Involvement of p114-RhoGEF and Lfc in Wnt-3a– and Dishevelled-Induced RhoA Activation and Neurite Retraction in N1E-115 Mouse Neuroblastoma Cells

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INTRODUCTION

The Wnt-induced planar cell polarity (PCP) signaling pathway is essential for polarized cell migration and morphogenesis. Dishevelled (Dvl) and its binding protein Daam1 mediate RhoA activation in this pathway. WGEF, a member of the Rho-geanine nucleotide exchange factor (Rho-GEF) family, was shown to play a role in Wnt-induced RhoA activation in Xenopus embryos. However, it has remained unknown which member(s) of a Rho-GEF family are involved in Wnt/Dvl-induced RhoA activation in mammalian cells. Here we identified p114-RhoGEF and Lfc (also called GEF-H1) as the Rho-GEFs responsible for Wnt-3a–induced RhoA activation in N1E-115 mouse neuroblastoma cells. We screened for Rho-GEF–silencing short-hairpin RNAs (shRNAs) that are capable of suppressing Dvl-induced neurite retraction in N1E-115 cells and found that p114-RhoGEF and Lfc shRNAs, but not WGEF shRNA, suppressed Dvl- and Wnt-3a–induced neurite retraction. p114-RhoGEF and Lfc shRNAs also inhibited Dvl- and Wnt-3a–induced RhoA activation, and p114-RhoGEF and Lfc proteins were capable of binding to Dvl and Daam1. Additionally, the Dvl-binding domains of p114-RhoGEF and Lfc inhibited Dvl-induced neurite retraction. Our results suggest that p114-RhoGEF and Lfc are critically involved in Wnt-3a– and Dvl-induced RhoA activation and neurite retraction in N1E-115 cells.

The Wnt family of secreted proteins play crucial roles in numerous developmental and physiological processes by regulating cell proliferation, migration, morphogenesis, and polarity formation (Logan and Nusse, 2004). Wnt signals are transduced via at least three distinct pathways: a canonical β-catenin–dependent pathway and two noncanonical β-catenin–independent pathways, comprising a planar cell polarity (PCP) pathway and a Ca2+ pathway (Veeman et al., 2003; Kikuchi et al., 2009). Dishevelled (Dvl) is a key cytoplasmic component that is activated downstream of the Wnt receptor Frizzled (Fz) to transduce both canonical and noncanonical Wnt pathways (Wharton, 2003; Wallingford and Habas, 2005). In the canonical Wnt pathway, Dvl mediates β-catenin stabilization via suppression of GSK-3β activity, and the stabilized β-catenin is translocated into the nucleus where it stimulates the transcription of Wnt-responsive genes (Logan and Nusse, 2004). In the noncanonical Wnt-PCP pathway, Dvl mediates activation of Rho family small GTPases, RhoA and Rac1, and their downstream kinases, Rho-associated kinase (ROCK) and c-Jun N-terminal kinase, respectively. This leads to actin cytoskeletal remodeling, which controls cell migration and PCP formation (Veeman et al., 2003; Habas et al., 2003). The Wnt-PCP pathway is required for the establishment of PCP of wing hairs, bristles, and ommatidia in Drosophila (Shulman et al., 1998; Adler, 2002). This pathway also functions in the processes of polarized cell motility and morphogenesis in vertebrates, including the convergent extension cell movements for anterior-posterior axis formation during Xenopus gastrulation and the planar polarized orientation of stereocilia of sensory hair cells in the mammalian inner ear (Jones and Chen, 2007; Seifert and Mlodzik, 2007). The Dvl-mediated activation of RhoA and ROCK is also involved in Wnt-3a–induced neurite retraction in rat PC-12 pheochromocytoma and mouse N1E-115 neuroblastoma cells (Kishida et al., 2004).

Rho family GTPases are inactive in the GDP-bound state and active in the GTP-bound state, and in their active form...
these proteins can associate with downstream effector proteins to induce a diverse cellular response (Etienne-Manneville and Hall, 2002). Guanine nucleotide exchange factors (GEFs) stimulate the conversion of Rho GTPases from their GDP-bound form to their active GTP-bound form (Schmidt and Hall, 2002; Rossman et al., 2005). In the human genome, there are at least 69 distinct members of the DbI-related Rho-GEF gene family, and these are thought to stimulate the GDP–GTP exchange of Rho family GTPases (Rossman et al., 2005). They possess a conserved Dbl homology (DH) domain responsible for Rho-GEF activity, and the DH domain is usually followed by a pleckstrin homology (PH) domain that mediates membrane association. In addition to the DH and PH domains, the Dbl-family Rho-GEFs contain a variety of protein-binding domains that are thought to be involved in the distinct regulation of GEF activity or the localization of individual Rho-GEFs (Rossman et al., 2005). Because of the large number of Rho-GEF genes in mammalian genomes, the functional roles of each of the individual Rho-GEFs in various cellular events have not been elucidated.

In the Wnt/PCP pathway, Wnt/Fz/Dvl-induced RhoA activation requires a formin-related protein, termed the Dvl-associated activator of morphogenesis 1 (Daam1) (Habas et al., 2001). When Fz is activated by the Wnt signal, it induces the translocation of Dvl to the plasma membrane and the formation of the Dvl-Daam1 complex. Because Daam1 does not have the ability to activate RhoA directly, it is predicted to function as a scaffold protein that recruits Rho-GEF for RhoA activation (Habas et al., 2001). A recent study showed that WGEF (named in this manner because of its weak similarity to GEPF), a member of the Dbl-related Rho-GEFs, connects Dvl to Rho activation in the Wnt/PCP pathway and controls the convergent extension process in Xenopus gastrulation (Tanegashima et al., 2008). Xenopus WGEF can bind to Dvl and Daam1, and depletion of WGEF caused axis elongation defects and inhibition of convergent extension in Xenopus embryos (Tanegashima et al., 2008). However, it is unknown whether WGEF, or other Rho-GEFs, are involved in Dvl-induced RhoA activation in other cells, tissues, and species.

