Rap1-PDZ-GEF1 interacts with a neurotrophin receptor at late endosomes, leading to sustained activation of Rap1 and ERK and neurite outgrowth

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Neurotrophins, such as NGF and BDNF, induce sustained activation of Rap1 small G protein and ERK, which are essential for neurite outgrowth. We show involvement of a GDP/GTP exchange factor (GEF) for Rap1, PDZ-GEF1, in these processes. PDZ-GEF1 is activated by GTP-Rap1 via a positive feedback mechanism. Upon NGF binding, the TrkA neurotrophin receptor is internalized from the cell surface, passes through early endosomes, and arrives in late endosomes. A tetrameric complex forms between PDZ-GEF1, synaptic scaffolding molecule and ankyrin repeat-rich membrane spanning protein which interacts directly with the TrkA receptor. At late endosomes, the complex induces sustained activation of Rap1 and ERK, resulting in neurite outgrowth. In cultured rat hippocampal neurons, PDZ-GEF1 is recruited to late endosomes in a BDNF-dependent manner involved in BDNF-induced neurite outgrowth. Thus, the interaction of PDZ-GEF1 with an internalized neurotrophin receptor transported to late endosomes induces sustained activation of both Rap1 and ERK and neurite outgrowth.

Introduction

Formation and extension of axons and dendrites, so-called neurite outgrowth, is a crucial event in neuronal differentiation and maturation during development of the nervous system (Da Silva and Dotti, 2002; Horton and Ehlers, 2003). These morphological changes require reorganization of the cytoskeletal networks and its accompanying membrane expansion and contraction (Craig et al., 1995; Mitchison and Kirschner, 1998). Rap1 small G protein has been shown to be involved in neurite outgrowth and neuronal polarization through regulating the MAPK cascade and the cytoskeletal network (York et al., 1998; Kao et al., 2001; Wu et al., 2001; Schwamborn and Püschel, 2004). Rho small G protein has been shown to be involved in the morphological development of neurons through regulating actin dynamics (Jalink et al., 1993; Kozma et al., 1997; Bito et al., 2000; Da Silva et al., 2003). We previously showed that inactivation of Rho is required for the Rap1-induced neurite outgrowth (Yamada et al., 2005). Rap1-activated Rho GTPase-activating protein, RA-RhoGAP, transduces a signal from Rap1 to Rho and inactivates Rho. The precise temporary and spatially activation of Rap1 is important for regulating the activity of RA-RhoGAP to produce the neurite. However, the mechanism underlying how and where Rap1 is activated remains unclear.

Neurite outgrowth begins with the activation of membrane receptors of neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (Huang and Reichardt, 2001; Sofroniew et al., 2001; Zweifel et al., 2005). NGF is the prototypic neurotrophin, a group of structurally related signaling proteins that are crucial for the survival, differentiation, and maintenance of specific neuronal population (Snider, 1994). First, NGF binds to TrkA receptor and induces its dimerization, which then activates its own tyrosine kinase and gathers a signaling complex consisting of SOS, a GDP/GTP exchange factor (GEF) specific for Ras small G protein, Grb2, an adaptor protein for SOS, and She, a linker protein between Grb2 and TrkA receptor, on the plasma membrane (Kao et al., 2001; Wu et al., 2001). Ras is transiently activated on the plasma membrane and induces activation of the c-Raf-MEK-ERK pathway. On the other hand, activated TrkA receptor gathers another signaling complex consisting of C3G, a GEF specific for Rap1, CrkL, an adaptor protein for C3G, and FRS2, a linker protein.
between Crkl and TrkA receptor, on the plasma membrane (Kao et al., 2001; Wu et al., 2001). TrkA receptor and the FRS2-Crk-C3G complex associating with the receptor are internalized into clathrin-coated vesicles and transported to early endosomes (Wu et al., 2001). Rap1 is activated at the early endosomes and induces activation of the B-Raf-MEK-ERK pathway. It has been believed that Rap1 is also activated on the cytoplasmic side of the plasma membrane, but the direct evidence has not been obtained. Then, TrkA receptor proceeds further along the degradative pathway in a microtubule- and motor-dependent fashion, reaching late endosomes, followed by the physical destruction of TrkA receptor by proteolysis in lysosomes (Jullien et al., 2002; Saxena et al., 2005a,b). Thus, transport of TrkA receptor to late endosomes has long been recognized as a means to terminate the signaling via degradation of the activated TrkA receptor complex after their internalization from the cell surface.

Recent studies suggest that TrkA receptor, after binding NGF at the cell surface, passes through early endosomes and reaches to late endosomes within 30 min (Saxena et al., 2005a). However, there is still sustained activation of Rap1 after 30 min (Kao et al., 2001; Wu et al., 2001). Like the activated TrkA receptor complex at early endosomes, TrkA receptor at late endosomes might be competent in the sustained activation of Rap1, but it remains unknown whether, or if so how, Rap1 is activated at late endosomes. Here, we addressed these issues and showed that activated TrkA receptor, which was transported to late endosomes, recruited PDZ-GEF1, a GEF specific for Rap1, by forming a complex through Trk receptors associating ankyrin repeat-rich membrane spanning (ARMS) and synaptic scaffolding molecule (S-SCAM). This tetramer complex then induced the sustained activation of Rap1 and ERK, eventually causing neurite outgrowth. ARMS is a tetraspan transmembrane protein and directly interacts with Trk receptors through each transmembrane domain (Kong et al. 2001; Arevalo et al. 2004). S-SCAM is a synaptic scaffolding molecule with six PDZ domains (Hirao et al., 1998).

Results

Activation of PDZ-GEF1 by GTP-Rap1

As PDZ-GEF1 has a Rap1-binding RA domain, we first examined whether the binding of GTP-Rap1 to the RA domain affects the GEF activity of PDZ-GEF1. We generated recombinant proteins of FLAG-tagged full-length PDZ-GEF1 (FLAG-PDZ-GEF1) and its RA domain-deficient mutant FLAG-PDZ-GEF1ΔRA and examined their GEF activity. FLAG-PDZ-GEF1 indeed showed GEF activity toward Rap1 as reported (de Rooij et al., 1999; Liao et al., 1999, 2001; Ohtsuka et al., 1999), while FLAG-PDZ-GEF1ΔRA showed much less effect on Rap1 (Fig. 1 A). The constitutively active form of GST-Rap1A enhanced the PDZ-GEF1 activity two-fold more than control GST did (Fig. 1 B), whereas it did not affect the GEF activity of FLAG-PDZ-GEF1ΔRA (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200610073/DC1). In addition, GST-Rap-binding domain (RBD) of RalGDS, a specific inhibitor of GTP-Rap1, inhibited the PDZ-GEF1 activity (Fig. 1 C). These results indicate that PDZ-GEF1 is activated by GTP-Rap1 and suggests that once PDZ-GEF1 is activated, its activation is amplified by GTP-Rap1 in a positive feedback mechanism.

Involvement of PDZ-GEF1 in the NGF-induced sustained activation of Rap1 at late endosomes

As PDZ-GEF1 has an auto-amplification GEF activity toward Rap1, we examined whether PDZ-GEF1 is involved in the NGF-induced sustained activation of Rap1 and ERK. We first performed loss-of-function experiments by use of RNA interference (RNAi) method for PDZ-GEF1. Immunoblotting showed that the amount of PDZ-GEF1 was markedly reduced by the knockdown of PDZ-GEF1 (Fig. 2 A). The amounts of other proteins, including TrkA receptor, another Rap1 GEF C3G, Rap1, Ras, and ERK remained unchanged (unpublished data). The knockdown of PDZ-GEF1 significantly decreased the activation of Rap1, but not Ras, after NGF stimulation as estimated by the pull-down assay (Fig. 2 A and unpublished data). The knockdown of PDZ-GEF1 did not inhibit the activation of ERK at 5 min after NGF stimulation, but it significantly decreased it at 30 and 60 min. The reason why we measured the activation of Rap1 and ERK at 30 and 60 min was that (1) it was reported that activation of Rap1 at 5 min does not contribute to transient activation of ERK at 5 min (York et al., 1998; Kao et al., 2001; Wu et al., 2001; Sasagawa et al., 2005); (2) that a substantial portion of the transient activation of ERK at 5 min mainly depends on Ras, but not on Rap1, whereas the activation of Rap1 at later time points, such as 30 and 60 min, but not at 5 min, contributes to the sustained activation of ERK at 30 and 60 min; and (3) that this sustained activation of ERK is essential for neurite outgrowth.

