Small GTPase Proteins Rin and Rit Bind to PAR6 GTP-dependently and Regulate Cell Transformation*

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Mitsunobu Hoshino‡§, Tamotsu Yoshimori§, and Shun Nakamura‡

From the ‡Department of Biochemistry and Cellular Biology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1, Ogawahigashi, Kodaira, Tokyo 187-8502 and the §Department of Cell Genetics, National Institute of Genetics, 1111, Yata, Mishima, Shizuoka 411-8540, Japan

The novel small GTPases Rin and Rit are close relatives of Ras, and recent studies show that they play a role in mediating neuronal differentiation. However, the direct effectors of Rin and Rit have yet to be fully characterized. Here we showed that Rin and Rit directly bind to the PDZ domain of PAR6, a cell polarity-regulating protein, in a GTP-dependent manner both in vivo and in vitro. Moreover, Rin and Rit can form a ternary complex consisting of PAR6 and Rac/Cdc42, members of the Rho family of small GTPases modulating cell growth and polarity. This ternary complex synergistically potentiates cell transformation in NIH3T3 cells, and the interaction between Rin/ Rit and the PDZ domain of PAR6 is important for this effect. These results suggest that the Rin/Rit-PAR6-Rac/ Cdc42 ternary complex may work physiologically in the cells, such as in tumorigenesis.

The Ras superfamily of small GTPases has been implicated in a wide variety of cellular processes, such as cell proliferation and differentiation (1). It functions as a molecular switch by cycling from the inactive GDP-bound state to the active GTP-bound state (1). Activation of small GTPases requires guanine nucleotide exchange factors, and inactivation was promoted by GTPase-activating proteins that accelerate the rate of intrinsic GTPase activity (2). In the active GTP-bound state, they interact with many cellular targets to exhibit their biological effects (1, 2).

The newly discovered GTPases Rin and Rit have been classified into the Ras superfamily (3–5). They share high sequence identity with Ras and have a highly conserved but distinct G2 effector region (3). Moreover, they display characteristics not observed in other members of the Ras superfamily, such as calmodulin binding activity and the lack of a typical C-terminal prenylation motif (CAAX motif) required for membrane association (3). Rin is expressed only in neurons, whereas Rit is ubiquitously expressed (3). We demonstrated previously that Rin is activated by stimulation with a growth factor using a Rin pull down assay system (6) and that Rit induces neurite outgrowth through the activation of endogenous Rac/Cdc42 and its association with calmodulin in rat pheochromocytoma PC12 cells (7). Rit also inhibits growth factor withdrawal-mediated apoptosis and induces neurite extension in pheochromocytoma cells (8). The Raf guanine nucleotide exchange factor-like 3/Ras pathway modulator was reported to be an effector of GTP-bound Rit and Ras, to activate the small GTPase Ral and to inhibit Elk-1-dependent gene induction (9, 10). These studies raised the possibility that Rin and Rit play important roles in the control of the calcium/calmodulin-mediated signaling pathways. However, the Raf guanine nucleotide exchange factor-like 3/Ras pathway modulator is the only effector of Rit that has been characterized so far, and the intracellular functions or signal transduction pathways of Rin and Rit, especially the effectors, largely remain to be elucidated.

Cell polarity is critical for many cellular functions. Polarized epithelial cells are organized into apical and basolateral surface domains (11). Recent studies reveal that the partitioning-defective protein PAR6 is a pivotal component of the cell polarity-regulating molecular machinery (12, 13). PAR6 is an evolutionarily conserved protein with a semi-Cdc42/Rac interactive binding (CRIB) domain, which is necessary but not sufficient for binding to the GTP-bound active form of Rac/Cdc42, and a PDZ domain (15–16). The PDZ domain consists of ~100 amino acids and mediates protein-protein interactions with other PDZ domains or with specific C-terminal motifs (17). These proteins localize to the subapical region and are involved in the formation or maintenance of tight junctions in epithelial Madin-Darby canine kidney cells (18, 19).

