

# IQGAP3, a novel effector of Rac1 and Cdc42, regulates neurite outgrowth

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## Summary

**Rac1 and Cdc42, members of the Rho family GTPases, control diverse cellular processes such as cell migration and morphogenesis through their effectors. Among the effectors, IQGAP1 plays pivotal roles in the establishment of cytoskeletal architecture and intercellular adhesions in various cells. However, its roles remain to be clarified, especially in neuronal cells. We have identified IQGAP3 as a novel member of the IQGAP family, which is highly expressed in brain. We found that IQGAP3, an effector of Rac1 and Cdc42, associates directly with actin filaments and accumulates asymmetrically at the distal region of axons in hippocampal neurons. The depletion of IQGAP3 impairs neurite or axon outgrowth in neuronal cells with**

**the disorganized cytoskeleton, but depletion of IQGAP1 does not. Furthermore, IQGAP3 is indispensable for Rac1/Cdc42-promoted neurite outgrowth in PC12 cells. Taken together, these results indicate that IQGAP3 can link the activation of Rac1 and Cdc42 with the cytoskeletal architectures during neuronal morphogenesis.**

Supplementary material available online at  
<http://jcs.biologists.org/cgi/content/full/120/4/567/DC1>

Key words: IQGAP, Rho family GTPases, Cytoskeleton, Axon outgrowth

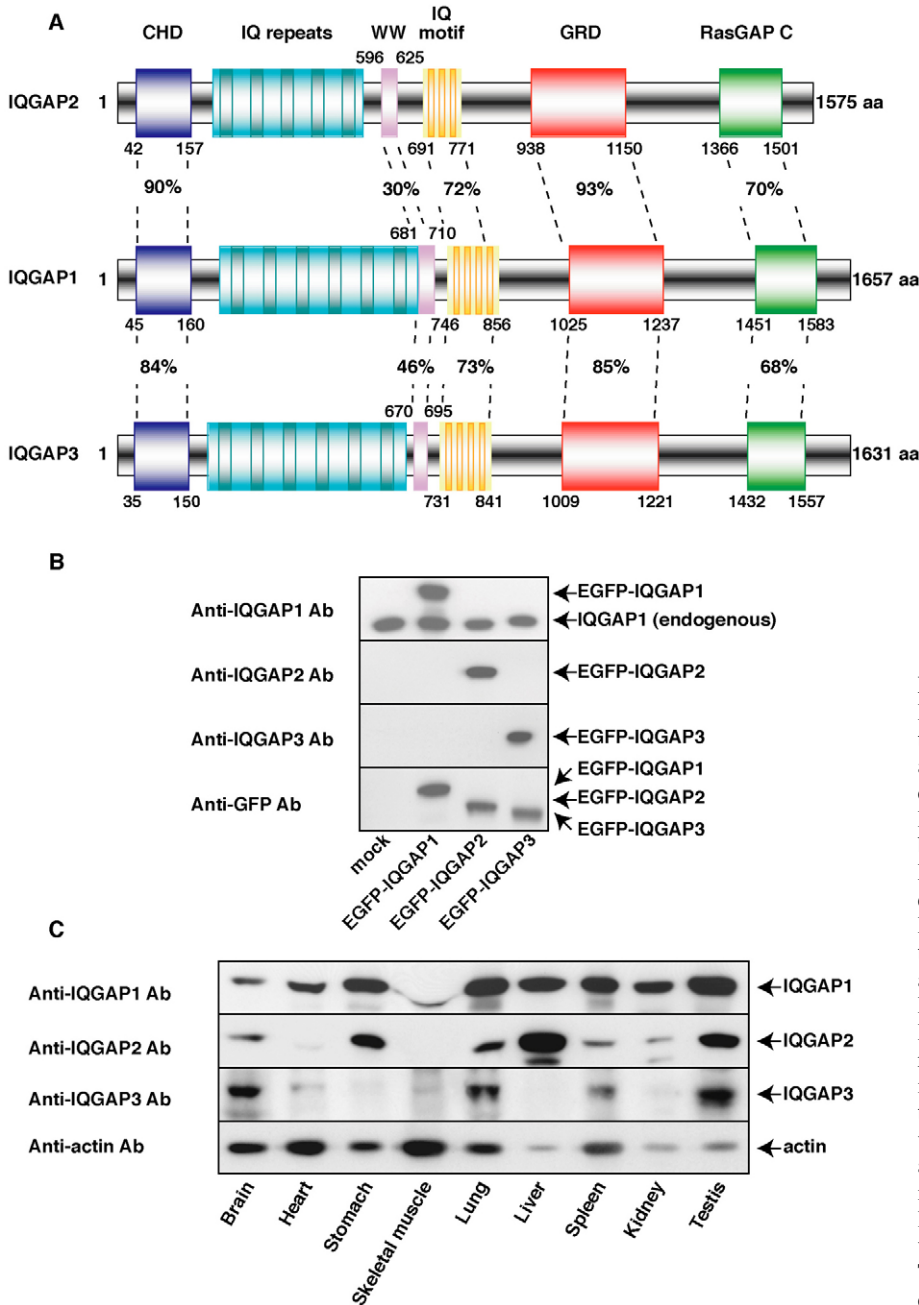
## Introduction

The Rho family GTPases play critical roles in various situations such as cell growth, metastasis, morphogenesis, organogenesis and pathogenesis (Fukata and Kaibuchi, 2001; Van Aelst and Cline, 2004; Govek et al., 2005; Jaffe and Hall, 2005). They function as molecular switches in cells by cycling between the activated GTP-bound and inactivated GDP-bound forms. Their nucleotide state is controlled by three classes of regulators: guanine nucleotide exchange factor, GTPase-activating protein (GAP) and GDP dissociation inhibitor (GDI). These regulators certify that the activation and inactivation of the Rho family GTPases are tightly regulated spatiotemporally to generate specific and localized signals (Kaibuchi et al., 1999; Jaffe and Hall, 2005). Activated GTP-bound forms of the Rho family GTPases transmit intracellular signals through direct interactions with their effectors, which elicit specific physiological responses.

Some members of the Rho family GTPases, which include Rac1, Cdc42 and RhoA, have been studied extensively for the last decade. They affect the organization of the cytoskeleton, cellular adhesions, membrane trafficking and gene transcription by interacting with their specific effectors, which have been identified by genetic or biochemical approaches (Kaibuchi et al., 1999; Jaffe and Hall, 2005). It has been shown clearly in cultured cells that these GTPases control the rearrangement of actin filaments. The polymerization of actin filaments is regulated by Rac1 and Cdc42 to form lamellipodia and filopodia, respectively. Their effectors include Par-6, N-

WASP, IQGAPs, PAK and WAVE. RhoA facilitates the assembly of contractile actin and myosin, stress fibers, and the retraction of migrating cells through its effectors, Rho-kinase/ROCK/ROK and mDia. Accumulating evidence has shown that the Rho family GTPases affect the dynamics of microtubules at the front of the motile cells to promote their migration (Fukata et al., 2003; Rodriguez et al., 2003; Raftopoulou and Hall, 2004; Watanabe et al., 2005). Because of their ability to generate positional cues and the protrusive force of the plasma membrane (Ridley et al., 2003; Raftopoulou and Hall, 2004), Rac1 and Cdc42 especially play important roles in cell polarization and migration.