In this study, we identified Rho-GEFs that are involved in the Wnt/PCP pathway in mammalian cells. Previous studies have shown that Dvl activates RhoA and ROCK, thereby inhibiting serum starvation-induced neurite outgrowth in N1E-115 mouse neuroblastoma cells (Kishida et al., 2004). To identify Rho-GEFs that are responsible for Dvl-induced RhoA activation, we constructed effective short-hairpin RNAs (shRNAs) targeting 14 Rho-GEFs (including WGEF) that are expressed in N1E-115 cells and controls with a high efficacy of gene silencing were selected. Each screening was repeated three times, and clones with a high efficacy of gene silencing were confirmed by nucleotide sequence analysis (target sequences are listed in Supplemental Table S1).

Materials and Methods

Materials and Antibodies

Recombinant mouse Wnt-3a was purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibodies against LARG (sc-25638; Santa Cruz Biotechnology, Santa Cruz, CA), p115-Rho-GEF (H-165; Santa Cruz), and green fluorescent protein (GFP) (A16455; Molecular Probes, Eugene, OR) were used at 1:2000, peroxidase-conjugated goat anti-rabbit IgG (sc-2004; Santa Cruz), AlexaFluor-594-conjugated goat anti-mouse IgG (A11071; Molecular Probes, Eugene, OR) at 1:1000, and 4′,6-diamidino-2-phenylindole (DAPI) at 1 mg/ml. For immunofluorescence, cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. For Western blotting, cells were lysed with 90% (v/v) lysis buffer (250 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate). Plasmid DNA was isolated from mammalian cells using thepeq-10 method (Tohoku University), respectively. Expression plasmids encoding for GFP were constructed by subcloning WGEF cDNA into the pUC4-K vector (Clontech). A stable cell line expressing the cDNA encoding Halog-GFP carrying the tetracycline-responsive element (TRE) was generated by co-transfection with pBabe-puro (Clontech) and pUC4-K-Halo-GFP constructs.

Cell Culture and Transfection

NIE-115 (provided by Dr. S. Narumiya, Kyoto University), G4P–293 (Clontech, Cambridge, UK), and HEK293T cells (American Type Culture Collection, Rockville, MA) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10%, 10%, and 10% fetal calf serum, respectively. Jurkat F-cells (Cell Resource Center, Tohoku University) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Wnt-3a–producing L cells were provided by Dr. S. Takada (National Institute for Natural Sciences, Okazaki, Japan) and maintained in DMEM supplemented with 10%, 15%, and 10% fetal calf serum. Wnt-3a–producing L cells were provided by Dr. S. Takada (National Institute for Natural Sciences, Okazaki, Japan) and maintained in DMEM supplemented with 10%, 15%, and 10% fetal calf serum.

Reverse Transcription (RT)-PCR

To examine the expression profile of Rho-GEF mRNAs, total RNA isolated from N1E-115 cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) was reverse-transcribed to yield single-stranded cDNA using the SuperScript Choice System (Invitrogen). cDNA was subjected to PCR amplification for 30 cycles with each cycle consisting of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s. The specific primers used for amplifying the 16 Rho-GEF cDNAs are listed in Supplemental Table S1.

Plasmid Construction

The cDNAs for human LARG (KIAA0382), p115-Rho-GEF (KIAA0521), and Lfc (KIAA0651) were provided by Dr. T. Nagase (Kazusa DNA Research Institute, Kisarazu, Japan). To examine the expression profile of Rho-GEF mRNAs, total RNA isolated from N1E-115 cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) was reverse-transcribed to yield single-stranded cDNA using the SuperScript Choice System (Invitrogen). cDNA was subjected to PCR amplification for 30 cycles with each cycle consisting of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s. The specific primers used for amplifying the 16 Rho-GEF cDNAs are listed in Supplemental Table S1.

Construction of an shRNA Library and Screening for Effective shRNA Constructs

To construct an shRNA library targeting each Rho-GEF, the partial cDNA (400 base pairs) of Rho-GEF was obtained by PCR amplification from the pNUL-Rho-GEF plasmid. An shRNA library was then produced from this cDNA fragment, according to the enzymatic production of RNAi library (EPRIL) method, as described previously (Shirane et al., 2004). For screening of effective shRNAs, an shRNA-carrying retrovirus was produced in 96-well plates. Plasmids were prepared using a Perfectprep Plasmid 96 Vac (Eppendorf, Hamburg, Germany), according to the manufacturer’s protocol. GIP-293 packaging cell lines were transfected with 200 ng of the shRNA-carrying pPAM-U6 plasmid or pPUL-Rho-GEF, and 17 ng of the VSV-G-encoding plasmid using FuGENE6. Two days after transfection, the retrovirus-containing culture medium was collected. Jurkat cells (1.0 × 10⁶ cells per ml) suspended in 50 μl medium were coincubated with 50 μl of the medium containing retrovirus particles (25 μl each of medium containing retrovirus carrying the shRNA constructs and pPUL-Rho-GEF) in 96-well white polystyrene plates (Corning, Comin, NJ). Two days later, the cells were lysed with 90 μl of Bright-Glo Assay System reagents (Promega), and the relative levels of luciferase activity were measured by measuring luminescent intensity using an LMax II luminometer (Molecular Devices, Sunnyvale, CA). The relative reduction of luciferase activity was used as a measure of silencing efficiency. The authenticity of all effective shRNA constructs was confirmed by nucleotide sequence analysis (target sequences are listed in Supplemental Table S2).

