstrate NRG1-stimulated migration from reaggregates of control siRNA-transfected Schwann cells. Video 5 demonstrates vehicle-stimulated migration from reaggregates of Rac1 siRNA-transfected Schwann cells. Video 6 illustrates NRG1-stimulated migration from reaggregates of Rac1 siRNA-transfected Schwann cells. Video 7 illustrates vehicle-stimulated migration from reaggregates of Cdc42 siRNA-transfected Schwann cells. Video 8 shows NRG1-stimulated migration from reaggregates of Cdc42 siRNA-transfected Schwann cells. Video 9 shows vehicle-stimulated migration from reaggregates of Dock7 siRNA-transfected Schwann cells. Video 10 represents NRG1-stimulated migration from reaggregates of Dock7 siRNA-transfected Schwann cells.

We thank Drs. E.M. Shooter and Y. Kaziro for their participation in insightful discussions and for providing encouragement. We thank Drs. S. Kusakawa and S. Takashima for their participation in helpful discussions, M. Matsuda and K.S. Ravichandran for providing plasmids, and H. Aizawa and M. Yamamoto for assistance in the time-lapse imaging studies.

We thank Arun Prasad for his support with time-lapse imaging and A. Gallo and D. Wu for assistance in the time-lapse imaging studies. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japan Society for the Promotion of Science, and the Ministry of Human Health and Welfare of Japan, and was supported partially by research grants from the Astellas Metabolic Disease Foundation, the Human Science Foundation of Japan, the Kamo Medical Foundation, the Kato Bioscience Foundation, the Kowa Life Science Foundation, the Nakajima Foundation, the Samuro Kakuchi Memorial Foundation, and the Takeda Science Foundation.

Submitted: 6 September 2007
Accepted: 25 March 2008

References