The IQGAP family comprises a limited number of known proteins, but they are widely distributed among various species (Briggs and Sacks, 2003; Mateer et al., 2003; Noritake et al., 2005). IQGAP1 and IQGAP2 were identified as the molecules harboring the RasGAP-related domain (GRD) (Weissbach et al., 1994; Brill et al., 1996; Hart et al., 1996; Kuroda et al., 1996; McCallum et al., 1996). They possess some conserved domains, including the calponin homology domain (CHD), which is responsible for their binding to actin filaments (Bashour et al., 1997; Fukata et al., 1997), and the IQ motif, which associates with calcium/calmodulin (Weissbach et al., 1994; Brill et al., 1996; Hart et al., 1996; Ho et al., 1999). It has not been shown that GRD within the IQGAPs facilitates the GTPase activity; rather, this domain interacts directly with and stabilizes activated Rac1 and Cdc42 (Brill et al., 1996; Swart-Mataraza et al., 2002; Noritake et al., 2004). This effect, presumably, is due to the lack



**Fig. 1.** IQGAP3 is a novel member of the IQGAP family. (A) Schematic representation of the IQGAP family. The domain structures and amino acid homologies are indicated. CHD, calponin homology domain; IQ repeats, IQGAP-specific repeats; IQ motif, a calmodulin binding motif; GRD, RasGAP-related domain; RasGAP C, RasGAP C terminus. (B) Total extracts from the indicated EGFP-fused IQGAP-expressing COS7 cells were subjected to immunoblot analysis with each anti-IQGAP antibody. Each antibody detected tagged IQGAP in a specific manner. Note that EGFP-IQGAP3 migrated further than IQGAP2. The results shown are representative of four independent experiments. (C) The expression of the IQGAP family in various rat tissues. Actin was monitored as a loading control. Equal amounts of total proteins were subjected to immunoblot analysis, except for IQGAP1 from lung and spleen, for which only half amount of was loaded because of its high level expression. The results represent three independent experiments.

of an arginine finger, which is involved evolutionarily in GAP catalysis, within the IQGAPs (Scheffzek et al., 1998). Thus, IQGAPs function as effectors for Rac1 and Cdc42. Intensive studies on IQGAP1 have shown its involvement in various cellular processes (Briggs and Sacks, 2003; Mateer et al., 2003; Noritake et al., 2005). In motile cells, IQGAP1 regulates the arrangement of actin filaments and microtubules, either directly or indirectly, downstream of Rac1 and Cdc42 (Fukata et al., 2002; Mataraza et al., 2003; Watanabe et al., 2004). These regulations are necessary for effective migration. IQGAP1 also controls the E-cadherin-mediated intercellular adhesion both positively and negatively in a Rac1- and a Cdc42-dependent manner (Fukata and Kaibuchi, 2001; Mateer et al., 2003; Izumi et al., 2004; Noritake et al., 2005). Despite the important roles of these compounds, mice lacking IQGAP1 exhibit only the

abnormality of hyperplasia (Li et al., 2000), suggesting that the other family members or molecules compensate for its function. In addition, although IQGAP1 appears to be involved in neuronal morphogenesis (Li et al., 2005; Kholmanskikh et al., 2006), the roles of the IQGAP family in neuronal cells remain to be clarified.

Here, we identified IQGAP3 as a novel member of the IQGAP family. We found that IQGAP3, similar to the other IQGAPs, is an effector of Rac1 and Cdc42. IQGAP3 interacts directly with actin filaments through its CHD at the amino terminus. In PC12 cells, IQGAP3 regulates neurite outgrowth specifically and positively downstream of Rac1 and Cdc42. Furthermore, both IQGAP2 and IQGAP3 are required for axon outgrowth in hippocampal neurons, but IQGAP1 is dispensable. We also found that IQGAP2 and IQGAP3 have

the distinct ability to promote axon outgrowth. Taken together, we show that IQGAP3 regulates the organization of the cytoskeleton under the regulation of Rac1 and Cdc42 in neuronal cells.

## Results

### Identification of IQGAP3 as a novel member of the IQGAP family

Human IQGAP1 protein has 59% identity to IQGAP2, and their domain structures are well conserved (Briggs and Sacks, 2003; Mateer et al., 2003; Noritake et al., 2005). We searched for the homologous sequences to IQGAP1 in the human genome. We found it in chromosome 1 (1q22), except for *IQGAP1* (15q26.1) and *IQGAP2* (5q13.3). By reverse transcription (RT)-PCR, we obtained the cDNA of the highly IQGAP1-related protein (GenBank accession no.: AY253300, AAP06954). This protein exhibits a high degree of homology with the other IQGAPs in the conserved domains (Fig. 1A). Thus, the protein was designated 'IQGAP3'.

We generated the antibody against IQGAP2 and IQGAP3 (see Materials and Methods). Each antibody detected exogenously expressed or purified IQGAP in a specific and a dose-dependent manner (Fig. 1B and supplementary material Fig. S1). We note that enhanced green fluorescent protein (EGFP)-IQGAP3 migrated further than IQGAP2 presumably because of its structure. Taken together with the observation that the immunoreactivity was specifically decreased by each small-interference RNA (siRNA) (see below), these results indicate that these IQGAP antibodies are the isoform-specific antibodies. The tissue lysates were blotted with these antibodies. The IQGAP family had different expression patterns (Fig. 1C). IQGAP3 was highly expressed in brain, lung and testis. Although the band of IQGAP1 was distorted

or masked in skeletal muscle, the pattern of IQGAP1 and IQGAP2 was comparable with those from previous reports (Weissbach et al., 1994; Brill et al., 1996; Li et al., 2000; Zhou et al., 2003; Izumi et al., 2004). IQGAP3 was expressed in brain (Fig. 1C), raising the possibility that this is where it acts.

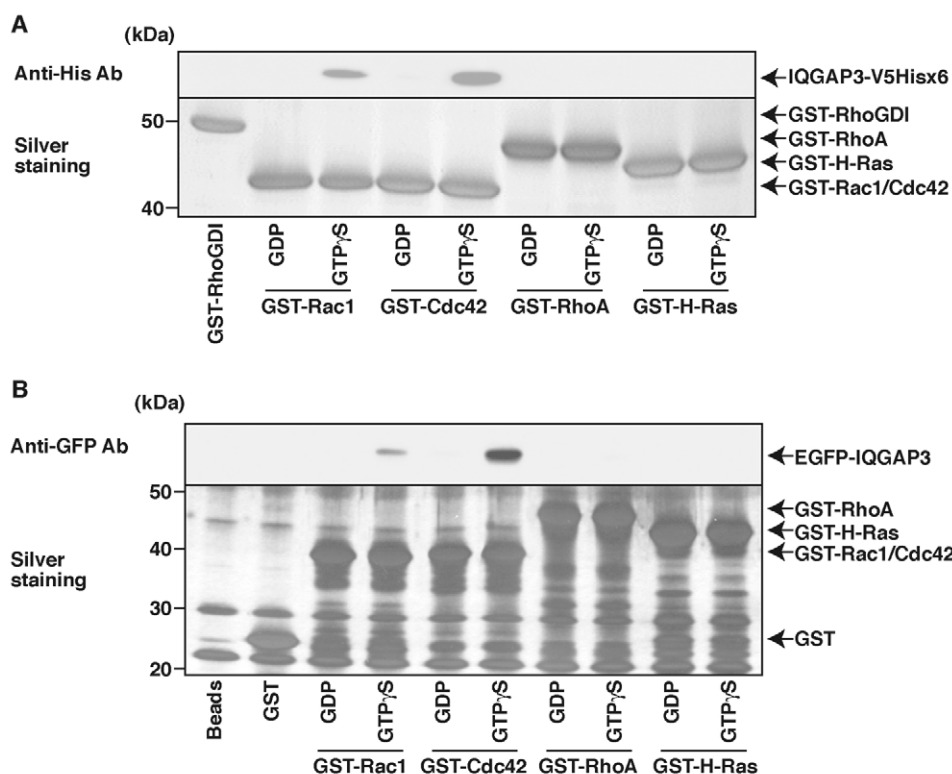
### IQGAP3 is a novel effector of Rac1 and Cdc42

IQGAP1 and IQGAP2 interact directly with activated Rac1 and Cdc42, and function as the scaffold proteins (Briggs and Sacks, 2003; Mateer et al., 2003; Noritake et al., 2005). We examined whether IQGAP3 interacts directly with small GTPases using an in vitro binding assay. IQGAP3-V5Hisx6, similar to IQGAP1, co-eluted with GTP $\gamma$ S-loaded Rac1 and Cdc42, but not with GTP $\gamma$ S-loaded RhoA or H-Ras (Fig. 2A). Similar results were obtained by using maltose-binding protein (MBP)-IQGAP3-C (749-1631 aa), which included GRD (supplementary material Fig. S2).