Neurite-bearing Cell Scoring Assay

NIE-115 cells were plated on glass coverslips pretreated with 1 μg/ml poly-L-lysine, cultured for 24 h, and transfected with CFP expression plasmids and/or shRNA plasmids using Lipofectamine 2000. After incubation for 24 h, cells were serum-starved for 24 h and then fixed with 4% formaldehyde.
hydro in phosphate-buffered saline. The CFP-positive cells with neurites longer than one cell body length were scored as neurite-bearing cells. More than 50 cells were evaluated for each experiment. All data are indicated as means ± SD. To examine the effects of Wnt-3a-induced neurite retraction, Wnt-3a conditioned medium (CM) was prepared as described previously (Shibamoto et al., 1998).

**Immunoprecipitation and Immunoblot Analysis**

Cells were lysed with ice-cold lysis buffer (50 mM HEPES, pH 7.4, 130 mM NaCl, 0.5% Nonidet P-40, 5% glycerol, 1 mM MgCl₂, 1 mM MnCl₂, 1 mM Na₃VO₄, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml leupeptin). Cell lysates were subjected to immunoprecipitation and immunoblot analysis, as described previously (Ohashi et al., 2000).

**Pull-Down Assay of RhoA Activity**

An active GTP-bound form of RhoA was analyzed via pull-down assays using glutathione-S-transferase (GST) fusion proteins of the Rho-binding domain (RBD) of rhoetkin, as described previously (Ren et al., 1999; Nishita et al., 2002). N1E-115 cells were cotransfected with CFP-Dvl-1 and shRNAs targeting LARG, p114-RhoGEF, or Lfc and cultured for 48 h. Cell lysates were then incubated with GST-RBD bound to glutathione-Sepharose. The bead pellets were washed and analyzed by immunoblotting using an anti-RhoA antibody.

**Statistical Analysis**

Statistical data are expressed as means ± SD of three independent experiments. When appropriate, one-tailed Student’s t-test was applied. In all cases, p < 0.05 was considered statistically significant.

**RESULTS**

**Expression Profile of Rho-GEFs in N1E-115 Cells**

The human genome encodes at least 69 Db1-like Rho family GEFs, of which 16 members were reported to exhibit GEF activity preferentially activating RhoA (Rossman et al., 2005). As the first step to identify Rho-GEFs that are involved in Dvl-induced RhoA activation in N1E-115 cells, we analyzed the expression profile of these 16 Rho-GEF transcripts in N1E-115 cells by RT-PCR analysis, using primers listed in Supplemental Table S1. RT-PCR analyses revealed that 13 Rho-GEFs (p115-RhoGEF, PDZ-RhoGEF, LARG, p114-RhoGEF, Lbc/AKAP, Lfc/GEF-H1, Net1, ECT2, Scambio, p164-RhoGEF, Vsm-RhoGEF, CDEF/Farp1, and TrioC) were expressed in N1E-115 cells, but the expression of the other three Rho-GEFs (XPLN, Tim1, and NGEF/Ephexin) was not detected (Figure 1A). Because WGEF was recently reported to be involved in RhoA activation in the Wnt-PCP pathway in Xenopus embryos (Tanegashima et al., 2008), we also examined the expression of the mouse ortholog of WGEF in N1E-115 cells by RT-PCR analysis and detected the expression of WGEF in N1E-115 cells (Figure 1A).

**Selection of Effective shRNA Constructs Targeting Rho-GEFs**

To identify which members of the Rho-GEF family are involved in Dvl-induced RhoA activation, we planned to search for the Rho-GEF shRNAs that suppress Dvl-induced neurite retraction in N1E-115 cells. To obtain effective shRNA constructs against each of the Rho-GEFs expressed in N1E-115 cells, we developed a screening method schematically shown in Figure 1B. Using an EPRIL method (Shirane et al., 2004), we produced an shRNA library consisting of an array of different shRNA constructs from the PCR-amplified cDNA fragment (~400 base pairs) of each Rho-GEF. Each shRNA construct from the EPRIL library was introduced into Jurkat cells together with the reporter gene construct, encoding a chimeric cDNA composed of luciferase and the cDNA fragment of the target gene (Zhao et al., 2005). If any shRNA construct effectively suppresses expression of its target gene, luciferase expression will be simultaneously reduced. Thus, the silencing effect of each shRNA on target gene expression can be easily monitored by measuring luciferase activity. Using this reporter assay, two effective shRNA constructs targeting each of the 13 mouse Rho-GEFs were selected (Figure 1C), and the target sequences are listed in Supplemental Table S2. The target specificity of the selected shRNA constructs was assessed by luciferase reporter assays. The shRNA constructs for LARG, p114-RhoGEF, and Lfc suppressed the expression of the corresponding RhoGEF-luciferase chimeric proteins but not the expression of the irrelevant RhoGEF-luciferase chimeric proteins (Supplemental Figure S1), indicating that each of the selected shRNAs has the target specificity. Immunoblot analyses of endogenous LARG, p114-RhoGEF, Lfc, and p115-RhoGEF proteins revealed that the shRNA constructs selected by our method effectively suppressed expression of their target protein in N1E-115 cells (Figure 1D and Supplemental Figure S2). We also constructed an shRNA plasmid targeting mouse WGEF. When coexpressed with HA-tagged WGEF in N1E-115 cells, the shRNA targeting WGEF effectively suppressed the expression of HA-WGEF (Figure 1D).