Consistent with the results of the pull-down assay, the fluorescence resonance energy transfer (FRET) imaging assay showed that the knockdown of PDZ-GEF1 reduced the activation of Rap1 at the perinuclear region of the cells after NGF stimulation (Fig. 2 Ba). To rescue the knockdown of PDZ-GEF1, we generated siRNA-resistant PDZ-GEF1 expression vector (Fig. 2 Bb). Expression of siRNA-resistant PDZ-GEF1 potently rescued the activation of Rap1 at the perinuclear region of the cells at 30 min after NGF stimulation (Fig. 2 Bc). Rap1 has been shown to localize at various intracellular organelles, such as the Golgi complex, early endosomes, and late endosomes, in cultured fibroblasts and epithelial cells (Beranger et al., 1991; Pizon et al., 1994). To examine the localization of endogenous Rap1 in PC12 cells, we performed subcellular fractionation from the post-nuclear supernatant (PNS) of PC12 cells. A significant amount of Rap1 was recovered in the EEA1-positive early endosomal fraction and the Rab7-positive late endosomal fraction (Fig. 2 Ca). To examine the localization of GTP-Rap1 in PC12 cells, we performed the subcellular fractionation in the presence of GST-RalGDS-3xRBD, which preferentially binds to GTP-Rap1 (Herrmann et al., 1996). GST-RalGDS-3xRBD was recovered in the late endosomal fraction at 30 min after NGF stimulation, indicating that Rap1 was activated at late endosomes at 30 min after NGF stimulation (Fig. 2 Cb). The huge amount of GST-RalGDS-3xRBD, which did not bind to Rap1, remained in the bottom PNS and heavy membrane fractions.

The NGF-induced neurite outgrowth was reduced in the PDZ-GEF1-knockdown PC12 cells (Fig. 2 D). This knockdown effect was rescued by expression of siRNA-resistant PDZ-GEF1 (Fig. 2 E). Moreover, PC12 cells overexpressing PDZ-GEF1 or...
PDZ-GEF1-ΔRA displayed marked neurite outgrowth at 12 h after NGF stimulation as compared with control cells (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200610073/DC1). Consistent with the difference in the GEF activities between PDZ-GEF1 and PDZ-GEF1-ΔRA shown in Fig. 1 Ab, the effect of PDZ-GEF1-ΔRA on neurite outgrowth was less effective than that of PDZ-GEF1. Collectively, these results indicate that PDZ-GEF1 is involved in the NGF-induced sustained activation of Rap1 and ERK, which eventually causes neurite outgrowth.

Before NGF stimulation, FLAG-PDZ-GEF1 diffusely localized at the cytoplasm, but it was recruited to LBPA-positive late endosomes at 30 min after NGF stimulation (Fig. 3). FLAG-PDZ-GEF1 was not recruited to EEA1-positive early endosomes at either 5 or 30 min. In contrast to PDZ-GEF1, C3G was recruited to EEA1-positive early endosomes at 5 min after NGF stimulation (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200610073/DC1). C3G was not recruited to LBPA-positive late endosomes at either 5 or 30 min. These results indicate that PDZ-GEF1 is recruited to late endosomes in response to NGF and suggest that this Rap1 GEF is involved in the NGF-induced sustained activation of Rap1 at late endosomes.

**Involvement of transport of TrkA receptor to late endosomes in the NGF-induced recruitment of PDZ-GEF1**

We next examined whether transport of TrkA receptor to late endosomes recruits PDZ-GEF1 there. Bafilomycin is known to be an inhibitor of V-ATPase-regulating protein trafficking from
Figure 2. Involvement of PDZ-GEF1 in the NGF-induced sustained activation of Rap1 and neurite outgrowth in PC12 cells. (A) The activation level of Rap1 and the phosphorylation level of ERK in PC12 cells. (Aa) Effects of the knockdown of PDZ-GEF1 on the activation of Rap1 and ERK after NGF stimulation.
Recruitment of PDZ-GEF1 to late endosomes in a NGF-dependent manner. PC12 cells were transfected with pFLAG-CMV2-PDZ-GEF1. PC12 cells were double-stained with the anti-PDZ-GEF pAb and the anti-EEA1 mAb (left panels) or with the anti-PDZ-GEF pAb and the anti-LBPA mAb (right panels). Insets are enlarged images of boxed areas. The area of colocalization was quantified in the right panel. Bars, 10 μm. The results shown are representative of three independent experiments.

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Results indicate that transport of TrkA receptor to late endosomes is required for the recruitment of PDZ-GEF1 to late endosomes. Bafilomycin inhibited the activation of Rap1 at 5 min and more potently at 30 and 60 min as estimated by the pull-down assay (Fig. 4 Ca). In contrast, bafilomycin did not inhibit the activation of Ras at 5 min (Fig. 4 Cb). Bafilomycin did not inhibit the activation of ERK at 5 min, but significantly decreased it at 30 and 60 min. Bafilomycin reduced the NGF-induced activation of Rap1 at the perinuclear region of the cells as estimated in PC12 cells. PC12 cells were transfected with control RNA (C) or PDZ-GEF1 siRNA #1 (KD). The amount of GTP-Rap1 was measured by the pull-down assay using GST-RalGDS. The amount of phospho-ERK was measured by immunoblotting with the anti-phospho-ERK mAb. The immunoblot bands were quantified in the right panel. In the top panel, representative ratio images of YFP/CFP at indicated time points after NGF stimulation are shown. In the intensity-modulated display mode, eight colors from red to blue were used to represent the YFP/CFP ratio, with the intensity of each color indicating the mean intensity of YFP and CFP. The upper and lower limits of the ratio range are shown in the right panel. Bars, 10 μm. In the bottom panel, YFP/CFP ratios of three representative datasets were expressed by measuring the increase over the reference value used in the top panel. (Ab) Expression of FLAG-siRNA-resistant PDZ-GEF1 in PDZ-GEF1-knockdown PC12 cells. PC12 cells were transfected with an empty pSUPER vector (Control) or pSUPER-retro-PDZ-GEF1 (PDZ-GEF1-KD). In the top panel, representative ratio images of YFP/CFP at indicated time points after NGF stimulation are shown. In the intensity-modulated display mode, eight colors from red to blue were used to represent the YFP/CFP ratio, with the intensity of each color indicating the mean intensity of YFP and CFP. The upper and lower limits of the ratio range are shown in the right panel. Bars, 10 μm. In the bottom panel, YFP/CFP ratios of three representative datasets were expressed by measuring the increase over the reference value used in the top panel. (Bb) FRET analysis on the localization of the activation of Rap1 in PC12 cells. (Ba) FRET analysis in PDZ-GEF1-knockdown PC12 cells. Bars, 10 μm. In the right panel, YFP/CFP ratios of three representative datasets were expressed by measuring the increase over the reference value used in the top panel. (Bc) FRET analysis in rescued PDZ-GEF1-knockdown PC12 cells. Representative ratio images of YFP/CFP at 30 min after NGF stimulation are shown. In the intensity-modulated display mode, eight colors from red to blue were used to represent the YFP/CFP ratio, with the intensity of each color indicating the mean intensity of YFP and CFP. The upper and lower limits of the ratio range are shown in the right panel. Bars, 10 μm. In the bottom panel, YFP/CFP ratios of three representative datasets were expressed by measuring the increase over the reference value used in the top panel. (Cb) Localization of GTP-Rap1 in PC12 cells. The PNS fraction from PC12 cells was subjected to sucrose gradient centrifugation. Equal amounts of proteins from different fractions were subjected to immunoblotting with indicated Abs. PNS: post-nuclear supernatant fraction, HM: heavy membrane fraction, EE: early endosomal fraction, LE: late endosomal fraction. (D) Neurite outgrowth in the NGF-treated PC12 cells. PC12 cells were transfected with control scramble siRNA (Control) or PDZ-GEF1 siRNA#1-3 (PDZ-GEF1-KD). Left panel: representative DIC images. Right panel: percentage of cells with neurites. Asterisks indicate statistical significance (t test; *, P < 0.01). Bars, 10 μm. The results shown are representative of three independent experiments.
by the FRET assay (Fig. 4 D). Bafilomycin reduced the NGF-induced neurite outgrowth (Fig. 4 E). Collectively, these results indicate that the NGF-induced internalization and transport of TrkA receptor to late endosomes is involved in the NGF-induced sustained activation of Rap1 and ERK and neurite outgrowth by recruiting PDZ-GEF1 to late endosomes.