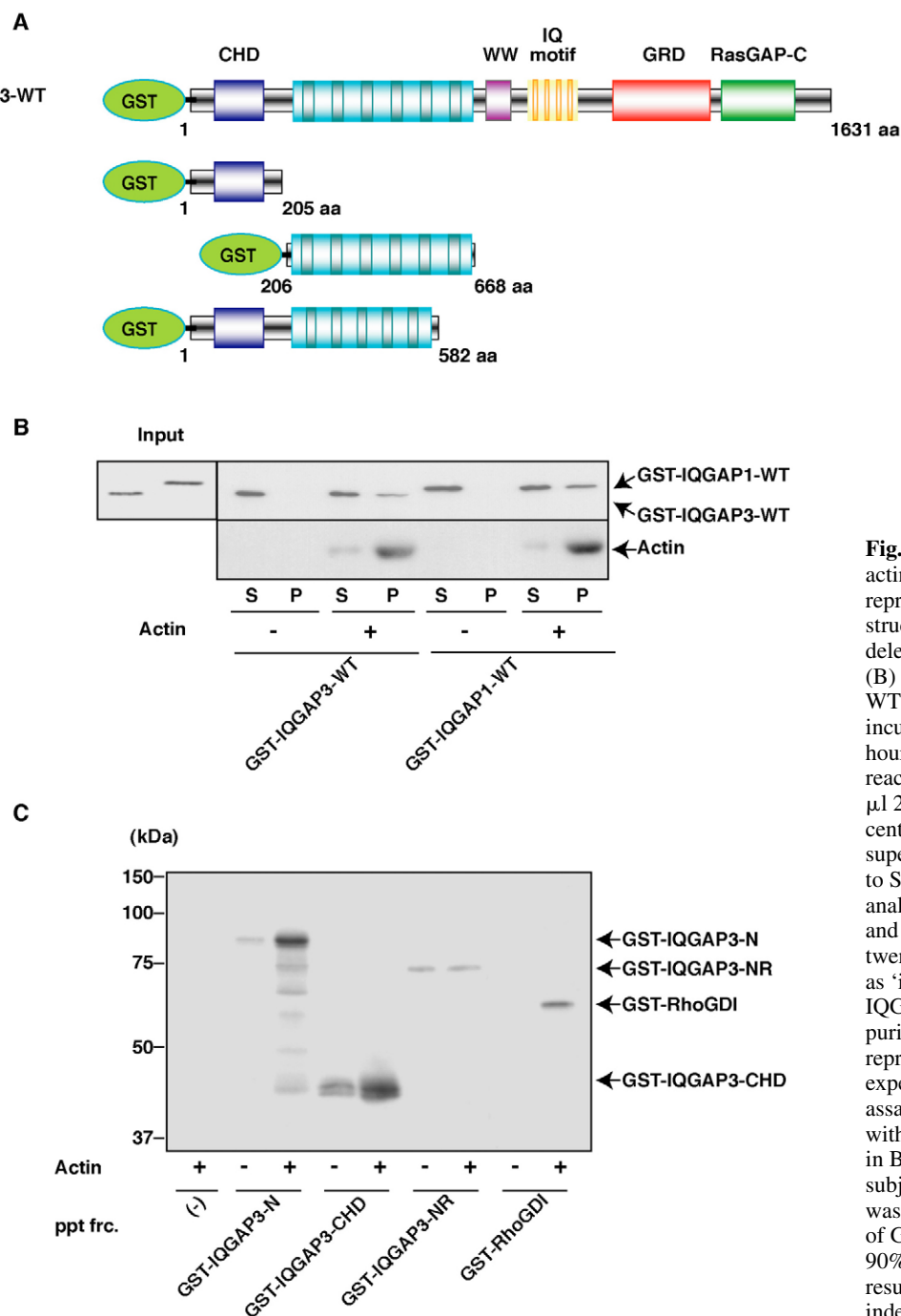
The pull-down assay was also employed to examine the interaction of IQGAP3 with the Rho family. EGFP-IQGAP3 bound specifically to GTP $\gamma$ S-loaded Rac1 and Cdc42 (Fig. 2B). Similar to IQGAP1 and IQGAP2 (Brill et al., 1996; Hart et al., 1996; Kuroda et al., 1996; McCallum et al., 1996), IQGAP3 bound more to Cdc42 than to Rac1 in these assays. Its average recovery for Cdc42 was more than twice that from Rac1. These results indicate that IQGAP3 interacts directly with activated Rac1 and Cdc42. Thus, IQGAP3 is a novel effector of Rac1 and Cdc42.

### IQGAP3 interacts directly with actin filaments

IQGAP3 has a CHD in its amino terminus, which is also present in several actin-binding proteins including the other IQGAPs. To determine whether IQGAP3 interacts directly with F-actin, we performed a co-sedimentation assay (Fig. 3A).



**Fig. 2.** IQGAP3 is an effector for Rac1 and Cdc42. (A) IQGAP3-V5Hisx6 was mixed with the affinity beads coated with the indicated GST-fused small GTPases. Bound IQGAP3-V5Hisx6 was co-eluted with GST-fused proteins by the addition of glutathione. The eluates were subjected to SDS-PAGE, followed by immunoblot analysis with anti-His antibody (top) and silver staining (bottom). The results shown are representative of three independent experiments. (B) COS7 cells expressing EGFP-IQGAP3 were lysed, and the supernatants were incubated with the beads coated with the indicated GST-fused small GTPases. The eluates were subjected to SDS-PAGE, followed by immunoblot analysis with anti-GFP antibody (top) and silver staining (bottom). The results shown are representative of three independent experiments.



**Fig. 3.** IQGAP3 interacts directly with actin filaments. (A) Schematic representation of GST-IQGAP3. The structure of IQGAP3 and its various deletion mutants are represented. (B) GST-IQGAP3-WT or GST-IQGAP1-WT was mixed with F-actin, followed by incubation at room temperature for 1 hour. After the incubation, 50  $\mu$ l of each reaction mixture was layered onto a 100- $\mu$ l 20% (w/v) sucrose barrier and centrifuged. Two fifteenths of the supernatants or the pellets was subjected to SDS-PAGE, followed by immunoblot analysis with anti-GST antibody (top) and anti-actin antibody (bottom). One twentieth of the total proteins was shown as 'input'. GST-IQGAP1-WT and GST-IQGAP3-WT were approximately 70% purity. The results shown are representative of three independent experiments. (C) The cosedimentation assay of the indicated IQGAP3 mutants with F-actin was performed as described in B. Two fifths of each pellet sample was subjected to SDS-PAGE. GST-RhoGDI was used as a negative control. The purity of GST-IQGAP3-CHD, -NR, and -N was 90%, 80% and 80%, respectively. The results shown are representative of three independent experiments.