**Knockdown of LARG, p114-RhoGEF, or Lfc Suppresses Dvl-Induced Neurite Retraction**

N1E-115 cells extend neurites under serum-starved conditions (Jalink et al., 1994; Kozma et al., 1997), but treatment of the cells with Wnt-3a or overexpression of Dvl suppresses serum starvation-induced neurite outgrowth through the activation of the RhoA-ROCK pathway (Kishida et al., 2004). To identify Rho-GEFs that are involved in the Wnt/PCP pathway, we examined the effect of each Rho-GEF shRNA on Dvl-induced neurite retraction in N1E-115 cells. The cells were cotransfected with the shRNA construct targeting each of the Rho-GEFs and the plasmid encoding CFP or CFP-tagged Dvl-1. The transfected cells were cultured for 24 h, serum-starved for 24 h, and then fixed (Figure 2A). To quantitate the effects of shRNAs on neurite outgrowth, we scored the percentage of neurite-bearing cells with neurite(s) longer than one cell body length in CFP-positive cells (Figure 2B). Under the serum-starved conditions, more than 60% of cells transfected with control CFP extended neurites, but expression of CFP-Dvl-1 significantly reduced the percentage of neurite-bearing cells to <30% (Figure 2B). Similar to the results in a previous report (Kishida et al., 2004), Dvl-1 fragments (1-367) and (165-670), but not (1-167), suppressed serum starvation-induced neurite outgrowth (Supplemental Figure S3), indicating the importance of the central region of Dvl-1 for its neurite retraction activity.

Interestingly, cotransfection of the shRNAs targeting LARG, p114-RhoGEF, or Lfc with CFP-Dvl-1 significantly increased the percentage of neurite-bearing cells, compared with the cells cotransfected with control shRNA (Figure 2, A and B). In contrast, no significant change in Dvl-induced neurite retraction was observed by cotransfection of the other Rho-GEF shRNAs (Figure 2B). WGEF shRNA had no apparent effect on Dvl-induced neurite retraction in N1E-115 cells (Figure 2, A and C). Cotransfection of shRNA-resistant LARG, p114-RhoGEF, or Lfc cDNA with the corresponding Rho-GEF shRNA reversed the inhibitory effects of Rho-GEF shRNAs on Dvl-induced neurite retraction (Supplemental Figure S4). In addition, knockdown of LARG, p114-RhoGEF, Lfc, or WGEF had no significant effect on the percentage of neurite-bearing cells.
in CFP-expressing (Dvl-1 nontransfected) control cells (Supplemental Figure S5). These results suggest that LARG, p114-RhoGEF, and Lfc are the Rho-GEFs involved in Dvl-induced neurite retraction, and other Rho-GEFs, including WGEF, are not related to this process in N1E-115 cells. As previously reported (Kishida et al., 2004), Y-27632, an inhibitor of ROCK, blocked Dvl-induced neurite retraction (data not shown), indicating that Dvl-1-induced neurite retraction involves ROCK activation.

Effects of the Knockdown of RhoGEFs on Wnt-3a- or Lysophosphatic Acid-Induced Neurite Retraction

Previous studies showed that both Wnt-3a and lysophosphatidic acid (LPA) induce neurite retraction in N1E-115 cells (Jalink et al., 1994; Kozma et al., 1997; Kishida et al., 2004). To determine the functional specificity of Rho-GEFs, we examined whether the knockdown of LARG, p114-RhoGEF, or Lfc affects Wnt-3a- or LPA-induced neurite retraction in N1E-115 cells. Treatment of the cells with CM of Wnt-3a-producing L cells significantly decreased the number of neurite-bearing cells, but CM of control L cells had no effect (Figure 3). Knockdown of LARG, p114-RhoGEF, or Lfc by shRNA transfection markedly inhibited Wnt-3a CM-induced neurite retraction (Figure 3). Similar results were obtained when we used purified recombinant Wnt-3a in place of Wnt-3a CM (Supplemental Fig. S6). These results indicate that these three Rho-GEFs are involved in Wnt-3a-induced neurite retraction. In contrast, when we analyzed the effects of Rho-GEF knockdown on LPA-induced neurite retraction in N1E-115 cells, LARG shRNA significantly blocked LPA-induced neurite retraction but p114-RhoGEF and Lfc shRNAs had only partial or no effects (Figure 3). In addition, WGEF shRNA had no apparent effect on Wnt-3a- or LPA-induced neurite retraction (Supplemental Figure S7). These results suggest that p114-RhoGEF and Lfc preferentially function in Wnt-3a-in-
duced neurite retraction, but LARG plays a more general role in neurite retraction, being responsive to two distinct types of extracellular cues.