NGF-induced formation of a tetramer complex of PDZ-GEF1, S-SCAM, ARMS, and TrkA receptor

We next attempted to understand the molecular mechanism how PDZ-GEF1 induces the activation of Rap1 at late endosomes in response to NGF. We previously showed that PDZ-GEF1 directly binds S-SCAM (Ohtsuka et al., 1999), whereas it was shown that TrkA receptor directly binds an endosomal protein ARMS (Yano and Chao, 2005; Arevalo et al. 2006). Consistent with our earlier observation, S-SCAM was coimmunoprecipitated with PDZ-GEF1 before or after NGF stimulation (Fig. 5 A). ARMS and TrkA receptor were also coimmunoprecipitated with PDZ-GEF1, but this coinmunoprecipitation was strengthened after NGF stimulation. However, presumably due to the reconstitution of the complex during the procedure of cell lysis, we did not get the strong difference in the amount of the complex between 5 min and 60 min after NGF stimulation. These results suggest that PDZ-GEF1 forms a tetramer complex with S-SCAM, ARMS, and TrkA receptor after NGF stimulation.

As ARMS has a PDZ-binding motif, RESIL, at the cytoplasmic tail, we examined by the pull-down assay whether ARMS directly binds to S-SCAM or PDZ-GEF1. The GST fusion protein containing the C-terminal region of ARMS (GST-ARMS-C-term) bound to S-SCAM, but not to PDZ-GEF1 (Fig. 5 B and unpublished data). GST-ARMS-C-terminal lacking the last three amino acids in the RESIL motif (GST-ARMS-C-terminal-ΔSIL) did not bind to S-SCAM. The PDZ4 domain of S-SCAM bound to GST-ARMS-C-terminal (Fig. 5 C, a and b). Under the conditions where Myc-S-SCAM was coimmunoprecipitated with FLAG-ARMS, Myc-S-SCAM lacking the PDZ4 domain (S-SCAM-ΔPDZ4) was not coimmunoprecipitated (Fig. 5 Cc). These results indicate that S-SCAM directly binds ARMS and that this binding is mediated by the PDZ4 domain of S-SCAM and the PDZ-binding motif of ARMS. Collectively with the earlier observations that PDZ-GEF1 binds to the PDZ1 domain of S-SCAM and that ARMS directly binds to TrkA receptor in a NGF-dependent manner, these results indicate that PDZ-GEF1 binds to S-SCAM to form a binary complex in a NGF-independent manner, which binds to TrkA receptor through ARMS to form a tetramer complex in a NGF-dependent manner.

Formation of the tetramer complex at late endosomes

We then examined whether this tetramer complex of PDZ-GEF1, S-SCAM, ARMS, and TrkA receptor is formed at late endosomes in intact PC12 cells. The immunofluorescence signal for ARMS was mainly concentrated at LBPA-positive late endosomes, not at early endosomes, before and after NGF stimulation, indicating that ARMS is an intrinsic late endosomal protein (Fig. 6 A). The signals for PDZ-GEF1 and S-SCAM localized diffusely at the cytoplasm before NGF stimulation (Fig. 6 B, a and c). The signal for TrkA receptor localized at the cell surface plasma membrane and a vesicular-like structure, presumably, early endosomes, before NGF stimulation (Fig. 6 Bb).

The signals for PDZ-GEF1, S-SCAM, and TrkA receptor became concentrated at ARMS-positive late endosomes after NGF stimulation. As the signals for PDZ-GEF1 and S-SCAM colocalized well before and after NGF stimulation, these two molecules seem to form a binary complex, consistent with the above coimmunoprecipitation results (Fig. S5A, available at http://www.jcb.org/cgi/content/full/jcb.200610073/DC1). Collectively, these results suggest that upon binding of NGF, TrkA receptor is internalized and transported to ARMS-positive late endosomes, interacts with ARMS, and then recruits the PDZ-GEF1-S-SCAM complex mainly to ARMS-positive late endosomes, resulting in the tetramer complex formation.

To further examine the recruitment of PDZ-GEF1 to late endosomes biochemically, we performed immunosolization of PDZ-GEF1-containing vesicles using PDZ-GEF1 pAb-coated magnetic beads from the PNS fraction from NGF- nonstimulated and -stimulated PC12 cells. Rab7 was recovered in the immunosolated PDZ-GEF1-containing vesicles, whereas EEA1 was not recovered in the immunosolated vesicles (Fig. 6 Bd). Rab7 and ARMS were more predominantly recovered after NGF stimulation. These results have provided another line of evidence that the tetramer complex of TrkA receptor, ARMS, S-SCAM, and PDZ-GEF1 is formed mainly at late endosomes in a NGF-dependent manner.

Necessity of ARMS and S-SCAM for the NGF-induced recruitment of PDZ-GEF1 to late endosomes and neurite outgrowth

To confirm that ARMS and S-SCAM are required for the recruitment of PDZ-GEF1 to late endosomes, we performed...
loss-of-function experiments by use of the RNAi method for ARMS or by use of a dominant negative mutant for S-SCAM. The recruitment of FLAG-PDZ-GEF1 to late endosomes was reduced in the ARMS-knockdown PC12 cells (Fig. 7, A and B, and Fig. S5 B). Expression of Myc-S-SCAM did not inhibit the recruitment of FLAG-PDZ-GEF1 to late endosomes, but expression of
Myc-S-SCAM-ΔPDZ4 reduced the recruitment of FLAG-PDZ-GEF1 to late endosomes (Fig. 7 C). Expression of Myc-S-SCAM, but not Myc-S-SCAM-ΔPDZ4, slightly induced the recruitment of FLAG-PDZ-GEF1 to late endosomes even in the absence of NGF (Fig. S5A).

We next examined whether ARMS and S-SCAM are indeed involved in the NGF-induced neurite outgrowth. The NGF-induced neurite outgrowth was reduced in the ARMS-knockdown PC12 cells (Fig. 7 D). The NGF-induced neurite outgrowth was reduced in the PC12 cells expressing Myc-S-SCAM-ΔPDZ4 (Fig. 7 E). Collectively, these results indicate that ARMS and S-SCAM are essential for the NGF-induced recruitment of PDZ-GEF1 to late endosomes and neurite outgrowth.