The Rho GTPase family proteins Rho, Rac, and Cdc42 participate in actin cytoskeleton dynamics, cell growth, and tumorigenesis (20, 21). In Swiss 3T3 fibroblasts, Rho regulates growth factor-stimulated stress fiber formation, whereas Rac and Cdc42 regulate growth factor-stimulated membrane ruffling and filopodium formation, respectively (20, 21). To date, many Rho family effector proteins have been identified. For example, GTP-bound Rac and Cdc42 bind to p21-activated kinases through their CRIB domain and control the regulation of the actin cytoskeleton (20, 21). Recently, it was also demonstrated that GTP-bound Cdc42 functions together with PAR6 in asymmetric cell division in Caenorhabditis elegans (22, 23).

Here we show that Rin and Rit directly bind to the PDZ domain of PAR6 in a GTP-dependent manner and that they can form a ternary complex with PAR6 and the active form of Rac/Cdc42. These interactions are important for cell transformation or tumorigenesis in NIH3T3 fibroblast cells.

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¶ To whom correspondence should be addressed: Tel.: 81-42-346-1722; Fax: 81-42-346-1752; E-mail: hoshinom@ncnp.go.jp.

1 The abbreviations used are: CRIB, Cdc42/Rac interactive binding; GMPPNP, 5'-guanylyl imidodiphosphate; GST, glutathione S-transferase; PBS, phosphate-buffered saline; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; aPKC, atypical protein kinase C.
EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-c-Myc 9E10 antibody and anti-PAR6 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-hemagglutinin (HA) high affinity antibody (3F10) was from Roche Applied Science. Anti-Rin antibody and anti-Rit antibody were from Calbiochem. pGEX vector, glutathione-Sepharose beads, and protein G-Sepharose beads were from Amersham Biosciences. All chemical reagents were purchased from Sigma, unless otherwise specified.

Expression Plasmids—Myc-Rin and HA-Rin mammalian expression pEF-Bos vectors were constructed as described previously (6). The mouse Rit and PAR6 expression constructs were produced as pEF-Bos or pCMV mammalian expression vectors with HA tag or Myc tag. Several PDZ domain mutants of PAR6 (PAR6-ΔPDZ mutant and PAR6 PDZ point mutant containing four alanine substitutions, R167A/P168A/L169A/G170A), Rin effector mutant (RinY57C; Rin-EM), and Rit effector mutant (RitY58C; Rit-EM) were generated by PCR-based site-directed mutagenesis, as described previously (14). The coding region of all constructs was confirmed by DNA sequencing. The glutathione S-transferase (GST)-fused PAR6 construct was generated by inserting PAR6 cDNA into the pGEX vector, according to the manufacturer’s instructions.

Two-hybrid Screening and X-Gal Assay—Two-hybrid screening was performed as described previously (6). In X-gal assay, fresh colonies were transferred to a sterile nitrocellulose filter, submerged in a liquid nitrogen pool, and thawed. Then it was put on a pre-soaked filter in the