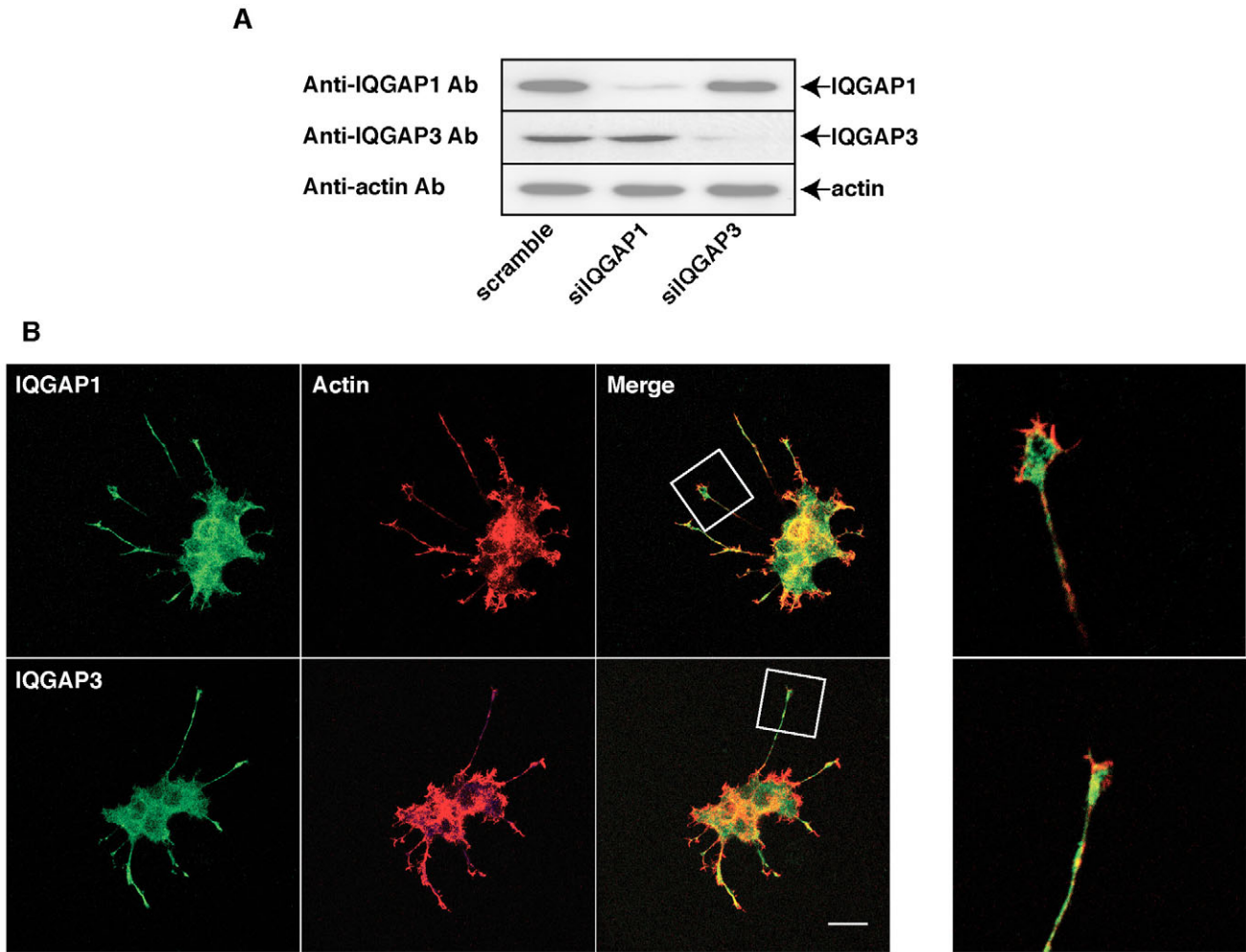
GST-IQGAP3-WT, as well as GST-IQGAP1-WT, was sedimented in the presence of F-actin, but not in the absence of F-actin (Fig. 3B). The recovery rate of IQGAP3 was similar to that of IQGAP1.

IQGAP3 deletion mutants (Fig. 3A) were also employed in this cosedimentation assay. GST-IQGAP3-CHD and -N were incrementally sedimented in the presence of F-actin (Fig. 3C). The recovery rates of IQGAP3-CHD and -N in the pellets were about 30 and 20 times greater, respectively, than that of control GST-RhoGDI. GST-IQGAP3-NR was only sedimented at the background level (Fig. 3C). The affinity column chromatography also showed actin as the interacting molecule

with GST-IQGAP3-N (supplementary material Fig. S3). These results indicate that IQGAP3 interacts directly with F-actin through its CHD.

#### IQGAP3 is necessary for neurite outgrowth in PC12 cells

PC12 cells have been used frequently as a model for neurite outgrowth induced by nerve growth factor (NGF). We first investigated the expressions of IQGAPs, and found that both IQGAP1 and IQGAP3 were expressed in PC12 cells (Fig. 4A), whereas IQGAP2 was undetected (data not shown). The expression levels of IQGAP1 and IQGAP3 were decreased



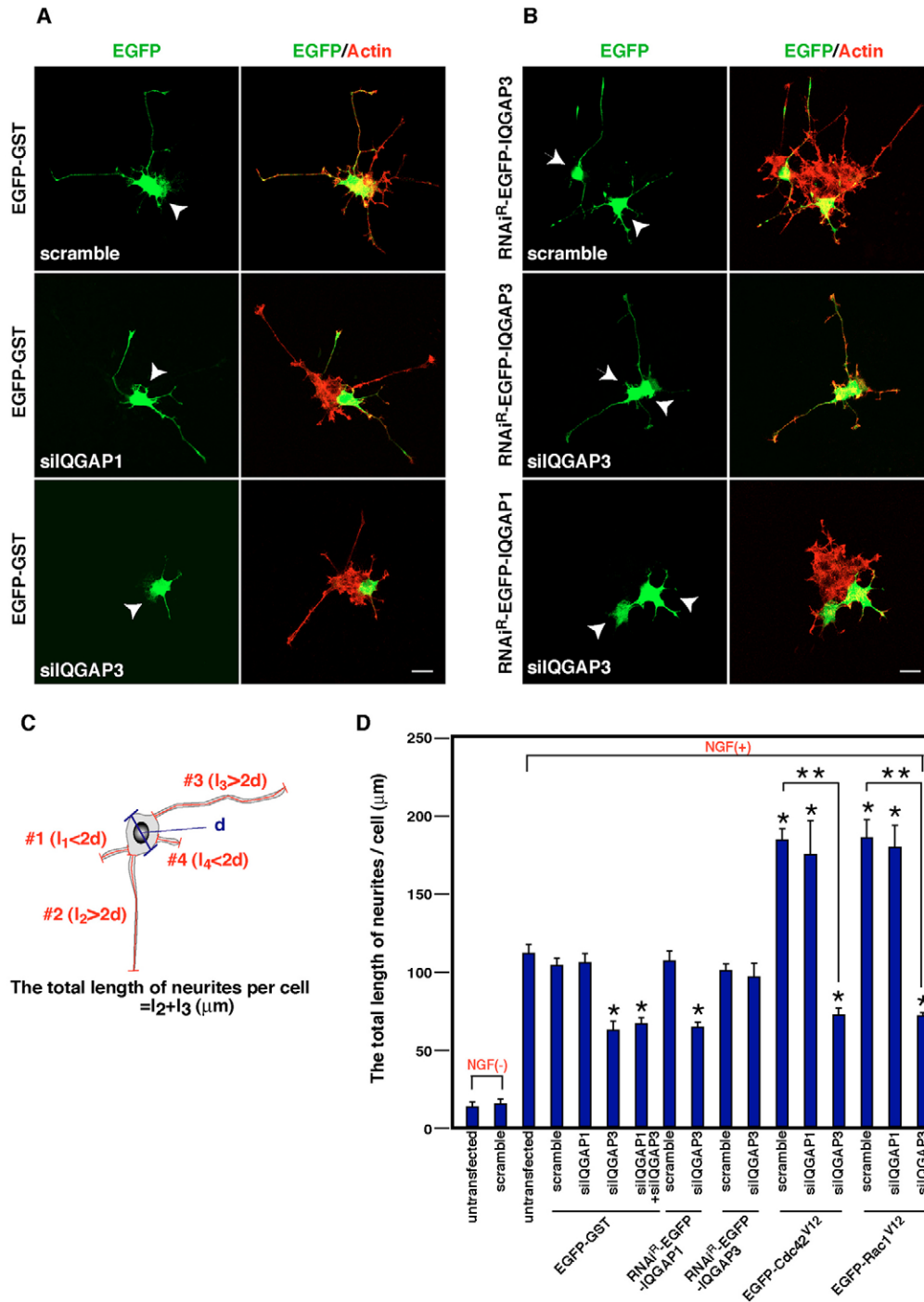
**Fig. 4.** Localizations of the IQGAP family in PC12 cells. (A) Total extracts from the indicated siRNA-transfected PC12 cells were collected after the treatment with NGF for 48 hours, and were subjected to immunoblot analysis with the indicated antibodies. The expression levels of IQGAP1 and IQGAP3 were decreased specifically. The results shown are representative of five independent experiments. (B) PC12 cells were treated with NGF for 48 hours, followed by immunostaining with the indicated anti-IQGAP antibodies and Texas Red-phalloidin. The regions in white boxes are shown magnified in the far right panels. The results shown are representative of five independent experiments. Bar, 20  $\mu$ m.

specifically by IQGAP1-specific siRNA (siIQGAP1) and IQGAP3-specific siRNA (siIQGAP3), respectively (Fig. 4A). Under these conditions, the transfection efficiency of fluorescently labeled siRNA was approximately 90% (data not shown). In PC12 cells, IQGAP1 and IQGAP3 localized diffusely in the cytoplasm, and often accumulated at the tip of a neurite (Fig. 4B). We examined the effect of IQGAP1 or IQGAP3 depletion on neurite outgrowth. The cells with siIQGAPs and EGFP-GST were treated with NGF for 48 hours. The transfected cells were identified using anti-GFP antibody. The control cells elongated their neurites in response to NGF (Fig. 5A,B). The transfection of siIQGAP1 did not affect neurite outgrowth, whereas neurite elongation was impaired in the cells with siIQGAP3 (Fig. 5A,D). The depletion of both IQGAP1 and IQGAP3 did not show the additive effects (Fig. 5D).