Involvement of C3G and PDZ-GEF1 in the NGF-induced neurite outgrowth

To understand the relationship between C3G and PDZ-GEF1 in the NGF-induced neurite outgrowth, we first performed loss-of-function experiments by use of the RNAi method for C3G (Fig. 8 A). The inhibitory effect of the knockdown of C3G on neurite outgrowth was reduced in the ARMS-knockdown PC12 cells (Fig. 7 D). The NGF-induced neurite outgrowth was reduced in the ARMS-knockdown PC12 cells expressing Myc-S-SCAM-ΔPDZ4 (Fig. 7 E). Collectively, these results indicate that ARMS and S-SCAM are essential for the NGF-induced neurite outgrowth.

Figure 6. Localization of the ARMS-S-SCAM-PDZ-GEF1 complex at late endosomes in a NGF-dependent manner. (A) Localization of ARMS at the late endosomes. PC12 cells were stimulated by NGF for 30 min and double-stained with the anti-ARMS pAb and the anti-EEA1 mAb or with the anti-ARMS pAb and the anti-LBPA mAb. Insets are enlarged images of boxed areas. The area of colocalization was quantified in the right panel. Bars, 10 μm. (B) Recruitment of PDZ-GEF1, S-SCAM, and TrkA receptor to ARMS-positive late endosomes. (Ba) Recruitment of PDZ-GEF1 to ARMS-positive late endosomes. PC12 cells were transfected with pFLAG-CMV2-PDZ-GEF1. PC12 cells were double-stained with the anti-PDZ-GEF pAb and the anti-ARMS mAb. The area of colocalization was quantified in the right panel. (Bb) Recruitment of TrkA receptor to ARMS-positive late endosomes. PC12 cells were double-stained with the anti-TrkA receptor pAb and the anti-ARMS mAb. The area of colocalization was quantified in the right panel. (Bc) Recruitment of S-SCAM to ARMS-positive late endosomes. PC12 cells were double-stained with the anti-S-SCAM pAb and the anti-ARMS mAb. The area of colocalization was quantified in the right panel. (Bd) Immunoisolation of PDZ-GEF1-containing vesicles. PDZ-GEF1-containing vesicles were immunoisolated with the anti-PDZ-GEF1 pAb or the anti-rabbit IgG (control IgG)-coated magnetic beads from the PNS fraction of PC12 cells. The bound proteins were analyzed by immunoblotting with indicated Abs. Arrowhead indicates a light chain of rabbit IgG. Arrow indicates Rab7. The PDZ-GEF1-pAb immunoreactive nonspecific bands were observed in the control IgG coated-magnetic beads presumably due to the nonspecific reaction of the secondary Ab against the contaminating proteins interacted with the magnetic beads. The immunoblot bands were quantified in the right panel. The results were obtained from the same experiments and the same gels. The results shown are representative of three independent experiments.
neurite outgrowth was less effective than that of the knockdown of PDZ-GEF1 alone (Fig. 8 C). In addition, overexpression of C3G enhanced the NGF-induced neurite outgrowth less effectively than that of PDZ-GEF1 (Fig. S2). The effect of the knockdown of C3G was rescued by expression of siRNA-resistant C3G (Fig. 8 C). The double knockdown of C3G and PDZ-GEF1 reduced neurite outgrowth more potently than the knockdown of PDZ-GEF1 or C3G alone (Fig. 8 D). In addition, the inhibitory effect of the double knockdown of C3G and PDZ-GEF1 on neurite outgrowth was similar to that of the knockdown of Rap1 (Fig. 8 E and F). These results indicate C3G and PDZ-GEF1 are main Rap1GEFs in the NGF-induced neurite outgrowth in PC12 cells.
Figure 8. Involvement of C3G and PDZ-GEF1 in the NGF-induced neurite outgrowth. (A) Efficiency of the knockdown of C3G by three different siRNAs. PC12 cells were transfected with control siRNA (Cont), C3G siRNA #1-3, or scramble RNA (Scr). The immunoblot bands were quantified in the right panel. (B) Inhibition of neurite outgrowth in the C3G-knockdown PC12 cells. PC12 cells were transfected with scrambled RNA (Control) or C3G siRNA #1-3 (C3G-KD). Left panel: representative DIC images. Right panel: percentage of cells with neurites. (C) Rescue of the inhibitory effect of the knockdown of C3G on neurite outgrowth. (Ca) Expression of FLAG-siRNA-resistant C3G in the C3G-knockdown PC12 cells. PC12 cells were first transfected with C3G siRNA #2 or control RNA and cultured for 2 d. Then cells were transfected with FLAG-siRNA-resistant C3G. The total cell lysates were subjected to SDS-PAGE, followed by immunoblotting with the anti-C3G pAb. (Cb) Neurite outgrowth in rescued PC12 cells. PC12 cells were cotransfected with pSUPER-retro-C3G and pRed NLS-FLAG vector (C3G-KD) or pRed NLS-FLAG siRNA-resistant C3G (C3G-rescue). Left panel: representative DIC images. Right panel: percentage of cells with neurites. (D) Effect of the double knockdown of C3G and PDZ-GEF1 on neurite outgrowth. PC12 cells were transfected with control siRNA (Control), PDZ-GEF1 siRNA #1 (PDZ-GEF-KD), C3G siRNA #1 (C3G-KD), or PDZ-GEF1 siRNA #1 and C3G siRNA #1 (PDZ-GEF-KD, C3G-KD). Top panels: representative DIC images. Bottom panels: percentage of cells with neurites and the length of neurite. (E) Efficiency of the knockdown of Rap1 by three different siRNAs. PC12 cells were transfected with control siRNA (Cont) or Rap1 siRNAs #1-3. The immunoblot bands were quantified in the right panel. (F) Inhibition of neurite outgrowth in the Rap1-knockdown PC12 cells. PC12 cells were transfected with control siRNA (Control) or Rap1 siRNA #1-3 (Rap1-KD). Left panels: representative DIC images. Right panels: percentage of cells with neurites and the length of neurite. Asterisks indicate statistical significance (*, P < 0.01). Bars, 10 μm.
The inhibitory effect of the knockdown of C3G on the NGF-induced sustained activation of Rap1 was less effective than that of the knockdown of PDZ-GEF1 (Fig. 9 A). The knockdown of PDZ-GEF1 alone did not completely abolish the NGF-induced sustained activation of Rap1. The double knockdown of C3G and PDZ-GEF1 more potently abolished the NGF-induced sustained activation of Rap1 and ERK. The inhibitory effect of the double knockdown of C3G and PDZ-GEF1 on the sustained activation of ERK was similar to that of the knockdown of Rap1 (Fig. 9, A and B). These results indicate that C3G and PDZ-GEF1 work cooperatively in the NGF-induced sustained activation of Rap1.