FIG. 1. Rin binds to PAR6. A, yeast L40 cells carrying β-galactosidase reporter genes were cotransformed with both bait (empty vector, Rin-CA gene, or Rin-dominant negative (DN) gene) and prey (Par6 gene) constructs. The activity of β-galactosidase was examined by the X-gal assay, as described under “Experimental Procedures.” B and C, COS-7 cells were transfected with a Myc-tagged pEF-Bos vector encoding wild type Rin (B) or RinY57C (Rin-EM, C) using Lipofectamine 2000. After 48 h, cells were washed with an ice-cold PBS buffer and lysed with an ice-cold lysis buffer. Cell lysates were cleared by centrifugation and incubated in the presence of 10 mM EDTA and 0.5 mM nucleotide (either GDP (D) or GMPPNP (T)) at 30 °C for 2.5 min in a total volume of 250 μl. Then MgCl2 (20 mM) was added, and the solution was incubated at 30 °C for 1 min. This was followed by a pull down assay with 10 μg of GST protein, GST-PAR6 full-length protein, GST-PAR6 PDZ domain, GST-PAR6-ΔPDZ mutant, or GST-PAR6-PDZ point mutant protein, as described previously (6). After 2 h, the beads were washed twice, and bound Myc-Rin proteins were visualized by immunoblot analysis using an anti-Myc antibody or an anti-Rin antibody. Data are representative of three independent experiments, which gave essentially identical results. D, COS-7 cells were transfected with a Myc-tagged pEF-Bos vector encoding full-length PAR6 using Lipofectamine 2000. A pull down assay using GST or GST-Rin protein (either GDP (D) or GMPPNP (T) preloaded) was performed as described (B), and bead-bound Myc-PAR6 proteins were visualized by immunoblot analysis using an anti-Myc antibody. Data are representative of three independent experiments, which gave essentially identical results. E, recombinant HA-Rin protein (0.5 μg) was incubated in the presence of 10 mM EDTA and 0.5 mM nucleotide (either GDP (D) or GMPPNP (T)) at 30 °C for 2.5 min in a total volume of 250 μl. MgCl2 (20 mM) was added, and the solution was incubated at 30 °C for 1 min and then subjected to a pull down assay using GST or GST-PAR6 full-length protein as described (B). Recombinant HA-Rin protein ran as a doublet, both on Coomassie staining and in Western blotting. Data are representative of three independent experiments, which gave essentially identical results.
Rin and Rit Bind to PAR6

Z buffer/X-gal solution (100 ml of Z buffer, 0.27 ml of 2-mercaptoethanol, 1.67 ml of 20 mg/ml X-gal solution; Z buffer: 16.1 g/liter Na2HPO4·7H2O, 5.5 g/liter NaH2PO4·H2O, 10 mM KCl, 1 mM MgSO4, pH 7.0), and the filters were incubated at 37 °C. The colors of colonies were examined periodically.

**RESULTS**

**Rin Binds to the PDZ Domain of PAR6 in a GTP-dependent Manner**—First we carried out a yeast two-hybrid screening for proteins that interact with the constitutively active form of Rin (Rin-CA) (6). After sequencing these positive clones, we found several partial cDNA fragments of the PDZ domain of PAR6 protein. To confirm the interaction between Rin and PAR6 within yeast cells, X-gal assay in the yeast L40 strain was performed. As shown in Fig. 1A, two independent transformants containing Rin-CA (bait) and Par6 (prey) genes developed blue color on the filter at 37 °C. However, both cotransformants of empty bait vector and Par6 genes and that of Rin-dominant negative and Par6 genes remained white, indicating that the interaction between Rin-CA and PAR6 in the yeast cells is specific (Fig. 1A). Next we examined whether Rin actually binds to PAR6 GTP-dependently using a GST-PAR6 pull down assay system. COS-7 cells transfected with Myc-tagged wild type Rin protein were lysed, and the lysates were used as Rin bait. These cell lysates were preloaded with either GDP or GMPPNP (a non-hydrolyzable GTP analogue) and incubated in the presence of GST-PAR6-prebound glutathione-Sepharose beads. Fig. 1B shows that GMPPNP-preloaded Rin tightly binds to the GST-full-length PAR6 protein after 2× buffer wash and that GDP-bound Rin has no affinity for it. Rin did not bind to GST proteins (Fig. 1B). In order to define the PDZ domain in PAR6 responsible for the interaction with the GTP-Rin protein, we constructed a fusion protein comprising GST and the PDZ domain of PAR6. As shown in Fig. 1B, both GDP-bound and GMPPNP-bound Rin associated with the PDZ domain. Moreover, Rin protein failed to associate with either the PAR6ΔPDZ mutant (PDZ domain-deleted mutant of PAR6) or PAR6ΔPDZ point mutant (PAR6 mutant containing alanine mutations of four consecutive residues within the PDZ domain) (Fig. 1B). These residues were reported to be critical for interaction with target proteins, such as Par3 (14). The dependence of GTP on the association with the full-length PAR6 suggests that PAR6 may be an effector molecule of Rin. To investigate whether PAR6 is an effector of Rin, a pull down assay using a Rin effector mutant (RinY57C; Rin-EM) was performed. The cognate Y40C mutant in Ras was point-mutated in the effector region, shown not to bind to effector molecules, and regarded as an effector mutant (1). As shown in Fig. 1C, Rin-EM did not associate with PAR6. Not GDP-preloaded but GMPPNP-preloaded GST-Rin protein successfully pulled down the full-length PAR6 (Fig. 1D). To determine whether an active form of Rin directly associates with PAR6, we used recombinant Rin protein purified from E. coli instead of COS-7 cell lysates. As shown in Fig. 1E, recombinant Rin protein bound to PAR6 in a GTP-dependent manner. These results suggest that Rin directly associates with PAR6 GTP-dependently and that the PDZ domain of PAR6 is necessary and sufficient for the association.