We examined whether the RNAi-resistant mutants (RNAi<sup>R</sup>) can rescue neurite outgrowth in IQGAP3-depleted PC12 cells. The expression levels of these RNAi<sup>R</sup> mutants were not

decreased by the siRNAs (data not shown). RNAi<sup>R</sup>-IQGAP3 rescued the impaired neurite outgrowth caused by siIQGAP3, whereas RNAi<sup>R</sup>-IQGAP1 could not (Fig. 5B,D). To determine the functional region of IQGAP3, the chimeric IQGAP1 and IQGAP3 proteins were employed in this rescue experiment (supplementary material Fig. S4A). The inhibitory effect was rescued by RNAi<sup>R</sup>-IQGAP (1N-3C), but not by RNAi<sup>R</sup>-IQGAP (3N-1C) (supplementary material Fig. S4B). These results suggest that IQGAP3 is necessary for neurite outgrowth, and that the functional difference between IQGAP1 and IQGAP3 arises from the carboxyl region.

We next examined whether IQGAPs regulate neurite outgrowth downstream of Rac1 and Cdc42. PC12 cells were co-transfected with constitutively active Rac1 (Rac1<sup>V12</sup>) or Cdc42 (Cdc42<sup>V12</sup>) with siIQGAPs, followed by incubation with NGF. The expression of Rac1<sup>V12</sup> or Cdc42<sup>V12</sup> promoted neurite outgrowth. The depletion of IQGAP3 suppressed the Rac1/Cdc42-promoted neurite outgrowth, but that of IQGAP1 did not (Fig. 5D). In the absence of NGF, the overexpression



**Fig. 5.** IQGAP3 is necessary for neurite outgrowth in PC12 cells. (A) EGFP-GST and indicated siRNA were cotransfected into PC12 cells. The cells treated with NGF for 48 hours were labeled with anti-GFP antibody and Texas Red-phalloidin. Arrowheads indicate the cotransfected cells that were identified by anti-GFP antibody. The transfection of siIQGAP3 impaired neurite outgrowth, but that of scramble or siIQGAP1 did not. The results represent five independent experiments. Bar, 20  $\mu\text{m}$ . (B) PC12 cells cotransfected with indicated RNAi<sup>R</sup>-IQGAPs and siRNA were treated with NGF for 48 hours. The cells were labeled with anti-GFP antibody and Texas Red-phalloidin. Arrowheads indicate the cotransfected cells which were identified by anti-GFP antibody. RNAi<sup>R</sup>-IQGAP3 specifically rescued the impaired neurite outgrowth by siIQGAP3, but RNAi<sup>R</sup>-IQGAP1 did not. The results shown are representative of five independent experiments. Bar, 20  $\mu\text{m}$ . (C) Schematic diagram of the quantification of the total length of neurites per cell. For each transfected PC12 cell, we determined the total length of neurites for which the lengths exceeded twice of the diameter of its cell body. See also Materials and Methods.  $l$ , length;  $d$ , diameter of the cell body. (D) Quantification of the total length of neurites. The total length of neurites was measured in the cells with or without NGF. Data represent the means  $\pm$  s.d. of five independent experiments.  $n > 150$ . Single and double asterisks indicate the difference in the value of each of the control cells at  $P < 0.01$ .

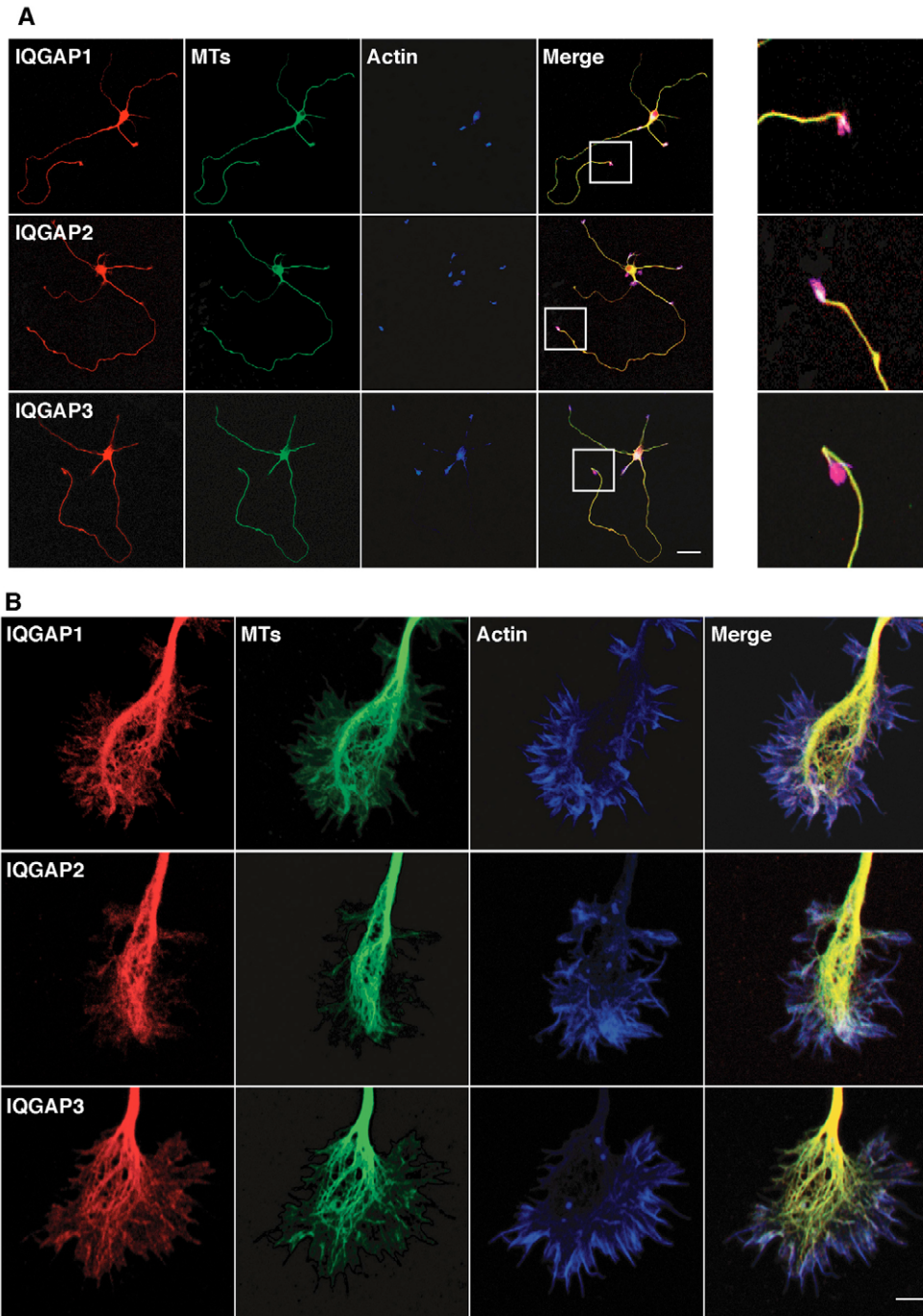
of Rac1<sup>V12</sup> or Cdc42<sup>V12</sup> affected their morphology, but failed to induce typical neurites (supplementary material Fig. S5), similar to the previous reports (Daniels et al., 1998; Katoh et al., 2000). These results suggest that IQGAP3 positively regulates neurite outgrowth downstream of Rac1 and Cdc42 in PC12 cells.

#### IQGAPs localize at the tips of axon in hippocampal neurons

We next employed primary cultured hippocampal neurons to examine the roles of IQGAP3 in more physiological

conditions. The neurons show a polarized morphology with a single elongated axon and some minor processes at stage 3 (Craig and Banker, 1994; Arimura and Kaibuchi, 2005).