Involvement of PDZ-GEF1 in the BDNF-induced neurite outgrowth of hippocampal neurons

To validate the role of PDZ-GEF1 in PC12 cells, we finally examined whether PDZ-GEF1 is involved in the BDNF-induced neurite outgrowth in rat primary cultured hippocampal neurons. It has been reported that BDNF, but not NGF, enhances axon elongation in hippocampal neurons (Ip et al., 1993; Morfini et al., 1994; Labelle and Leclerc, 2000; Yoshimura et al., 2005). BDNF binds to its receptor, TrkB receptor, in hippocampal neurons (Huang and Reichardt 2001; Sofroniew et al., 2001). Retrograde transport of BDNF-TrkB and the signaling complex associating with it is...
involved in the axon elongation and cell survival signal (Howe and Mobley, 2004; Zweifel et al., 2005). Hippocampal neurons at 2.0 days in vitro were stimulated by BDNF for indicated periods of time. PDZ-GEF1 was recruited to LBPA-positive late endosomes at 30 min after BDNF stimulation (Fig. 10 A). PDZ-GEF1 was not recruited to EEA1-positive early endosomes. Hippocampal neurons from E18 rats were cotransfected with GFP as a morphological marker along with pSUPER-retroPDZ-GEF1 (PDZ-GEF1-KD) by electroporation. Transfected neurons were cultured for 1.5 d in the presence of BDNF, and allowed to extend axons and minor neurites. Transfected neurons were visualized by the GFP fluorescence (green) and immunostained with the anti-PDZ-GEF1 pAb (red). The fluorescence intensity of PDZ-GEF1 of the neurons expressing GFP was compared with that of the untransfected neurons in the same field of view in the right panel. (Bb) Effect of the knockdown of PDZ-GEF1 on axon outgrowth. The morphology of transfected neurons was visualized by the GFP fluorescence (green) and immunostained with the anti-Tau-1 mAb (red) and the anti-MAP2 pAb (blue). Bars, 20 μm. (Bc) Quantitative analysis of the number of neurite per transfected neuron. An average neurite number (± SEM) of between 40 and 60 analyzed neurons. (Bd) Quantitative analysis of the length of axon per transfected neuron. An average axon length (± SEM) of between 40 and 60 analyzed neurons. The mean value (± SEM) of three independent experiments is shown. Asterisks indicate statistical significance (t test; *, P < 0.01; **, P < 0.05). The results shown are representative of three independent experiments.

Discussion
We first showed here that NGF induced the sustained activation of Rap1 at late endosomes and neurite outgrowth in cultured

| Figure 10. Involvement of PDZ-GEF1 in the BDNF-induced neurite outgrowth in hippocampal neurons. (A) Localization of endogenous PDZ-GEF1 at late endosomes in hippocampal neurons. Hippocampal neurons at 2.0 days in vitro were stimulated by BDNF for 30 min in the absence of serum and stained with indicated Abs. Insets are enlarged images of cell bodies. The area of colocalization was quantified in the right panel. Bars, 20 μm. (B) Inhibition of axon outgrowth in the PDZ-GEF1-knockdown hippocampal neurons. (Bb) Expression level of PDZ-GEF1 in knockdown neurons. Hippocampal neurons from E18 rats were cotransfected with GFP as a morphological marker along with pSUPER-retroPDZ-GEF1 (PDZ-GEF1-KD) by electroporation. Transfected neurons were cultured for 1.5 d in the presence of BDNF, and allowed to extend axons and minor neurites. Transfected neurons were visualized by the GFP fluorescence (green) and immunostained with the anti-PDZ-GEF1 pAb (red). The fluorescence intensity of PDZ-GEF1 of the neurons expressing GFP was compared with that of the untransfected neurons in the same field of view in the right panel. (Bb) Effect of the knockdown of PDZ-GEF1 on axon outgrowth. The morphology of transfected neurons was visualized by the GFP fluorescence (green) and immunostained with the anti-Tau-1 mAb (red) and the anti-MAP2 pAb (blue). Bars, 20 μm. (Bc) Quantitative analysis of the number of neurite per transfected neuron. An average neurite number (± SEM) of between 40 and 60 analyzed neurons. (Bd) Quantitative analysis of the length of axon per transfected neuron. An average axon length (± SEM) of between 40 and 60 analyzed neurons. The mean value (± SEM) of three independent experiments is shown. Asterisks indicate statistical significance (t test; *, P < 0.01; **, P < 0.05). The results shown are representative of three independent experiments.
PC12 cells. The activation of Rap1 at late endosomes was catalyzed by PDZ-GEF1. Earlier FRET studies showed that Rap1 was activated at peri-nuclear regions and it was practically difficult to distinguish the sites of the activation of Rap1 at the plasma membrane, early endosomes, and late endosomes by this method (Mochizuki et al., 2001; Obha et al., 2003). We could not directly show the activation of Rap1 at late endosomes but indirectly showed it by immunofluorescence microscopy and immunocolocalization analysis: PDZ-GEF1 was recruited to late endosomes in response to NGF. PDZ-GEF1 was not recruited to early endosomes, whereas C3G was recruited to early endosomes, but not to late endosomes. Thus, these two GEFs for Rap1 play roles in different compartments.

As Rap1 is first activated by C3G at the plasma membrane and early endosomes after NGF stimulation (Kao et al., 2001; Wu et al., 2001), activated Rap1 may affect the GEF activity of PDZ-GEF1. We found that the inhibitory effect of the knock-down of C3G on the NGF-induced sustained activation of Rap1 and neurite outgrowth was less effective than that of the knock-down of PDZ-GEF1. The knockdown of PDZ-GEF1 alone did not completely abolish the NGF-induced sustained activation of Rap1 or neurite outgrowth. The double knockdown of C3G and PDZ-GEF1 completely abolished the NGF-induced sustained activation of Rap1 and neurite outgrowth similar to the knock-down of Rap1. Thus, C3G and PDZ-GEF1 seem to work cooperatively in the NGF-induced sustained activation of Rap1 and neurite outgrowth. We found that C3G was recruited to early endosomes, but not to late endosomes. The activation of Rap1 was observed on late endosomes, not on early endosomes, at 30 min after NGF stimulation. Moreover, inhibition of the transport of TrkA receptor form early to late endosomes reduced the activation of Rap1 at 30 min after NGF stimulation. Collectively, we propose the following model for the activation of Rap1 by C3G and PDZ-GEF1: (1) Upon NGF stimulation, C3G first activates Rap1 on early endosomes; (2) GTP-Rap1 on early endosomes is transported to late endosomes with TrkA receptor; and (3) Transported GTP-Rap1 activates PDZ-GEF1 and induces the sustained activation of Rap1 on late endosomes, eventually inducing neurite outgrowth.

The long-distance retrograde NGF signaling from axon terminals to cell bodies is crucial for neuronal survival and plasticity (Howe and Mobley 2004; Zweifel et al., 2005). It was shown that the internalization and endosomal trafficking of TrkA receptor are important for the long-distance retrograde NGF signaling (York et al., 2000; Ginty and Segal, 2002; Howe and Mobley, 2004). TrkA receptor continues to transduce a signal after its internalization into endosomes (York et al., 2000; Ginty and Segal, 2002; Howe and Mobley, 2004). Certain specialized endosomal organelles might represent signaling platforms from which specific pathways emerge (Howe et al., 2001; Sorkin and Von Zastrow, 2002). Earlier studies demonstrated that late endosomes are the major endosomal population labeled by endocytosed iodinated NGF in cell bodies of sympathetic neurons (Claude et al., 1982). As the interaction of TrkA receptor and NGF is resistant to decreasing pH values within the endosomal pathway (Zapf-Colby and Olefsky, 1998), it appears likely that NGF is still bound to the extracellular domain of TrkA receptor within late endosomes. Accordingly, immunoelectron microscopic study found phospho-TrkA receptor (pTrkA) immunoreactivity in late endosomes of sciatic-nerves (Bhattacharyya et al., 2002) and biochemical study found pTrkA in late endosomes in PC12 cells (Saxena et al., 2005a). Rab7, a member of the Rab family small G proteins, which regulates TrkA receptor transport from late endosomes to lysosomes, was shown to regulate the persistence of TrkA receptor at late endosomes and TrkA receptor signaling (Saxena et al., 2005b). Consistent with these earlier observations, we showed here by use of bafilomycin, an agent which inhibits the vesicular traffic from early endosomes to late endosomes, that the NGF-induced internalization and transport of TrkA receptor to late endosomes was essential for the sustained activation of Rap1 at late endosomes, the activation of ERK, and neurite outgrowth. Collectively with our findings, like TrkA receptor in clathrin-coated vesicles (Howe et al., 2001) and early endosomes (Delcroix et al., 2003), TrkA receptor in late endosomes is competent of signaling. In addition, there are several reports that receptor tyrosine kinases (RTKs) induce signaling from late endosomes. For instance, it is known that late endosomes contain phospho-EGF receptor (pEGFR and its activated downstream signaling components (Oksvold et al., 2001). The MAPK scaffold p14 localizes to the outer limiting membrane of late endosomes, and this localization is essential for EGFR signaling (Wunderlich et al., 2001). There is also in vivo evidence pointing toward the importance of late endosomes in the regulation of neuronal TGF-β signaling (Sweeney and Davis, 2002). Thus, late endosomes appear to be ideally suited for regulating spatial and temporal compartmentalization of signal transduction, beyond its conventional role in cargo degradation. Our report is the first to demonstrate that late endosomes represent functional TrkA receptor signaling platforms. This system might play a role in continuing activation state of NGF signaling during transport of TrkA receptor from axon terminals to cell bodies.