**PAR6 Coimmunoprecipitated Rin**—To investigate whether PAR6 interacts with Rin in vivo, COS-7 cells were cotransfected with pEF-BoS expression vectors encoding Myc-tagged PAR6 and a HA-tagged wild type Rin or constitutively active form of Rin (Rin-CA) and were subjected to coimmunoprecipitation using an anti-Myc antibody. Fig. 2A shows that Myc-full-length PAR6 coimmunoprecipitated both wild type HA-Rin and...
Rit protein (0.5 μg/H9262 antibody. Data are representative of three independent experiments, which gave essentially identical results. COS-7 cells were transfected with a Myc-tagged pEF-Bos vector encoding wild type Rit (A) or RitY58C (Rit-EM, B) using Lipofectamine 2000. A pull down assay was performed as described in Fig. 1B legend. Bead-bound Myc-Rit proteins were visualized by immunoblot analysis using an anti-Myc antibody or an anti-Rit antibody. Data are representative of three independent experiments, which gave essentially identical results. COS-7 cells were transfected with a Myc-tagged pEF-Bos vector encoding full-length PAR6 using Lipofectamine 2000. A pull down assay using GST or GST-Rit protein (either GDP (D) or GMP-PNP (T) preloaded) was performed as described in Fig. 1B legend. Bead-bound Myc-PAR6 proteins were visualized by immunoblot analysis using an anti-Myc antibody. Data are representative of three independent experiments, which gave essentially identical results. Recombinant Rit protein (0.5 μg) was assayed for its association with PAR6 as described in Fig. 1E legend. The 35 K protein is an unidentified protein, but does not contain Rit sequence. It was confirmed that this protein could not be recognized with anti-Rit antibody by Western blotting. Data are representative of three independent experiments, which gave essentially identical results. COS-7 cells were transfected with a Myc-tagged pCMV vector encoding constitutively active H-Ras (H-Ras-CA) using Lipofectamine 2000. A pull down assay using GST, GST-PAR6 full-length, or GST-Raf1-Ras binding domain (encoding residues 51–131 of the Raf-1 used as a positive control) protein was performed as described in the Fig. 1B legend. Bead-bound Myc-H-Ras-CA proteins were visualized by immunoblot analysis using an anti-Myc antibody. Data are representative of three independent experiments, which gave essentially identical results.

To investigate this interaction in a mammalian cell, we examined whether Myc-full-length PAR6 coimmunoprecipitated Rit protein using Myc-PAR6 and HA-Rit (both wild type and constitutively active mutant)-coexpressing cell lysates (Fig. 4A). Myc-PAR6-ΔPDZ mutant failed to coimmunoprecipitate the Rit protein (Fig. 4A). As shown in Fig. 4B, Rit protein was coimmunoprecipitated with endogenous PAR6 protein by using Rit-expressing cell lysates. These results suggest that Rit directly associates with the PDZ domain of PAR6 dependent on GTP both in vitro and in vivo.