We first examined the expressions of IQGAPs, and found that all members were expressed, but IQGAP2 was most abundant in the hippocampus (data not shown). The neurons were usually cultured on poly-D-lysine (PDL) and laminin. For visualization of the fine structures within the growth cone, the cells were cultured on PDL alone (see Materials and Methods). To investigate the overall distribution of the IQGAP family, the neurons were labeled with each anti-IQGAP antibody at 3 days



**Fig. 6.** IQGAPs localize at the tips of axons in hippocampal neurons. (A) Localization of the IQGAP family in stage 3 hippocampal neurons. The cells were labeled with each anti-IQGAP antibody (red), anti-unique  $\beta$ -tubulin (TUJ1) antibody (green), and Alexa Flour 647-phalloidin (blue). The regions in white boxes are shown magnified (right). The results shown are representative of five independent experiments. Bar, 20  $\mu$ m. (B) Detailed localization of the IQGAP family in the growth cones. The cells on PDL alone were labeled as described in A. The results shown are representative of six independent experiments. Bar, 5  $\mu$ m.

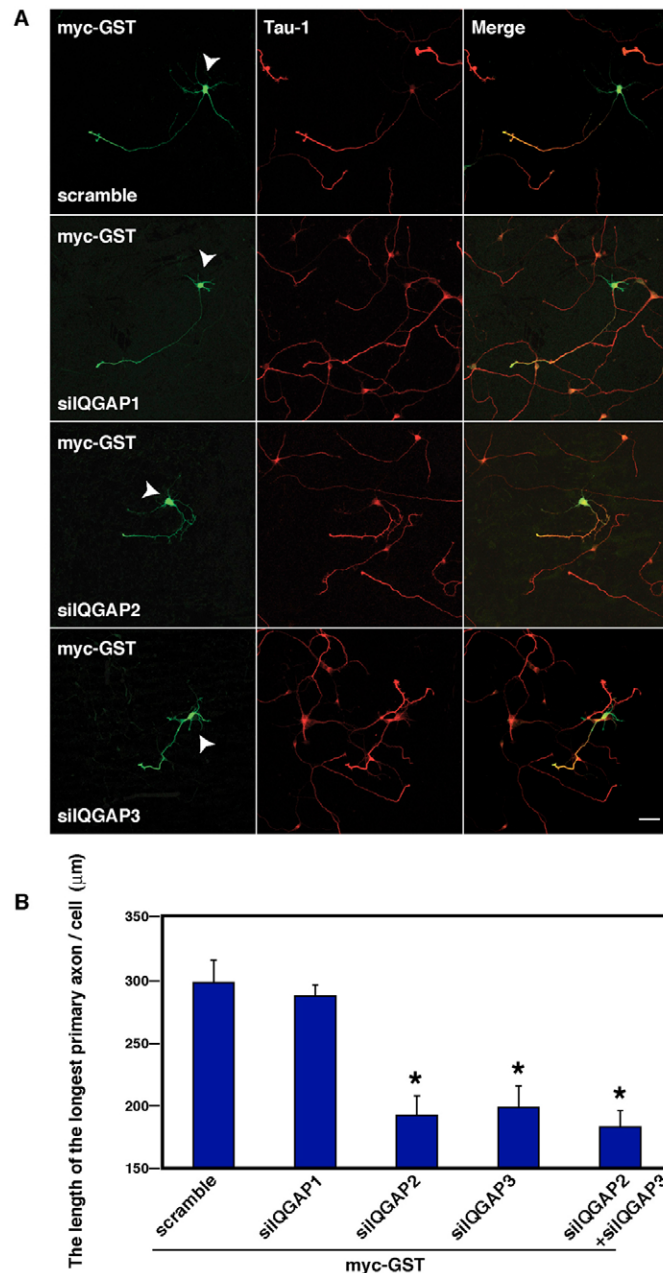
in vitro (DIV3) (Fig. 6A). A high level of staining of each IQGAP was observed in their cell bodies, the distal parts of axons, and the axon growth cones. The levels of IQGAPs were low in the minor processes of stage 3 neurons (Fig. 6A). In the growth cones on PDL alone, all IQGAPs concentrated mainly in the central region and colocalized with microtubules (Fig. 6B).

#### IQGAP3 is important for axon outgrowth in hippocampal neurons

We examined the effects of the depletion of IQGAPs on axon outgrowth. IQGAP2-specific siRNA (siIQGAP2) depleted the

expression of IQGAP2 in rat hepatoma cells (supplementary material Fig. S6). The neurons were co-transfected with siIQGAPs and Myc-GST, and were labeled at DIV3. The transfected cells and axons were identified by anti-Myc antibody and Tau-1 antibody (axon marker), respectively. The neurons with siIQGAP1 elongated their axons almost to the length of control cells (Fig. 7A,B), whereas axon elongation was impaired in neurons with either siIQGAP3 or siIQGAP2 (Fig. 7A,B). The depletion of both IQGAP2 and IQGAP3 did not show additive effects on the inhibition of axon outgrowth (Fig. 7B), suggesting that IQGAP2 and IQGAP3 stay mainly in a common regulatory pathway. We also examined the effects

of IQGAP depletion on neuronal growth cones on PDL alone. We found that the depletion of either IQGAP2 or IQGAP3 affected growth cone morphology, but depletion of IQGAP1



**Fig. 7.** IQGAP3 plays important roles for axon outgrowth in hippocampal neurons. (A) The neurons were cotransfected with Myc-GST and siRNA, followed by double staining with anti-Myc (green) and Tau-1 (red) antibodies at DIV3. Arrowheads indicate the cotransfected cells that were identified by anti-Myc antibody. Axon outgrowth was impaired in the neurons transfected with either siIQGAP2 or siIQGAP3. The results shown are representative of seven independent experiments. Bar, 20 μm. (B) Quantification of the length of the longest primary axon. The axon length was measured in the cells cotransfected with Myc-GST and the indicated siRNA. Data represent the means  $\pm$  s.d. of five independent experiments.  $n > 150$ . Asterisks indicate a significant difference from the value of the control cells at  $P < 0.01$ .

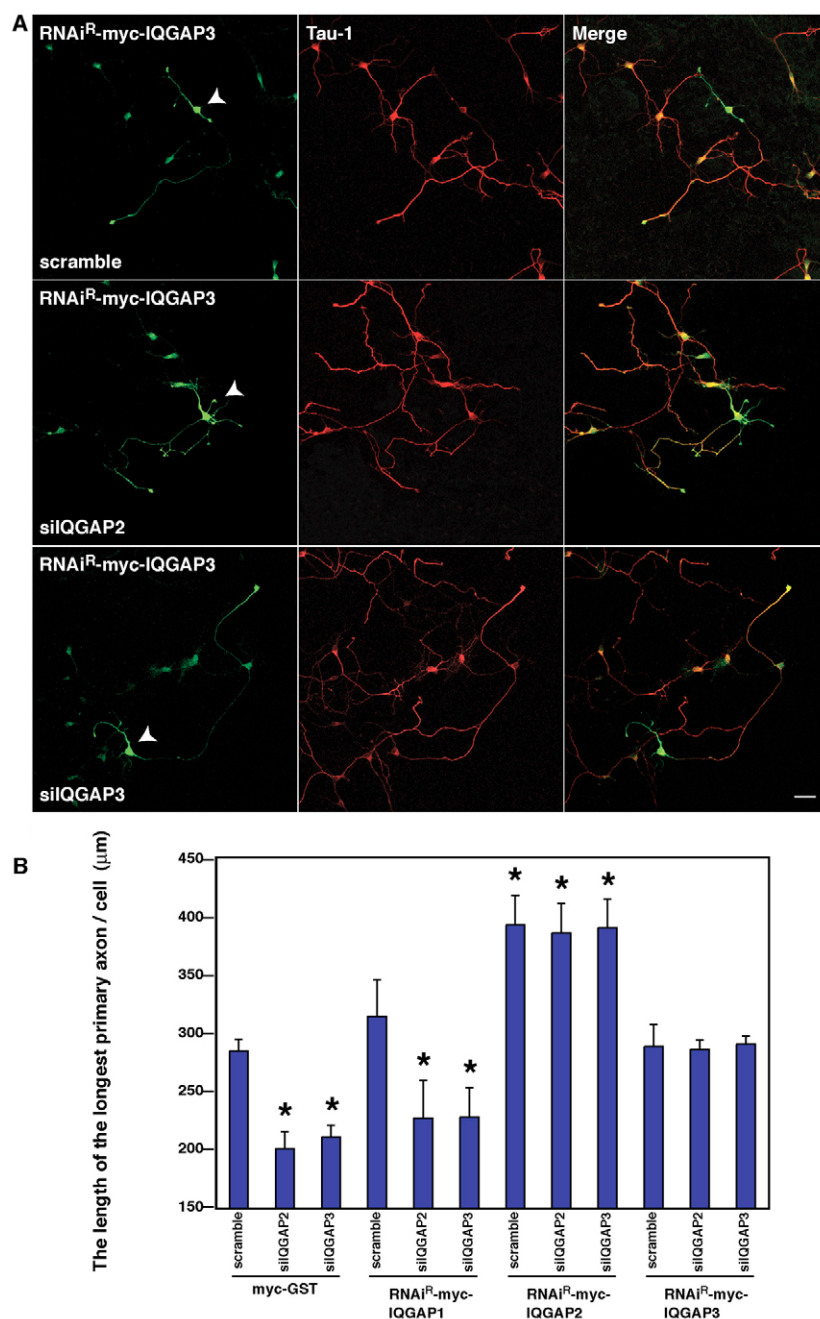
had less effect (supplementary material Fig. S7). In the IQGAP2- or IQGAP3-depleted neurons, the growth cone adopted a small club-like appearance with disorganized actin filaments. The IQGAP2- or IQGAP3-depleted cells often formed some protrusions along their growth cones and shafts. This morphology was also observed in the cells treated with cytoskeleton disrupting reagents, cytochalasin and colchicine (Dent and Gertler, 2003). These results indicate that IQGAP3 and IQGAP2 play important roles in the regulation of the cytoskeleton for axon outgrowth in hippocampal neurons.