We furthermore showed here that PDZ-GEF1 was recruited to late endosomes to form a complex with TrkA receptor, which was internalized and transported there, and that recruited PDZ-GEF1 induced sustained activation of Rap1 and ERK. NGF-bound TrkA receptor activates its own tyrosine kinase and gathers the signaling complex consisting of C3G, CrkL, and FRS2 on the plasma membrane (Kao et al., 2001; Wu et al., 2001). TrkA receptor and the FRS2-Crk-C3G complex associating with the receptor are internalized into clathrin-coated vesicles and move into early endosomes within 5 min (Kao et al., 2001). Rap1 is activated at early endosomes and activated Rap1 induces the activation of the MAPK cascade through activating B-Raf (Wu et al., 2001). TrkA receptor then passes through early endosomes without entering into the recycling pathway to the plasma membrane and reaches to late endosomes within 30 min. Like the activated TrkA receptor complex at early endosomes, the TrkA receptor complex containing ARMS, S-SCAM, and PDZ-GEF1 at late endosomes was competent in activation of Rap1. While the GEF activity of C3G is activated by its interacting partner Crk (York et al., 1998), it remains unknown how the activity of PDZ-GEF1 is regulated at late endosomes. PDZ-GEF1 binds to the PDZ1...
domain of S-SCAM through its C-terminal PDZ-binding motif and forms a stable complex (Ohtsuka et al., 1999). However, the binding of S-SCAM to PDZ-GEF1 does not affect GEF activity in vitro (Ohtsuka et al., 1999). The complex was recruited to ARMS-positive late endosomes through directly binding to ARMS, in a manner dependent on transport of TrkA receptor. The C-terminal PDZ-binding motif of PDZ-GEF1 contributed to direct the subcellular localization of PDZ-GEF1, leading to the augmented activation of Rap1 at late endosomes. Collectively, PDZ-GEF1 is recruited to late endosomes through its C-terminal PDZ-binding motif. PDZ-GEF1 then activates Rap1 at late endosomes and the GEF activity of PDZ-GEF1 is augmented by a positive feedback mechanism, resulting in the sustained activation of Rap1 at late endosomes. It may be noted that when TrkA receptor is transported from early endosomes to late endosomes, the FRS2-Crk-C3G complex associating with TrkA receptor remains at early endosomes, suggesting that this complex is dissociated from the receptor. The mechanism of this dissociation remains unknown and is an issue to be addressed in the future.

We finally showed here that the PDZ-GEF1-induced sustained activation of Rap1 at late endosomes was involved in the NGF-induced neurite outgrowth in PC12 cells and the BDNF-induced axon outgrowth in rat hippocampal neurons. What is the target protein(s) of Rap1 in the neurite outgrowth? Many downstream targets of Rap1 have been identified: they include c-Raf, B-Raf, RalGDS, afadin/AF-6, PI3-kinase, and RAPL (Bos et al., 2001; Kinashi and Katagiri, 2004; Nakanishi and Takai, 2004; Price and Bos, 2004). c-Raf and B-Raf are protein kinases connecting Ras to ERK; RalGDS is a regulator of another small G protein Ral; afadin/AF-6 is an actin filament- and nectin-binding protein at adherens junctions; PI3-kinase is a phosphatidylinositol kinase, and RAPL is a small protein which transduces a signal from Rap1 to integrin. Among them, for activating MAP kinase cascade, B-Raf was shown to be the target protein of Rap1 for the NGF-induced neurite outgrowth (Ohtsuka et al., 1996). Several transcription factors, which are downstream of the MAP kinase cascade, contribute to neurite outgrowth by gene expression. On the other hand, RAPL and its binding protein Mst1 were recently shown to be involved in transport of integrin LFA-1 and its activation in lymphocytes (Katagiri et al., 2006). Transport of transmembrane proteins from late endosomes to the plasma membrane is an emerging paradigm and generally accepted (Mellman and Steinman, 2001; Trajkovic et al., 2006; van Niel et al., 2006). Accordingly, activation of Rap1 at late endosomes might be involved in the transport of integrin from late endosomes to the plasma membrane and thereby regulate the activation of MAP kinase cascade indirectly. Moreover, we previously identified RA-RhoGAP as another direct downstream target of Rap1 (Yamada et al., 2005). RA-RhoGAP has the RA and GAP domains in addition to the PH and annexin-like repeat domains. It indeed shows a GAP activity specific for Rho and this GAP activity is enhanced by GTP-Rap1. The Rap1-RA-RhoGAP-Rho pathway plays an important role in the neurite outgrowth. Collectively, during the initiation of the neurite outgrowth, a neurite formation signal(s), such as NGF and BDNF, induces the C3G-mediated activation of Rap1 at endocytic vesicles or early endosomes in a growth cone of the nascent neurite. Subsequently, RA-RhoGAP is recruited and activated by Rap1 to cause the inactivation of Rho at the growth cone of the nascent neurite. Repression of the Rho-mediated signaling pathway induces rapid actin depolymerization of the growth cone and thereby extends the incipient neurite further. In addition, to replenish the requirement for neurite outgrowth, such as signaling molecules and cytoskeletal proteins, endocytic vesicles or early endosomes are transported to late endosomes to sustain the activation of Rap1 by PDZ-GEF1. The sustained activation of Rap1 prolongs the activation time of the MAPK cascade and results in the up-regulation of gene expression. Thus, the Rap1-RA-RhoGAP-Rho system and the PDZ-GEF-Rap1-B-Raf-MAPK system could cooperatively regulate neurite outgrowth. In future studies, it will be important to elucidate how these two systems are spatially and temporally activated during the neurite outgrowth.

Materials and methods

Expression vectors

pFLAG-CMV2-PDZ-GEF1 and pFLAG-CMV2-PDZ-GEF1-ΔRA were obtained from Dr. Toshio Kataoka [Kobe University, Kobe, Japan]. The RNAi-resistant mutant of FLAG-human PDZ-GEF1 (pErEdNLS-FLAG-PDZ-GEF1) was generated by mutagenesis of 5′-GAGAGATGTATGTTAGGAA-3′ to 5′-GGCGAAATGCTGTATGAA-3′ using the QuikChange site-directed mutagenesis kit [Stratagene]. The letters in the codons, which are different from the letters in the RNAi sequence of rat PDZ-GEF1, are underlined. pCMV-RA-PDZ-GEF1 was constructed using standard molecular biology methods. pCINE-Myc-S-SCAM-B, pCINE-Myc-S-SCAM-M, pCINE-Myc-S-SCAM-10, and pCINE-Myc-S-SCAM-12 were obtained from Dr. Yutaka Hata [Tokyo Medical and Dental University, Tokyo, Japan]. pCINE-Myc-S-SCAM-3pPDZ4 (aa 929–1010 deletion) was constructed using standard molecular biology methods. KIAA1250/ARMS cDNA was obtained from Dr. Takahiro Nagase [Kazusa DNA Research Institute, Chiba, Japan]. pCMV-FLAG-ARMS, pGEX4T-2-ARMS-Ct (aa 1616–1715), and pGEX4T-2-ARMS-Ct-A5IL (aa 1616–1712) were constructed using standard molecular biology methods. pCMV-FLAG-C3G was constructed using standard molecular biology methods. The RNAi-resistant mutant of FLAG-human C3G (pEREdNLS-FLAG-C3G) was generated by mutagenesis of 5′-GCTCTCCTATGAGGTATAC-3′ to 5′-GCTCTCCTATGAGGTATAC-3′ using the QuikChange site-directed mutagenesis kit [Stratagene]. The letters in the codons, which are different from the letters in the RNAi sequence of rat C3G, are underlined. pGEX4T-1-RalGDS-3xRBD was constructed using standard molecular biology methods.