In addition, we performed GST-PAR6 pull down assay using another Ras family small GTPase, H-Ras, to examine the specificity of PAR6-binding properties of Rin/Rit. Rin and Rit have a highly conserved but distinct G2 effector region (HDFPTIEDAY) with H-Ras in which histidine is substituted for tyrosine at position 32, and alanine is substituted for serine at position 39 (3). As shown in Fig. 3E, constitutively active H-Ras, which is referred to as a GTP-bound state of H-Ras protein, did not bind to the PAR6 protein but did show the affinity for the Ras-binding domain of Raf-1, a well known Ras effector. These results suggest that the PAR6 may be a Rin/Rit-specific effector.

Rin and Rit Can Form a Ternary Complex with PAR6 and Rac/Cdc42—It has been demonstrated that PAR6 interacts with Rac and Cdc42 in a GTP-dependent manner through a semi-CRIB motif and a PDZ domain (14–16). To test whether Rin and Rit are capable of forming a complex with PAR6 and Rac/Cdc42 and whether PAR6 is responsible for binding to both Rin/Rit and Rac/Cdc42, we carried out a GST-Rac/Cdc42 pull down assay using recombinant Rin/Rit and PAR6 proteins.
5 shows that GST-Rac/Cdc42 successfully pulled down both Rin and Rit only in the presence of full-length PAR6 protein GTP-dependently. GST-Rac/Cdc42 pulled down neither Rin nor Rit even in the presence of the PAR6-PDZ point mutant protein (Fig. 5). To investigate whether the ternary complex is formed in vivo, COS-7 cells were cotransfected with Myc-tagged Rac/Cdc42, HA-tagged PAR6, and HA-tagged Rin/Rit and subjected to coimmunoprecipitation using an anti-Myc antibody. Fig. 6, A and B, shows that HA-Rin/Rit interacted with Myc-Rac/Cdc42 only in the presence of full-length HA-PAR6 (Fig. 6, A for Rac and B for Cdc42). Taken together, these results suggest that Rin/Rit links to Rac/Cdc42 through PAR6 in a GTP-dependent manner and that it can form a ternary complex with PAR6 and Rac/Cdc42.

Rin and Rit Potentiate Cell Transformation Activity by Rac/Cdc42 through PAR6—It has been shown previously that PAR6 potentiates cell transformation activity by directly interacting with the constitutively active Rac/Cdc42 in NIH3T3 cells (16). To provide evidence that a ternary complex consisting of

**Fig. 4.** PAR6 communoprecipitated Rit. A, COS-7 cells were transfected with the pCMV HA-Rit vector and pEF-Bos Myc-PAR6 (either full-length or ΔPDZ mutant) vector either alone or in pairs using Lipofectamine 2000. After 48 h, cells were lysed with an ice-cold lysis buffer, immunoprecipitated (IP) with an anti-Myc antibody, washed twice with the lysis buffer, separated by SDS-PAGE, transferred to a membrane, and probed with an anti-HA antibody. Data are representative of three independent experiments, which gave essentially identical results. B, COS-7 cells were transfected with pEF-Bos wild type Myc-Rit vector. After 48 h, cells were lysed, immunoprecipitated with either a mock or anti-PAR6 antibody, washed twice with the lysis buffer, separated by SDS-PAGE, transferred to a membrane, and probed with an anti-Myc antibody. Data are representative of three independent experiments, which gave essentially identical results. IB, immunoblot.

**Fig. 5.** Rin/Rit, PAR6, and Rac/Cdc42 form a ternary complex GTP-dependently in vitro. Recombinant HA-Rin protein (0.5 µg) (A) or recombinant Rit protein (0.5 µg) (B) was incubated with either GDP or GTPyS in the presence or absence of recombinant PAR6 full-length or PDZ point mutant protein (1 µg), and a pull down assay was conducted using either GST, GST-Rac, or GST-Cdc42 protein (either GDP (D) or GTPyS (T) preloaded), as described in Fig. 1D legend. Data are representative of three independent experiments, which gave essentially identical results.