We next examined whether the RNAi<sup>R</sup>-IQGAPs mutants can rescue the phenotypes by siIQGAP2 or siIQGAP3 in the neurons. When neurons were transfected with RNAi<sup>R</sup>-IQGAP3 together with siIQGAP2 or siIQGAP3, the impaired axon outgrowth was rescued. However, RNAi<sup>R</sup>-IQGAP1 could not rescue impaired axon outgrowth caused by siIQGAP2 or siIQGAP3 (Fig. 8A). Under these conditions, RNAi<sup>R</sup>-IQGAP2 promoted axon outgrowth. The average axonal length of RNAi<sup>R</sup>-IQGAP2-expressing neurons was about 1.4 times longer than that of the control cells (Fig. 8B). The overexpression of RNAi<sup>R</sup>-IQGAP2 overcame the inhibitory effects of siIQGAP2 or siIQGAP3 on axon outgrowth (Fig. 8B). These results indicate that IQGAP3 partially compensates the functions of IQGAP2, but has lesser ability than IQGAP2 to promote axon outgrowth in hippocampal neurons.

## Discussion

In the present study, we identified IQGAP3 as a novel effector of Rac1/Cdc42 and an actin binding protein, similar to IQGAP1 (Figs 2, 3). We found that the depletion of IQGAP3 impaired neurite outgrowth of PC12 cells in response to NGF, whereas the depletion of IQGAP1 had no effect, despite their similar domain structures and destinations (Figs 1, 4, 5). How does IQGAP3 regulate neurite outgrowth in an isoform-specific fashion? By the use of the chimeric proteins, we showed that the carboxyl region of IQGAP3 is necessary for the rescue (supplementary material Fig. S4), suggesting that the functional differences between IQGAP1 and IQGAP3 reside in the carboxyl region. Their carboxyl regions are responsible for the bindings of the Rho family, CLIP-170, and adenomatous polyposis coli (Fig. 2 and data not shown), but we could not find any different bindings to them. Uncharacterized or unidentified binding molecules to the IQGAP3 carboxyl region may generate its functional difference to IQGAP1.

The redundant roles of IQGAPs were also observed in hippocampal neurons. In addition to the depletion of IQGAP3, that of IQGAP2 also impaired axon outgrowth. The simultaneous depletion of IQGAP2 and IQGAP3 did not show additive effects (Fig. 7). Thus, IQGAP2 and IQGAP3 are thought to stay in a common regulatory pathway. The expression of RNAi<sup>R</sup>-IQGAP3 could rescue impaired axon outgrowth resulting from transfection of either siIQGAP2 or siIQGAP3, but IQGAP2 had characteristic ability to promote axon outgrowth and overcame the inhibitory effects of siIQGAP2 or siIQGAP3 (Fig. 8). Thus, IQGAPs could regulate axon outgrowth partially in a specific manner. In support of these observations, in *Dictyostelium discoideum*, two IQGAP-related proteins have distinct but partially redundant functions (Faix et al., 2001). The structure of IQGAP2 showed small differences with respect to the other IQGAPs: IQGAP2 lacked



**Fig. 8.** The expressions of RNAi<sup>R</sup>-IQGAP2 and -IQGAP3 rescue axon outgrowth. (A) The neurons were cotransfected with the indicated siRNA and RNAi<sup>R</sup>-IQGAP3, followed by immunostaining with anti-Myc (green) and Tau-1 (red) antibodies at DIV3. Arrowheads indicate the cotransfected cells that were identified by the anti-Myc antibody. RNAi<sup>R</sup>-IQGAP3 rescued the impaired axon outgrowth created by siIQGAP2 or siIQGAP3. The results represent five independent experiments. Scale bar equals 20 μm. (B) Quantification of the length of the longest primary axon. The axon length was measured in the cells cotransfected with the indicated combinations. Data represent the means ± s.d. of five independent experiments.  $n > 150$ . Asterisks indicate a significant difference from the value of the control cells at  $P < 0.01$ .

PTP $\mu$ -mediated neurite outgrowth is impaired by the transfection of the peptide, which competes in the interaction of IQGAP1 with Rac1 and Cdc42, in chick nasal retina. We have shown here, by the depletion of endogenous IQGAPs (Figs 5, 7), that IQGAP3 is important for neurite outgrowth downstream of Rac1/Cdc42 in PC12 cells or hippocampal neurons, but IQGAP1 is not. Although the overexpression of IQGAP1 mutant and the inhibitory peptide might affect other IQGAPs as well as IQGAP1, the IQGAP family consistently plays important roles in neurite outgrowth. How do IQGAPs regulate neuronal morphology? The major assumptions come from the roles of IQGAP1 in previous reports. IQGAP1 stabilizes activated forms of Rac1 and Cdc42 in epithelial cells (Swart-Mataraza et al., 2002; Noritake et al., 2004). To promote cell motility, IQGAP1 regulates the organization of actin filaments and microtubules (Fukata et al., 2002; Mataraza et al., 2003; Watanabe et al., 2004). These mechanisms, together with Lis1, can also affect the motility of cerebellar granule neurons (Kholmanskikh et al., 2006). We also showed that the IQGAP2- or IQGAP3-depleted growth cones adopted a small club-like appearance with disorganized actin filaments and some protrusions (supplementary material Fig. S7). This shape has also been observed in cells with treated with cytochalasin, an actin filaments disrupting reagent, and colchicine, a microtubule depolymerizing reagent (Dent and Gertler, 2003). Furthermore, the inhibition of Rac1 results in an increased number of filopodia around the growth cones (Rajnick et al., 2006). Taken together, these results suggest that IQGAPs regulate the neuronal morphology of PC12 cells and hippocampal neurons, possibly via their abilities to reorganize the cytoskeletal architectures and to stabilize activated Rac1 and Cdc42. However, it remains to be addressed why IQGAP3 is required for neurite outgrowth, but IQGAP1 is dispensable, and why either IQGAP2 or IQGAP3 is necessary for axon outgrowth in hippocampal neurons.

one IQGAP-specific repeat and one IQ motif (Fig. 1A). Although the function of IQGAP-specific repeats remains unknown, the IQ motif binds to calmodulin and shows a discrete association with the myosin essential light chain (Weissbach et al., 1998; Ho et al., 1999). Our preliminary results showed that synapsin1 was identified as interacting specifically with IQGAP2 (data not shown). These qualitative differences may account for the specific roles of IQGAP2 in hippocampal neurons as well as the specific physiological roles among the members of the IQGAP family.