Antibodies

The GST-fusion fragment of PDZ-GEF1 [aa 1–250] was produced in Escherichia coli, purified, and used as an antigen to raise a pAb in rabbit. The rabbit anti-PDZ-GEF1 pAb was affinity purified by using MBP-PDZ-GEF1 (aa 1–250) immobilized on Amino-Link agarose beads [Pierce Chemical Co]. A rabbit anti-S-SCAM pAb was obtained from Dr. Yutaka Hata [Tokyo Medical and Dental University, Tokyo, Japan]. A mouse anti-IP3R mAb was obtained from Dr. Toshihide Kobayashi [RIKEN, Wako, Japan] and Dr. Tetsuya Sugimoto [University of Tokyo, Tokyo, Japan]. A rabbit anti-Rab7 pAb was obtained from Dr. Marino Zerial [Max Plank Institute, Dresden, Germany]. A rabbit anti-Rap1 pAb, a mouse anti-pan-Trk mAb [B-39], a rabbit anti-C3G pAb, a mouse anti-cMyc mAb [9E10], and a mouse anti-GST mAb [B-14] were purchased from Santa Cruz Biotechnology, Inc. A mouse anti-phospho-ERK mAb [E10] and a rabbit anti-ERK pAb were purchased from Upstate Biotechnology. A mouse anti-EEA1 mAb and a mouse anti-GM130 mAb were purchased from Transduction Laboratories. A mouse anti-FLAG mAb and a rabbit anti-FLAG pAb were purchased from Sigma-Aldrich. A mouse anti-Kidins220/ARMS mAb was purchased from Abcam. A rabbit anti-Kidins220/ARMs pAb was purchased from ABK. A mouse anti-Tau-1 mAb and a rabbit anti-MAP2 pAb were purchased from Chemicon International. A mouse anti-HA mAb was purchased from Babco.
Knockdown of PDZ-GEF1, ARMS, and C3G by the RNAi method

For PDZ-GEF1 knockdown, double-stranded 19-nucleotide RNA duplexes (Qiagen) for PDZ-GEF1 #1 (5′-GAGA5GGUAUGAGCCAAUCUA-3′), #2 (5′-GAGUA5CCGGAUAGCCAAUAUCUA-3′), #3 (5′-CCAGACCUAAGCCGAAAGCU-3′), #4 (5′-UGUA5AGGGAUAAGCCAAAUA-3′), or double-stranded 25-nucleotide RNA duplexes (StealthTM RNA-mediated interference; Invitrogen) for PDZ-GEF1 #3 (5′-CCAGACCUAAGCCGGGAAAGCU-3′), #4 (5′-UGUA5AGGGAUAAGCCAAAUA-3′) was transfected into PC12 cells using Lipofectamine 2000 reagent. For ARMS knockdown, double-stranded 25-nucleotide RNA duplexes (StealthTM RNA-mediated interference; Invitrogen) for ARMS #1 (5′-SAAGCCGGAUAGAGCCAAUAUA-3′), #2 (5′-GGGUCCAUAGCCGAAAAGCU-3′), or scramble RNA for ARMS #1 (5′-GAGUAGAAAGGAAGCAAAAA-3′) was transfected into PC12 cells using Lipofectamine 2000 reagent. For ARMS knockdown, double-stranded 25-nucleotide RNA duplexes (StealthTM RNA-mediated interference; Invitrogen) for ARMS #1 (5′-SAAGCCGGAUAGAGCCAAUAUA-3′), #2 (5′-GGGUCCAUAGCCGAAAAGCU-3′), or scramble RNA for ARMS #1 (5′-GAGUAGAAAGGAAGCAAAAA-3′) was transfected into PC12 cells using Lipofectamine 2000 reagent. For C3G knockdown, double-stranded 19-nucleotide RNA duplexes (Qiagen) for C3G #2 (5′-GAGUGCAAGGGAAAGCU-3′), #3 (5′-CCAGACCUAAGCCGAAAGCU-3′), #4 (5′-UGUA5AGGGAUAAGCCAAAUA-3′), or scramble RNA for C3G #1 (5′-GAGUAGAAAGGAAGCAAAAA-3′) was transfected into PC12 cells using Lipofectamine 2000 reagent. For ARMS knockdown, double-stranded 25-nucleotide RNA duplexes (StealthTM RNA-mediated interference; Invitrogen) for ARMS #1 (5′-SAAGCCGGAUAGAGCCAAUAUA-3′), #2 (5′-GGGUCCAUAGCCGAAAAGCU-3′), or scramble RNA for ARMS #1 (5′-GAGUAGAAAGGAAGCAAAAA-3′) was transfected into PC12 cells using Lipofectamine 2000 reagent. For C3G knockdown, double-stranded 19-nucleotide RNA duplexes (Qiagen) for C3G #2 (5′-GAGUGCAAGGGAAAGCU-3′), #3 (5′-CCAGACCUAAGCCGAAAGCU-3′), #4 (5′-UGUA5AGGGAUAAGCCAAAUA-3′), or scramble RNA for C3G #1 (5′-GAGUAGAAAGGAAGCAAAAA-3′) was transfected into PC12 cells using Lipofectamine 2000 reagent. For ARMS knockdown, double-stranded 25-nucleotide RNA duplexes (StealthTM RNA-mediated interference; Invitrogen) for ARMS #1 (5′-SAAGCCGGAUAGAGCCAAUAUA-3′), #2 (5′-GGGUCCAUAGCCGAAAAGCU-3′), or scramble RNA for ARMS #1 (5′-GAGUAGAAAGGAAGCAAAAA-3′) was transfected into PC12 cells using Lipofectamine 2000 reagent. For C3G knockdown, double-stranded 19-nucleotide RNA duplexes (Qiagen) for C3G #2 (5′-GAGUGCAAGGGAAAGCU-3′), #3 (5′-CCAGACCUAAGCCGAAAGCU-3′), #4 (5′-UGUA5AGGGAUAAGCCAAAUA-3′), or scramble RNA for C3G #1 (5′-GAGUAGAAAGGAAGCAAAAA-3′) was transfected into PC12 cells using Lipofectamine 2000 reagent.

Immunofluorescence and confocal microscopy

Staining was performed as follows: PC12 cells were seeded onto polylysine coated coverslips in 24-well plates a day before the experiment. PC12 cells were cultured in DME with serum for 24 h, serum-starved for 16 h, treated with bafilomycin for 2 h, and stimulated by NGF for 30 min. The cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.05% Saponin for 30 min at room temperature. Images were captured using a Carl Zeiss confocal laser scanning microscope using a 63× oil immersion objective lens (model LSM 510/V3.2; Carl Zeiss Microlmaging, Inc.) or a confocal laser scanning microscope (model TE2000; Nikon) using a 60× oil immersion objective lens. The fluorescence area and intensity of each protein in PC12 cells were measured by use of NIH Image software. Collected data were exported as 8-bit TIFF files and processed using Adobe Photoshop 7.0.