**Fig. 6.** Ternary complex formation in vivo. COS-7 cells were transfected with pEF-Bos Myc-Rac (A) or Myc-Cdc42 (B) vector, pEF-Bos HA-PAR6 full-length, and pEF-Bos HA-Rin/pCMV HA-Rit vectors using Lipofectamine 2000. After 48 h, cells were lysed with an ice-cold lysis buffer, immunoprecipitated (IP) with an anti-Myc antibody, washed twice with the lysis buffer, separated by SDS-PAGE, transferred to a membrane, and probed with an anti-HA antibody. Data are representative of three independent experiments, which gave essentially identical results. IB, immunoblot.
Rin/Rit, PAR6, and Rac/Cdc42 might have a functional role in the cells, a focus-forming assay was carried out in NIH3T3 cells. As shown in Fig. 7, A and B, Rin-CA/Rit-CA, PAR6, or Rac-CA/Cdc42-CA alone showed weak transformation activity (Fig. 7, A for Rac and B for Cdc42). In contrast, cotransfection of Rin-CA/Rit-CA with PAR6 and Rac-CA/Cdc42-CA resulted in synergistic transformation activity, but either deletion of the PDZ domain of PAR6 or the Rin/Rit effector mutant significantly abolished this synergistic effect (Fig. 7, A and B). These results indicate that Rin and Rit show synergistic cell transformation activity by interacting with Rac/Cdc42 and PAR6 through the PDZ domain of PAR6 and that this ternary complex does have a functional role in vivo, at least in NIH3T3 cells.

Fig. 7. Rin/Rit, PAR6, and Rac/Cdc42 synergistically form foci in NIH3T3 cells. NIH3T3 cells were transfected with 5 μg each of pEF-Bos expression vector encoding Myc-Rin-CA, Myc-Rin-EM, Myc-Rit-CA, Myc-Rit-EM, Myc-PAR6 full-length, Myc-PAR6-PDZ, Myc-Rac-CA (A), or Myc-Cdc42-CA (B). An appropriate amount of pEF-BOS empty vector was transfected simultaneously so that each transfection contained the same total amount of each vector construct. The transfected cells were maintained in DMEM with 10% bovine calf serum. Foci were counted at 3 weeks after transfection. Columns and vertical bars denote the means ± S.E., respectively (n = 3).

In this study we described the GTP-dependent interaction of Rin/Rit with the partition-defective protein PAR6 and the effect of this complex on biological functions such as tumorigenesis.

The brain-specific protein Rin and ubiquitously expressed Rit are very similar to each other and are members of the Ras superfamily (3–5). The effector domains of Rin and Rit are identical, suggesting that they share the greater role of an effector, even if some differences do exist. In order to identify the binding partner of Rin, we screened a mouse brain-derived cDNA library with the yeast two-hybrid system by using a constitutively active mutant of Rin as bait. With this system, we have repeatedly isolated a gene encoding the PDZ domain of...
PAR6, a pivotal component of the cell polarity-regulating molecular machinery. Two-hybrid analysis and in vitro binding assay demonstrated that the interaction of Rin/Rit with PAR6 requires its PDZ domain and that PAR6 shows preferential binding to the active, GTP-bound form. The PDZ domain is a modular protein interaction domain and is shown to either form a homo/heterodimer with another PDZ-containing protein or bind to a unique C-terminal consensus motif of target proteins (17). However, Rin/Rit has neither a PDZ domain nor a unique C-terminal binding motif. To our knowledge, it is the first time that a small GTP-binding protein is shown to bind directly to the PDZ domain in a GTP-dependent manner. Considering the GTP- and effector region-dependent association of Rin/Rit with PAR6, PAR6 is one of the effector molecules of Rin/Rit. We also showed that H-Ras fails to bind to PAR6; however, it remains to be examined whether another Ras family protein directly binds to the PDZ domain of the effector protein and whether Rin/Rit bind to another PDZ-containing protein besides PAR6.