**Knockdown of p114-RhoGEF or Lfc Suppresses Dvl- or Wnt3a-Induced RhoA Activation**

We next examined the effects of knockdown of LARG, p114-RhoGEF, or Lfc on Dvl- or Wnt-3a–induced RhoA activation in N1E-115 cells. RhoA activity was assessed by GST pull-down assays using the GST-tagged Rho-binding domain (RBD) of rhotekin, to which only the active GTP-bound form of RhoA binds (Ren et al., 1999; Nishita et al., 2002). The level of active RhoA increased about threefold in cells transfected with Dvl-1, compared with nontransfected cells. When N1E-115 cells were cotransfected with Dvl-1 and each of the Rho-GEF shRNAs, Dvl-induced RhoA activation was significantly suppressed by p114-RhoGEF or Lfc shRNA but not by LARG shRNA (Figure 4A). Similarly, Wnt-3a–induced RhoA activation was suppressed by p114-RhoGEF or Lfc shRNA, but not significantly by LARG shRNA (Figure 4B). These results suggest that p114-RhoGEF and Lfc, but not LARG, play major roles in Dvl- and Wnt-3a–induced RhoA activation in N1E-115 cells.

**Effects of Double Knockdown of p114-RhoGEF and Lfc on Dvl- or Wnt-3a–Induced RhoA Activation and Neurite Retraction**

To examine the relationships between the roles of p114-RhoGEF and Lfc in Wnt-3a and Dvl signaling, we analyzed the effects of double knockdown of p114-RhoGEF and Lfc on Dvl- or Wnt-3a–induced RhoA activation and compared them with the effects of p114-RhoGEF or Lfc single knockdown. As shown in Figure 4, p114-RhoGEF/Lfc double knockdown suppressed Dvl-1- or Wnt-3a–induced RhoA activation to the extent similar to those by p114-RhoGEF or Lfc single knockdown. Furthermore, p114-RhoGEF/Lfc double knockdown suppressed Dvl-1–induced neurite retraction to the extent similar to those by p114 or Lfc single knockdown (Supplemental Figure S8). These results suggest that p114-RhoGEF and Lfc function in a similar pathway and that these Rho-GEF activities may depend on each other.

**p114-RhoGEF and Lfc, but Not LARG, Bind to Dvl and Daam1**

We next examined whether p114-RhoGEF, Lfc, and LARG would physically interact with Dvl. When Flag-tagged Dvl-1
was coexpressed with CFP-tagged p114-RhoGEF, Lfc, or LARG in HEK293T cells and immunoprecipitated with an anti-Flag antibody, p114-RhoGEF and Lfc, but not LARG, were coprecipitated with Flag-Dvl-1 (Figure 5A). We also analyzed the interactions between Daam1 and each of the Rho-GEFs. When Myc-tagged Daam1 was coexpressed with each of the CFP-tagged Rho-GEFs in HEK293T cells and immunoprecipitated with an anti-Myc antibody, p114-RhoGEF and Lfc, but not LARG, were coprecipitated with Myc-Daam1 (Figure 5B). Precipitation of CFP-p114-RhoGEF and CFP-Lfc was not detected when we used the irrelevant antibodies for coimmunoprecipitation analyses (Supplemental Figure S9). We also analyzed the binding of WGEF to Dvl-1 and Daam1. CFP-WGEF was coprecipitated with Flag-Dvl-1 and Myc-Daam1 (Figure 5, A and B), as previously reported (Tanegashima et al., 2008). Additionally, Myc-Daam1 was coprecipitated with Flag-Dvl-1 (Figure 5C), as previously reported (Habas et al., 2001). We next examined the binding of endogenous Dvl-1 to endogenous p114-RhoGEF or Lfc in N1E-115 cells. When N1E-115 cells were treated with Wnt-3a CM or left untreated and cell lysates were immunoprecipitated with an anti-p114-RhoGEF or an anti-Lfc antibody, Dvl-1 was coprecipitated with each of these Rho-GEFs and the interactions between Dvl-1 and these Rho-GEFs increased after treatment with Wnt-3a CM (Figure 5D). These results suggest that p114-RhoGEF and Lfc are involved in Wnt-3a– and Dvl-1–mediated RhoA activation by associating with Dvl-1 and Daam1. In contrast, LARG did not bind to Dvl-1 or Daam1, and knockdown of LARG had no significant effect on Wnt-3a– or Dvl-1–induced RhoA activation, thus indicating that LARG is not directly involved in Wnt-3a– or Dvl-1–induced RhoA activation.

Mapping of the Binding Regions of Dvl, Daam1, p114-RhoGEF, and Lfc

Dvl proteins have three conserved domains, termed DIX, PDZ, and DEP (Lee et al., 1999). To determine which region of Dvl is involved in binding to p114-RhoGEF and Lfc, deletion mutants of Flag-Dvl-1 were coexpressed with CFP-p114-RhoGEF or CFP-Lfc, and their binding ability was analyzed by coimmunoprecipitation using an anti-Flag antibody. As shown in Figure 6A, both CFP-p114-RhoGEF and CFP-Lfc coprecipitated with Dvl-1 (amino acids 165-367), but not with Dvl-1-(1-167) or Dvl-1-(326-670), indicating that Dvl-1 binds to p114-RhoGEF and Lfc through the central PDZ-containing region (165-367). We also analyzed the binding of each Rho-GEF to the N- and C-terminal fragments of Daam1 [N-Daam1 (amino acids 1-235) and C-Daam1 (490-1078)] (Figure 6B). p114-RhoGEF and Lfc coprecipitated with Myc-tagged full-length Daam1 and HA-tagged N-Daam1 but not Myc-tagged C-Daam1, indicating that Daam1 binds to these Rho-GEFs through its N-terminal region.