Pull-down assay for GTP-Rap1 and GTP-Ras

The pull-down assay was performed as described previously (Sasagawa et al., 2005). PC12 cells were transfected with siRNA for 2 d and stimulated by NGF for indicated periods of time. The PC12 cells were washed with 1 ml of ice-cold PBS and lysed in Buffer A (50 mM Tris/HCl at pH 7.4, 150 mM NaCl, 5 mM MgCl2, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate). The samples were centrifuged at 100,000 × g for 10 min, and the supernatant was collected as the cell lysates. The PC12 cells were transferred to 75×10 cm culture flasks and the cell lysate (60 μg of protein) was incubated with GST-RalGDS Rap binding domain (RBD) (10 μg of protein) immobilized on glutathione-Sepharose beads (5 μl) for 30 min. After the beads were washed three times with Buffer A, GTP-Rap1 was detected by immunoblotting with an anti-Rap1 pAb. For the Ras pull-down assay, the cell lysate (600 μg of protein) was incubated with GST-c-Raf Ras binding domain (RBD) (30 μg of protein) immobilized on glutathione-Sepharose beads (50 μl) for 30 min. After the beads were washed three times with Buffer A, GTP-Ras was detected by immunoblotting with an anti-Ras mAb.

Detection of ERK activation

5% of the total cell lysates of PC12 cells was used for immunoblotting with either the anti-phospho-ERK mAb or the anti-ERK pAb to determine the amount of activated ERK (Phospho-ERK) or the total amount of ERK (Total-ERK), respectively, in each transfected cells.

Quantiﬁcation of neurites

PC12 cells were obtained from Dr. Shinya Kuroda (Tokyo University, Tokyo, Japan) and maintained in DMEM with 5% horse serum and 10% bovine calf serum (HyClone) in a 10% CO2 atmosphere. PC12 cells were cultured in DMEM with serum for 48 h and stimulated by NGF for 24 h. Images were captured using a confocal laser scanning microscope using a 60× oil immersion objective lens (model TE2000; Nikon). Collected data were exported as 8-bit TIFF files and processed using Adobe Photoshop software. The assay for neurite outgrowth was performed on 40–60 cells randomly chosen in each group. The number of primary neurites per cell was defined as the number of thin cell processes with a length longer than one cell diameter. The statistical signiﬁcance of differences between each group was analyzed by the two-tailed t test.

Rat hippocampal neurons were prepared form embryonic day (E) 18 Sprague-Dawley rats with slight modifications as described (Goslin and Banker, 1989). For cDNA transfection, before plating, the cells were resuspended in an optimized transfection solution for primary rat hippocampal neurons (Axamex). Each sample (2×103 cells in 100 μl) was transfected with 2 μg cDNA as indicated by using Nucleolectro electroperoration de- vice (Axamex), according to the optimized protocol for primary rat hippocampal neurons (Axamex). Immediately after transfection, the cells were cultured in 24-multicells at a density of 3×104 cells/well. After 1.5 d culture, neurons were processed for immunohistochemistry. Images were captured using a confocal laser scanning microscope using a 60× oil immersion objective lens (model TE2000; Nikon). Collected data were exported as 8-bit TIFF files and processed using Adobe Photoshop software. The morphometrical analysis for axons and minor neurites was performed on between 40 and 60 cells randomly chosen in each group. The number of primary neurites per cell was defined as the number of thin cell processes with a length longer than one cell diameter. The statistical significance of differences between each group was analyzed by the two-tailed t test.
Subcellular fractionation
PC12 cells (cultured on 3 × 10 cm plates) were cracked with a ball-bearing homogenizer in Buffer C (250 mM sucrose, 3 mM imidazole at pH 7.4, 1 mM EDTA, 10 μg/ml cycloheximide, and complete protease inhibitor cocktail [Roche]). Subsequently, a post-nuclear supernatant (PNS) was prepared by centrifugation at 3000 rpm for 10 min (Kubota S8000). The PNS was adjusted to 40.6% sucrose, loaded at the bottom of a 5:55ST centri- fugation tube (Hittachi), and overlaid sequentially with 35 and 30.5% sucrose solutions in 3 mM imidazole at pH 7.4, 1 mM EDTA, and 10 μg/ml cycloheximide. Subsequently, the tube was filled up with the homogenization buffer. The gradient was centrifuged at 35,000 rpm for 66 min using a p55ST rotor (Hittachi). After the centrifugation, the different interfaces and sucrose cushions were collected from top to bottom of the tube. Heavy membrane fraction was recovered in the 40.6/35% fraction. Early endo- somal fraction was recovered in the 35/30.5% fraction. Late endosomal fraction was recovered in the 30.5/8% fraction. BCA assays (Pierce Chemical Co.) were performed to determine the protein concentration of each fraction. Equal amounts of protein from each fraction were loaded on SDS-PAGE, followed by immunoblotting with the mouse anti-GM130 mAb, the mouse anti-EEA1 mAb, the rabbit anti-Rab7 pAb, and the rabbit anti-Rap1 pAb.

For detecting GTP-Rap1, PC12 cells (cultured on 3 × 10 cm plates) were cracked with a ball-bearing homogenizer in Buffer C (250 mM sucrose, 3 mM imidazole at pH 7.4, 1 mM EDTA, 10 μg/ml cycloheximide, complete protease inhibitor cocktail [Roche], and 100 μg/ml GSTrapGDS-3×RBD). Subsequently, the PNS was prepared and used for the subcellular fractionation as described above. Equal amounts of protein from each fraction were loaded on SDS-PAGE, followed by immunoblotting with the mouse anti-GST mAb, the rabbit anti-Rap1 pAb, and the mouse anti-Ras mAb.

Immunolocalization of PDZ-GEF1-containing vesicles
Immunolocalization of vesicles was performed as described previously (Saucan and Palade, 1994). In brief, PC12 cells (cultured on 4 × 10 cm plates) were starved for 16 h in phenol red-free DMEM/F12 medium containing 0.1% BSA, and either left unstimulated or stimulated by NGF for 30 min. Subsequently, the cells were cracked with a ball-bearing homogenizer in Buffer B. Subsequently, a PNS was prepared by centrifugation at 3,000 rpm for 10 min (Kubota S8000). Dynabeads Protein A (DYNAL Inc.) magnetic beads were coated with the anti-PDZ-GEF1 pAb or the control IgG at a density of 10 μg Ab per 50 μl beads according to manufacturer’s instructions. The anti-PDZ-GEF1 pAb-coated beads were added to the PNS and incubated with continuous rotation at 4°C for 2 h. The immunosolated beads were washed five times with Buffer B using a magnet and transferred to fresh tubes. The beads were finally resuspended in 80 μl of the SDS sample buffer for SDS-PAGE, followed by immunoblotting with the mouse anti-EEA1 mAb, the rabbit anti-Rab7 pAb, and the rabbit anti-Raps pAb.

Online supplemental material
Fig. S1 shows the effect of GTP-Rap1 on the GEF activity of PDZ-GEF1-∆Aa. Fig. S2 shows the overexpression of PDZ-GEF1 on neurite outgrowth. Fig. S3 shows the recruitment of C3G to early endosomes in a NGF-dependent manner. Fig. S4 shows the Inhibition of the transport of TRA receptor from early endosomes to late endosomes by bafilomycin. Fig. S5 shows the expression levels of S-SCAM and ARMS in S-SCAM-expressing and ARMS-knockdown PC12 cells, respectively. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200610073/DC1.

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