We found that the isolated PDZ domain interacted with both GTP- and GDP-Rin/Rit and that it did not show a GTP-dependent association with Rin. We tried to determine whether Cdc42 regulates the binding of GTP-bound Rin/Rit to PAR6 by using a GST-CRIB-PDZ domain pull down assay. However, we failed to clarify whether Cdc42 regulates the nucleotide dependence of the Rin/Rit association with PAR6 (data not shown). Another sequence in PAR6 might dictate the nucleotide-dependent association with Rin/Rit.

Moreover, by using a communoprecipitation method, we detected the interaction of PAR6 with Rin/Rit in vivo. These results suggest that PAR6 acts as a physiological effector for Rin/Rit. We found that wild type Rin/Rit as well as constitutively active Rin/Rit was coimmunoprecipitated with PAR6. We (6, 7) and Spencer et al. (8) observed previously that basal levels of GTP-bound wild type Rin/Rit remain quite high and that the amount of wild type Rin precipitated from the COS-7 cell lysates is almost the same as that of constitutively active Rin using a Rin pull down assay system (see Ref. 6 and data not shown). It may be that most Rin/Rit proteins remain in an active GTP-bound state in the cells.

We further demonstrated that Rin/Rit, PAR6, and Rac/Cdc42 form a ternary complex in vitro, and this complex functions biologically in a NIH3T3 focus-forming assay. In our previous study (7), Rin activated endogenous Rac/Cdc42 in PC12 cells. To examine whether Rin directly associates with Rac/Cdc42, we performed a communoprecipitation assay. However, we failed to detect any direct interaction between Rin and Rac/Cdc42. We further examined whether PAR6 has guanine nucleotide exchange factor activity by using a Rac/Cdc42 pull down assay. However, we did not find evidence that PAR6 is a Rac/Cdc42 activator (data not shown). There may be a linking molecule(s) that has guanine nucleotide exchange factor activity between Rin and Rac/Cdc42, distinct from PAR6. Further investigation is needed to identify the molecule(s) between Rin/Rit and Rac and the activator(s) of Rac/Cdc42 in this Rin/Rit-mediated signaling cascade.

In the focus-forming assay, we observed that the cell transformation complex is promoted by a ternary complex consisting of Rin/Rit, PAR6, and Rac/Cdc42 acting in concert. We observed that deletion of the PDZ domain of PAR6 almost completely abolished the synergistic effect of this ternary complex consisting of Rin/Rit, PAR6, and Cdc42. However, deletion of the PDZ domain partially, but not completely, abolished this synergistic effect of the ternary complex of Rin/Rit, PAR6, and Rac. The results raised the possibility that endogenous PAR6 in NIH3T3 cells could form a Rin/Rit/Rac complex even in the presence of the PAR6-ΔPDZ mutant, leading to the formation of foci. We cannot rule out the possibility that there may exist another signaling pathway leading to cell transformation that is independent of the formation of a Rin/Rit-PAR6-Rac complex. It was demonstrated previously that atypical protein kinase C (aPKC) can form a complex through the N-terminal region of PAR6 and that Par3 also can form a complex through the PDZ domain of PAR6 (24). Another study revealed that the Cdc42-PAR6 system might functionally contribute to cancer through aPKC-GSK-3-APC complex (25). Whether Rin/Rit participates in the aPKC-Par3-PAR6 complex, whether the kinase activity of aPKC potentiates the focus-forming activity of Rin/Rit, PAR6, and Rac/Cdc42, and whether aPKC adds some biological activity to the complex, such as the formation of cancer through the GSK-3-APC system, remain to be clarified.

Besides cell transformation, it might be that neuron-specific Rin is also involved in neuronal polarity. A previous study (26) showed that PAR6 is concentrated in the axon of the hippocampal neuron, especially at the tip, and that overexpression of PAR6 resulted in neurons with no axon and a loss of polarity. Whether Rin also colocalizes with PAR6 at the axonal tip of neurons remains to be clarified. It might be an interesting hypothesis that Rin and PAR6 cooperatively regulate neuronal polarity and axon formation.