Accumulating evidence has suggested that IQGAP1 regulates neurite extension in neuronal cells (Li et al., 2005; Phillips-Mason et al., 2006). The forced expression of mutant IQGAP1 can promote neurite outgrowth in N1E-115 cells.

Further analysis should shed light on how each IQGAP contributes to the establishment of neuronal morphology.

## Materials and Methods

### Materials and chemicals

The cDNA-encoding human IQGAP2 and pCAGGS vector were kindly provided by A. Bernards (Harvard Medical School, Charlestown, MA, USA) and M. Nakafuku (Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA), respectively. pEGFP-C1 vector was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA). Anti-IQGAP1 polyclonal antibody, raised against human IQGAP1 314-422 aa, was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-IQGAP polyclonal antibodies were raised against GST-IQGAP2 (213-589 aa) and GST-IQGAP3 (206-668 aa), respectively. To generate the specific antibodies, we purified the antibodies by sequential affinity column chromatography. Briefly, each anti-GST-IQGAP-NR serum was passed sequentially through the beads coated with two other GST-IQGAP-NR to absorb the cross-reactive antibodies. The pass through fraction, which includes the isoform-specific antibody, was further applied to the beads coated with its MBP-tagged antigen, and the isoform-specific antibody was eluted by acidic solution.

The following antibodies were used: anti-unique  $\beta$ -tubulin monoclonal antibody (TUJ1; Berkeley Antibody Company, Richmond, CA, USA); polyclonal anti-Myc antibody (A-14; Santa Cruz Biotechnology, Inc.); monoclonal Tau-1 antibody (Chemicon, Temecula, CA, USA). Cy2- or Cy3-conjugated secondary antibodies against mouse or rabbit immunoglobulin G were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). NGF was purchased from Upstate Biotechnology, Inc. (Charlottesville, VA, USA). Other materials and chemicals were obtained from commercial sources.

### Plasmid constructs

To obtain IQGAP3 cDNA, we isolated total RNA from HEK293 cells using TRIzol reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. We obtained partial cDNA fragments of IQGAP3 by RT-PCR using the primer sequences as determined from human genome sequences. After sequencing and confirming that these cDNAs encoded the sequence in chromosome 1 as well as IQGAP3, we ligated these overlapped fragments of IQGAP3 cDNA at corresponding restriction enzyme sites. The IQGAP3 cDNA and the amino acid sequence were submitted to GenBank database (AY253300 and AAP06954, respectively).

The constructs of pGEX-, pMal- and pEGFP-IQGAP1, full-length or mutants, were produced as described elsewhere (Noritake et al., 2004; Watanabe et al., 2004). To obtain constructs of the IQGAP family, we subcloned the corresponding cDNA fragments into pGEX-4T-1, pMal-c2, pEGFP-C1 and pCAGGS-Myc vectors. All tags were fused to the amino terminus of the proteins of interest.

### Preparation of recombinant proteins and in vitro binding assays

The expression and purification of GST fusion proteins was performed as described by Kuroda et al. (Kuroda et al., 1998). We purified full-length IQGAP1 and IQGAP3, tagged either with GST at the amino terminus or with V5 and Hisx6 at the carboxyl terminus, from overexpressing *Spodoptera frugiperda* cells as described by Fukata et al. (Fukata et al., 1997). An in vitro binding assay and the pull-down assay were described previously (Fukata et al., 1997; Kuroda et al., 1998).

### Actin co-sedimentation assay

The purification of F-actin and an actin co-sedimentation assay were performed as described by Fukata et al. (Fukata et al., 1997). Briefly, F-actin was mixed with the indicated recombinant protein and incubated at room temperature for 1 hour in buffer B [20 mM Tris-HCl at pH 7.4, 0.5 mM dithiothreitol, 2 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM EDTA, 10% (w/v) sucrose, 0.5 mM ATP, 10  $\mu$ g/ml leupeptin and 10  $\mu$ M (*p*-amidinophenyl)-methanesulfonyl fluoride]. After the incubation, 50  $\mu$ l of each reaction mixture was layered onto a 100- $\mu$ l sucrose barrier [20% (w/v) sucrose in buffer B] and centrifuged at 200,000 g for 1 hour at room temperature. The supernatants and pellets were separated and subjected to SDS-PAGE, followed by immunoblot analysis with the indicated antibodies.

### Small-interference RNA and RNAi<sup>R</sup> mutant

RNA oligomers containing 21 nucleotides were synthesized in sense and antisense directions corresponding to human IQGAP1 (5'-UGCCAUGGAUGAGAUUGGA-3'), rat IQGAP2 (5'-CACCGUCAGUGGUUAAGA-3'), rat IQGAP3 (5'-AAGUUCUUCGUUGCCAUCA-3'), with dTdT overhangs at each 3' terminus (Greiner-Japan, Tokyo, Japan). Each selected sequence was submitted to a BLAST search against the human genome to ensure that only the corresponding IQGAP gene was targeted. As a nonspecific siRNA control that we termed 'scramble', we used (5'-CAGUCGCGUUUGCGACUGG-3') with dTdT overhangs at the 3' terminus. Human IQGAP2 or IQGAP3 cDNA was used as an RNAi<sup>R</sup> mutant in rat PC12 cells and hippocampal neurons. The RNAi<sup>R</sup> IQGAP1 mutant was described previously (Watanabe et al., 2004).

### Cell culture

COS7 cells or PC12 cells were maintained in Dulbecco's modified Eagle's medium, which contained either 10% fetal bovine serum (FBS) or 5% FBS and 5% horse serum, respectively, at 37°C in an air-5% CO<sub>2</sub> atmosphere at constant humidity. Transfections were carried out with the use of Lipofectamine or Lipofectamine2000 reagent (Invitrogen) according to the manufacturer's instructions. PC12 cells were fixed with 3.0% formaldehyde in phosphate-buffered saline (PBS) for 10 minutes and then treated with PBS that contained 0.2% Triton X-100 and 2 mg/ml BSA for 10 minutes. The fixed cells were stained with the indicated antibodies and phalloidin.

### Culture of hippocampal neurons

A culture of hippocampal neurons prepared from E18 rat embryos using papain was performed as described previously (Inagaki et al., 2001). Neurons were seeded on coverslips with PDL (Sigma) alone or PDL and laminin (Iwaki, Tokyo, Japan) in neurobasal medium (Invitrogen) supplemented with B-27 supplement (Invitrogen) and 1 mM glutamine. Neurons were transfected using a calcium phosphate method before plating to analyze the morphology (Nishimura et al., 2004; Arimura et al., 2005; Kawano et al., 2005; Yoshimura et al., 2005). They were fixed after 3 days in vitro (DIV3) with 3.7% formaldehyde in PBS for 10 minutes at room temperature, followed by treatment for 10 minutes with 0.05% Triton X-100 on ice and 10% normal goat serum in PBS for 1 hour at room temperature. Neurons were then immunostained with the indicated antibodies and phalloidin.

### Immunofluorescence analysis

Fluorescence was examined using a confocal laser-scanning microscope (Carl Zeiss LSM 510; Carl Zeiss, Oberkochen, Germany) built around a Zeiss Axiocvert 100M. To measure the length of the neurite in PC12 cells, we co-transfected the cells with siRNA and the indicated plasmid, and cultured the cells for an additional 48 hours in the presence or absence of NGF at a final concentration 100 ng/ml. Transfected cells and neurite length were identified by the immunofluorescence of co-transfected plasmids. The co-transfection efficiency of siRNA and plasmid was nearly 50% judging from the immunofluorescence of GFP enhanced by anti-GFP antibody. According to the methods described by Schreiber et al. (Schreiber et al., 2004), we determined the total length of neurites for which the lengths exceeded twice of the diameters of their cell bodies, using LSM 510 software (Carl Zeiss). At least 150 cells in each condition were counted. The data are presented as means and s.d. of at least four independent experiments. To measure the axon lengths of hippocampal neurons, we co-transfected the neurons with siRNA and the indicated plasmid. The transfected neurons and their axons were identified by immunofluorescence of the co-transfected plasmid and Tau-1 antibody, respectively, at DIV3. About 75% of the cells showed the characteristic morphology with a single axon. The remaining neurons were still in stage 2 of development. The length of the longest primary axon was measured from the cell body only in Tau-1-positive neurons. At least 150 cells in each condition were counted. The data are presented as means and s.d. of at least four independent experiments.

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