To map the regions of p114-RhoGEF and Lfc required for binding to Dvl and Daam1, we constructed deletion mutants of CFP-p114-RhoGEF and CFP-Lfc and analyzed their binding abilities to Flag-Dvl-1-(165-367) or HA-N-Daam1 by coimmunoprecipitation assays. As shown in

Figure 3. Effects of knockdown of LARG, p114-RhoGEF, or Lfc on neurite retraction induced by Wnt-3a CM or LPA. N1E-115 cells were cotransfected with CFP and LARG, p114-RhoGEF, or Lfc shRNA at the ratio of 1:5 and serum-starved for 24 h. Cells were treated for 10 min with control (L cell) CM, Wnt-3a–producing L cell CM, or LPA. The percentages of neurite-bearing cells in CFP-positive cells were counted. Data represent means ± SD of three independent experiments (at least 50 cells in each experiment). *p < 0.01.

Figure 4. Effects of single knockdown of LARG, p114-RhoGEF, or Lfc, or double knockdown of p114-RhoGEF and Lfc, on RhoA activation induced by Dvl-1 (A) or Wnt-3a (B). In A, N1E-115 cells were cotransfected with CFP or CFP-Dvl-1 and shRNA constructs, cultured for 24 h, and serum-starved for 24 h. In B, N1E-115 cells transfected with shRNAs were cultured for 24 h, serum-starved for 24 h, and treated with control or Wnt-3a CM for 10 min. The level of active RhoA was analyzed by a GST-RBD pull-down assay. Relative activity of RhoA was normalized by the immunoblot intensity of RhoA in the total cell lysates. Data represent means ± SD of three independent experiments, with the level in control cells taken as 1.0. *p < 0.01.
Figure 5. Binding of p114-RhoGEF, Lfc, and WGEF, but not LARG, to Dvl and Daam1. (A) Binding to Dvl. Flag-tagged Dvl-1 was coexpressed with CFP-tagged Rho-GEFs in HEK293 cells. Cell lysates were immunoprecipitated with an anti-Flag antibody and immunoblotted with anti-GFP and anti-Flag antibodies. (B) Binding to Daam1. Myc-tagged Daam1 was coexpressed with CFP-tagged Rho-GEFs in HEK293 cells. Cell lysates were immunoprecipitated with an anti-Myc antibody and immunoblotted with anti-GFP and anti-Myc antibodies. (C) Binding of Daam1 to Dvl. Myc-Daam1 and Flag-Dvl-1 were coexpressed in HEK293 cells. Cell lysates were immunoprecipitated with an anti-Flag antibody and analyzed by immunoblotting with anti-GFP and anti-Flag antibodies. (D) Binding of endogenous proteins. N1E-115 cells were serum-starved for 24 h and treated with Wnt-3a CM for 10 min or left untreated. Cell lysates were immunoprecipitated with an anti-p114-RhoGEF or an anti-Lfc antibody and immunoblotted with anti-Dvl-1, anti-p114-RhoGEF, and anti-Lfc antibodies. All experiments were repeated twice, and similar results were obtained.

Figure 6. Mapping of the binding regions of Dvl and Daam1. (A) Binding of p114-RhoGEF and Lfc to the middle region of Dvl. Flag-tagged Dvl-1 fragments and CFP-tagged p114-RhoGEF or Lfc were coexpressed in HEK293 cells and analyzed as in A. In A and B, schematic structures of Dvl-1, Daam1, and their fragments are shown.

whether the DH/PH domain of Rho-GEFs would commonly have a potential to bind to Daam1. When Myc-Daam1 was coexpressed with CFP-tagged DH/PH domain of LARG, p114-Rho-GEF, Lfc, or WGEF and immunoprecipitated with an anti-Myc antibody, the DH/PH domain of each Rho-GEF was coprecipitated with Myc-Daam1, although the binding of the DH/PH domain of LARG was weaker than those of p114-RhoGEF and Lfc (Supplemental Figure S10). These results suggest that the DH/PH domains of these four Rho-GEFs commonly have the capacity to bind to Daam1 and that the Daam1-binding ability of the DH/PH domain of LARG may be masked in a full-length form by the intramolecular interaction.
Expression of the Dvl-Binding Fragment of p114-RhoGEF or Lfc Suppresses Dvl-Induced Neurite Retraction

We analyzed the effects of overexpression of the Dvl-binding fragment of p114-RhoGEF or Lfc on Dvl-induced neurite retraction. N1E-115 cells were cotransfected with Flag-Dvl-1 and CFP-tagged p114-RhoGEF or Lfc fragments and the percentages of neurite-bearing cells were analyzed. Coexpression of Dvl-binding fragments [p114-RhoGEF-(568-1016) or Lfc-(1-155)] significantly suppressed Dvl-induced neurite retraction, while coexpression of Dvl-nonbinding fragments [Lfc-(650-970)] did not affect on it (Figure 8, A and B). In addition, coexpression of p114-RhoGEF-(1-572) had no apparent effect on Dvl-induced neurite retraction, probably because this fragment contains the DH/PH domain and functions as an active form of p114-RhoGEF by truncation of the C-terminal extracatalytic region. These observations suggest that the Dvl-binding fragments of p114-RhoGEF and Lfc suppress Dvl-induced neurite retraction and function as the dominant-negative forms of Rho-GEFs.