In conclusion, we have identified a novel interaction between Rin/Rit and PAR6. However, many problems about the Rin/Rit-PAR6 signaling pathway, such as the regulation of neuronal polarity and cellular transformation, remain to be elucidated. Further study focusing on these problems will shed light on our understanding of the roles of the novel small GTPases Rin and Rit in tumorigenesis, cancer pathology, cellular polarity, and asymmetrical cell division.

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REFERENCES

1. Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M., and Satoh, T. (1991) Annu. Rev. Biochem. 60, 349–400
2. Bos, J. L. (1998) EMBO J. 17, 6766–6782
3. Lee, C.-H. J., Della, N. G., Chew, C. E., and Zack, D. J. (1996) J. Neurosci. 16, 6784–6794
4. Wes, P. D., Yu, M., and Montell, C. (1996) EMBO J. 15, 5839–5848
5. Reuther, G. W., and Der, C. J. (2000) Curr. Opin. Cell Biol. 12, 157–165
6. Hoshino, M., and Nakamura, S. (2002) Biochem. Biophys. Res. Commun. 295, 651–656
7. Hoshino, M., and Nakamura, S. (2003) J. Cell Biol. 163, 1067–1076
8. Spencer, M. L., Shao, H., and Andres, D. A. (2002) J. Biol. Chem. 277, 20160–20168
9. Shao, H., and Andres, D. A. (2000) J. Biol. Chem. 275, 26914–26924
10. Ehahrdt, G. R. A., Korherr, C., Wieler, J. S., Knaus, M., and Schrader, J. W. (2001) Oncogene 20, 188–197
11. Tsukita, S., Furuse, M., and Itoh, M. (2001) Nat. Rev. Mol. Cell Biol. 2, 285–293
12. Olino, S. (2001) Curr. Opin. Cell Biol. 13, 641–648
13. Henrique, D., and Schweisguth, F. (2003) Curr. Opin. Dev. 13, 341–350
14. Joberthy, G., Petersen, C., Gao, L., and Macara, I. G. (2000) Nat. Cell Biol. 2, 531–539
15. Lin, D., Edwards, A. S., Fawcett, J. P., Mbanalu, G. S., and Dawson, T. (2000) Nat. Cell Biol. 2, 540–547
16. Qu, R.-G., Abo, A., and Martin, G. S. (2000) Curr. Biol. 10, 697–707
17. Hung, A. Y., and Sheng, M. (2002) J. Biol. Chem. 277, 5699–5702
18. Gao, L., Joberthy, G., and Macara, I. G. (2001) Curr. Biol. 11, 221–225
19. Hirsch, T., Izumi, Y., Nagashima, Y., Tamai-Nagay, G., Kurihara, H., Sakai, T., Suzuki, Y., Yamanaka, T., Suzuki, A., Mizuno, K., and Ohno, S. (2002) J. Cell Biol. 151, 2485–2495
20. Lim, L., Manse, E., Leung, T., and Hall, C. (1996) Eur. J. Biochem. 242, 171–185
21. Bishop, A. L., and Hall, A. (2000) Biochem. J. 348, 241–255
22. Gotta, M., Abraham, M. C., and Ahringer, J. (2000) Curr. Biol. 11, 482–488
23. Kay, A. J., and Hunter, C. P. (2001) Curr. Biol. 11, 474–481
24. Suzuki, A., Yamanaka, T., Hirshe, T., Manabe, N., Mizuno, K., Shimizu, M., Akimoto, K., Izumi, Y., Ohnishi, T., and Ohno, S. (2001) J. Cell Biol. 152, 1183–1196
25. Ettienne-Manneville, S., and Hall, A. (2003) Nature 421, 753–756
26. Shi, S. H., Jan, L. Y., and Jan, Y. N. (2000) Cell 112, 63–75