Because previous studies showed that N-Daam1 suppressed Dvl-induced RhoA activation (Habas et al., 2001; Liu et al., 2008), we analyzed the effects of expression of N-Daam1 on Dvl-induced RhoA activation in N1E-115 cells. Coexpression of N-Daam1 with Dvl-1 significantly suppressed Dvl-1-induced RhoA activation, while full-length Daam1 had no apparent effect (Supplemental Figure S11), which indicates that N-Daam1 functions as a dominant-negative form of Daam1 for Dvl-induced RhoA activation in N1E-115 cells, as reported previously in other cell types (Habas et al., 2001).

DISCUSSION

RhoA activation is a key step in the Wnt-PCP signaling pathway. Both genetic and biochemical studies in Drosophila and mammalian cells demonstrated that Dvl mediates Wnt-induced RhoA activation (Strutt et al., 1997; Winter et al., 2001). However, the molecular mechanism of Dvl-mediated RhoA activation is not well understood. In this study, we identified Rho-GEFs that are responsible for Dvl-mediated RhoA activation in the mammalian Wnt-PCP pathway by analyzing the inhibitory effects of shRNA-assisted Rho-GEF depletion on Dvl-induced neurite retraction in N1E-115 cells. Of 14 Rho-GEF shRNAs (including WGEF shRNA) examined, only three shRNAs, targeting p114-RhoGEF, Lfc, and LARG, suppressed Dvl-induced neurite retraction. Interestingly, neither p114-RhoGEF nor Lfc shRNA had any significant effect on LPA-induced neurite retraction, but LARG shRNA did suppress the neurite-retracting activity of LPA. LARG was previously shown to be involved in LPA- and semaphorin 4D-induced RhoA activation and neurite retraction (Fukuhara et al., 2000; Aurandt et al., 2002; Perrot et al., 2002; Yamada et al., 2005). These results suggest that LARG is involved in multiple signaling pathways that lead to RhoA activation and neurite retraction, while p114-RhoGEF and Lfc function preferentially in Wnt3a-induced neurite retraction. We also showed that p114-RhoGEF and Lfc shRNAs significantly suppressed Dvl- or Wnt3a-induced RhoA activation, but LARG shRNA did not. Furthermore, p114-RhoGEF and Lfc, but not LARG (at least in a full-length form), can physically associate with Dvl and Daam1. Taken together, these results suggest that p114-RhoGEF and Lfc are critically involved in Wnt/Dvl-induced RhoA activation and neurite retraction in N1E-115 cells by forming a complex with Dvl and Daam1. In contrast, LARG is not directly involved in Dvl-induced RhoA activation and probably has a more general role in suppression of neurite outgrowth.

We showed that LARG shRNA suppressed Wnt/Dvl-induced neurite retraction but had little effect on Dvl-induced RhoA activation. This raises the question of how LARG is involved in Wnt/Dvl-induced neurite retraction

Figure 7. Mapping of the binding regions of p114-RhoGEF and Lfc. (A) Binding of p114-RhoGEF fragments to Dvl-1 and Daam1. CFP-tagged p114-RhoGEF fragments were coexpressed with Flag-Dvl-1-(165-367) or HA-N-Daam1 in HEK293 cells. Cell lysates were immunoprecipitated with an anti-Flag or an anti-HA antibody and analyzed by immunoblotting with an anti-GFP antibody. (B) Binding of Lfc fragments to Dvl-1 and Daam1. CFP-tagged Lfc fragments were coexpressed with Flag-Dvl-1-(165-367) or HA-N-Daam1 in HEK293 cells. Cell lysates were analyzed as in A. Schematic structures of p114-RhoGEF, Lfc, and their fragments are shown.
without significant change in RhoA activity in the whole cell. One possible explanation is that LARG contributes to the neurite retraction by activating any of the Rho family GTPases other than RhoA (such as RhoB or RhoC) or other cytoskeletal regulators that promote neurite retraction. In this case, LARG knockdown would not affect RhoA activity but could suppress Wnt/Dvl-induced neurite retraction by lowering the activity of neurite-retracting factors. Another possibility is that LARG is involved in RhoA activation only in the limited region of the cell. In this case, LARG knockdown would have little effect on the level of the total RhoA activity. In this respect, a recent study demonstrated that Dia1, one of the downstream effectors of RhoA, binds to and activates LARG. It has been proposed that Dia1, LARG, and RhoA constitute a positive feedback loop to potentiate the RhoA signaling pathway (Kitzing et al., 2007). This suggests the possibility that LARG contributes to neurite retraction by locally activating RhoA through this positive feedback mechanism. LARG may activate RhoA in the limited region of the cell, such as the growing tip of the neurites. It is possible that LARG shRNA suppresses Wnt/Dvl-induced neurite retraction by decreasing the local level of RhoA activity at the neurite tip via disconnecting the Dia1-LARG-RhoA feedback loop.

WGEF was shown to be involved in Rho activation in the Wnt-PCP pathway and to regulate convergent extension cell movements in *Xenopus* gastrulation (Tanegashima et al., 2008). Consistent with this report, we observed the binding of WGEF to Dvl and Daam1 by coprecipitation experiments. However, unlike p114-Rho-GEF or Lfc shRNA, WGEF shRNA had no apparent effect on Wnt/Dvl-induced neurite retraction in N1E-115 cells. Thus, WGEF is involved in Wnt/Dvl-induced RhoA activation in *Xenopus* embryos, but not in N1E-115 cells. Accordingly, Wnt/Dvl-induced RhoA activation appears to be mediated by distinct Rho-GEFs in a cell type–dependent manner. The ineffectiveness of WGEF shRNA in N1E-115 cells may be due to the low level of expression of WGEF protein in the cells, compared with those of p114-Rho-GEF and Lfc proteins. Alternatively, WGEF may require additional factors (that exist in *Xenopus* embryo cells, but not in N1E-115 cells) to function. Further studies are required to understand the mechanisms governing the cell type-specific functions of Rho-GEFs in the Wnt-PCP pathway.

Lfc is a microtubule-associated Rho-GEF, which is thought to mediate the cross-talk between microtubules and the actin cytoskeleton (Ren et al., 1998; Krendel et al., 2002; Birkenfeld et al., 2007). Lfc is highly expressed in the brain and regulates dendritic spine morphology (Ryan et al., 2005). However, the roles of Lfc and p114-RhoGEF in the regulation of neurite outgrowth were still to be elucidated. Our study demonstrated that Lfc and p114-RhoGEF are involved in Wnt/Dvl-induced neurite retraction in N1E-115 cells. p114-RhoGEF and Lfc have a similar domain structure, composed of the closely related DH-PH domains and the C-terminal coiled-coil domain, although Lfc contains the extra C1 domain in the N-terminal region (Rossman et al., 2005). Coprecipitation assays revealed that both p114-RhoGEF and Lfc bind to N-Daam1 through the DH-PH-containing region. Additionally, we ob-
served that the DH/PH domains of LARG and WGEF weakly bound to Daam1, which indicates that the DH/PH domains of these Rho-GEFs commonly have the potential to bind to Daam1 and the conformational change to relieve the DH/PH domain may be required for LARG to bind to Daam1. Interestingly, p114-RhoGEF binds to the PDZ-containing central region of Dvl through the C-terminal (568-1016) region, but Lfc binds to Dvl through the N-terminal (1-155) region. Overexpression of the Dvl-binding fragment of p114-RhoGEF or Lfc suppressed Dvl-induced neurite retraction, indicating the important role of the interactions between Dvl and these Rho-GEFs in Dvl-induced neurite retraction. These fragments probably function as the dominant-negative forms of Rho-GEFs by inhibiting Dvl binding to endogenous p114-RhoGEF and Lfc.

Based on the results in this study and those previously reported (Habas et al., 2001; Liu et al., 2008), we propose a model for the signaling of Wnt-3a–induced RhoA activation and the interactions of Dvl-1, Daam1, and p114-RhoGEF or Lfc in the ternary complex (Figure 9). In nonstimulated cells, Daam1 exists in an autoinhibited state by the intramolecular interaction between its N-terminal GBD and C-terminal DAD domains (Liu et al., 2008). Similarly, p114-RhoGEF and Lfc presumably exist in the autoinhibited states before cell stimulation (Schmidt and Hall, 2002; Meiri et al., 2009). Once cells are stimulated by Wnt-3a treatment or Dvl overexpression, both Daam1 and Rho-GEFs are activated by binding of Dvl, which relieves their autoinhibitory intramolecular interactions. Additionally, the N-terminal region of Daam1 interacts with the DH/PH domain of Rho-GEFs, which probably facilitates the GDP-GTP exchange activity of Rho-GEFs, by tethering RhoA near the DH/PH domain of Rho-GEFs and/or by stabilizing the active conformation of Rho-GEFs. As previously reported (Habas et al., 2001), overexpression of N-Daam1 suppressed Dvl-induced RhoA activation. This may be caused by the binding of N-Daam1 to the NAD domain of endogenous Daam1, which leads to the inhibition of the interaction between Daam1 and Dvl, or by the sequestering of endogenous RhoA by N-Daam1. More detailed biochemical and structural analyses are required to understand the molecular mechanisms of how the Dvl/Daam1/Rho-GEF ternary complex is regulated and stimulates RhoA activity in response to the Wnt signal.

Numerous studies have elucidated the specific roles of individual Rho-GEFs in various signaling pathways. However, considering the existence of a large number of Rho-GEF genes in mammalian genomes and the expression of many distinct Rho-GEF proteins in the same cell, more comprehensive studies are required. In particular, research is required to better understand the mechanisms whereby Rho activity is differentially regulated. In this study, we performed a search for Rho-GEFs involved in the Wnt-PCP pathway. The 14 Rho-GEFs that are expressed in N1E-115 cells were knocked down, and three Rho-GEF shRNAs were found to suppress Dvl-induced neurite retraction. This study constitutes the first step toward a genome-wide approach to knockdown Rho-GEF, using shRNAs targeting all known Rho-GEF candidate genes (including 69 Dbl-like genes, 11 Dock1-related genes and two SWAP70-like genes) (Rossman et al., 2005). We anticipate that this approach will lead to a more comprehensive understanding of the functional roles of Rho-GEFs in the regulation of Rho family GTases in various aspects of cell activity, such as cytoskeletal reorganization, polarity formation, gene transcription, cell growth, migration, neurite outgrowth, and tumor invasion. In this study, we used a high-throughput screening method to obtain shRNA constructs with effective gene-silencing activity. This method can be applied to any gene, even when the expression of the gene cannot be monitored directly, due to either a lack of specific antibodies or very low gene expression levels. Therefore, our approach will contribute toward efforts to knock down large numbers of genes simultaneously and can be applied to genome-wide knockdown experiments.

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Figure 9. A model for the Wnt-3a– and Dvl-induced RhoA activation. Wnt-3a induces the formation of a ternary complex, composed of Dvl-1, Daam1, and p114-RhoGEF or Lfc, which in turn stimulates RhoA activation and neurite retraction. Details are described in text.
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