Rit Mutants Confirm Role of MEK/ERK Signaling in Neuronal Differentiation and Reveal Novel Par6 Interaction

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Abstract

Rit is a novel member of the Ras superfamily of small GTP-binding proteins that regulates signaling pathways controlling cellular fate determination. Constitutively activated mutants of Rit induce terminal differentiation of pheochromocytoma (PC6) cells resulting in a sympathetic neuron-like phenotype characterized by the development of highly-branched neurites. Rit signaling has been found to activate several downstream pathways including MEK/ERK, p38 MAPK, Ral-specific guanine nucleotide exchange factors (GEFs), and Rit associates with the Par6 cell polarity machinery. In this study, a series of Rit effector loop mutants was generated to test the importance of these cellular targets to Rit-mediated neuronal differentiation. We find that Rit-mediated neuritogenesis is dependent upon MEK/ERK MAP kinase signaling but independent of RalGEF activation. In addition, *in vivo* binding studies identified a novel mechanism of Par6 interaction, suggesting that the cell polarity machinery may serve to spatially restrict Rit signaling.

Keywords

Introduction

The Ras superfamily of small GTPases is composed of more than 200 members that act as molecular switches at key regulatory nodes to direct a variety of cellular functions [1]. Within this varied superfamily, the Ras subfamily is particularly important for the regulation of cellular proliferation, differentiation, and survival [1–3]. The exchange of bound GDP for GTP, stimulated by external cues that activate guanine nucleotide exchange factors (GEFs), results in a conformational rearrangement of the G-protein exposing previously buried residues including a region known as the effector domain, which is primarily responsible for the interaction with, and activation of, downstream signaling partners [4–6]. Determining the identity of effector protein targets and understanding how their activation leads to alterations in cellular function are important goals in Ras-mediated signal transduction [7–10].

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One recently identified branch of the Ras subfamily is composed of Rit, Rin and *Drosophila* Ric proteins [11,12]. These GTPases contain a unique effector binding domain and lack the usual C-terminal signal for lipid addition, suggesting that they may function to regulate signaling pathways distinct from Ras [11,13]. Initially investigated for their abilities to promote tumor formation in NIH 3T3 fibroblasts, only a constitutively activated version of Rit, Rit[L79], was found to support tumorigenic growth in a manner distinct from that for oncogenic Ras [14,15]. However, like Ras, Rit binds and activates the Ral GEF, RGL3, to activate Ral GTPase signaling [16].

While only Rit was found to promote NIH 3T3 cell transformation, all members of this subfamily potently stimulate differentiation in rat pheochromocytoma (PC12) cells [17–21]. PC12 cells are a well-defined model system that has been used extensively to examine the extracellular stimuli and intracellular signaling cascades that regulate neuronal differentiation [22–28]. Nerve Growth Factor (NGF) stimulation promotes neurite outgrowth in PC12 cells, induces differentiation and survival of neurons *in vivo,* and leads to the activation of both Rit and Rin GTPases [24,29–33]. Selective knock-down of either Rit or Rin in PC6 cells, a sub-line of PC12, using small interfering RNA (siRNA) methods significantly decreases neurite outgrowth and activation of p38 MAP kinase signaling following NGF stimulation [29,30]. RNAi studies have also demonstrated a role for Rit in NGF-mediated ERK MAP kinase activation in PC6 cells [29].

More recently, Rit has been shown to interact with the cellular polarity machinery by directly binding to Par6 [34]. The Par3-Par6-atypical Protein Kinase C (aPKC) complex is a known scaffold for directing apical-basal polarity in epithelial cells and for axon-specification in neurons, at least in part through control of Rho family-mediated actin remodeling [35–37]. GTP-bound Rac1 and Cdc42 bind to the semi-Cdc42/Rac1 interactive binding (CRIB) domain of Par6 [38]. Interestingly, GDP-bound Rac1 also indirectly interacts with the Par3/6 scaffold, associating with the Rac activator Tiam 1, which is a component of the larger Par complex [39]. This association is necessary for tight junction formation in keratinocytes as well as directing neuronal polarity in hippocampal neurons [39,40]. Hoshino and colleagues have shown that Rit directly associates with the PDZ domain of Par6 in a GTP-dependent fashion, even though Rit does not contain a consensus PDZ recognition site at its C-terminus [34]. While Par6 association has been found to promote Rit-mediated NIH 3T3 cell transformation, the importance of Par6 association to Rit-directed neuronal differentiation has not been examined [34]. Since Rit-mediated neurite outgrowth has been proposed to require Rac/Cdc42 activation, association with the Par6 complex may play a critical role in both Rit-mediated Rho-family activation and neuronal differentiation [19].

While it is clear that Rit family GTPase signaling is important for appropriate PC6 cell and neuronal signaling, the contribution of individual Rit-mediated effector signaling pathways to this process remains unknown. To begin to understand the consequences of Rit-dependent activation of these distinct cellular signaling pathways, a series of alanine mutations was made to the effector domain of constitutively activated Rit (residues 48 to 59). These Rit effector mutant proteins were tested for their ability to induce PC6 cell differentiation and to associate with, and activate, known Rit effector targets.

Materials and Methods

Plasmids and reagents

Flag-tagged wild type, constitutively activated, and dominant negative Rit in p3xFLAG-CMV-10 vector, and Glutathione-S-Transferase-tagged activated Rit in the pEBG vector and wild-type Rit in the pGEX-KG vector have been described previously [29,41]. Hemagglutinin-tagged pKH3 Par6A and Par6B were the kind gift of Dr. Ian Macara, University of Virginia,

Charlottesville, VA. Par6C (or Par6α) was PCR isolated from Homo sapiens cDNA clone IMAGE: 4844008. ΔPDZ (residues 1-139) and ΔN (residues 154-345) mutants lacking the PDZ domain or the amino terminus, respectively, were isolated by PCR. Full length, ΔPDZ, and ΔN constructs were verified by sequence analysis by Agencourt Bioscience Corp. (Beverly, MA). The following commercially available antibodies were used: Flag (Sigma, Saint Louis, MO), GST (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), HA (12CA5), phospho-specific-ERK1/2 mouse monoclonal antibody and phospho-specific-p38 mitogen-activated protein kinase (MAPK) mouse monoclonal antibody (Cell Signaling, Beverly, MA). The GST-RGL3-RBD glutathione-agarose beads were prepared from bacterially-expressed GST-RGL3-RBD as described previously [16,17,29]. The full-length biotinylated human Par6C (or Par6α) cDNA-PinPoint Xa-3 and GST–tagged PKCι PB1 domain [PKCι-(1-113)] ⁻pGEX-6P plasmids were constructed previously [42].

Expression and purification of proteins

Expression and purification of biotinylated human Par6 protein has been described previously [43]. Cm^r plasmids carrying αA crystallin gene and GST-PKCι-(1-113) pGEX-6P were co-transformed into *E. coli* BL21(DE3) and transformants were grown in LB containing 35 μg/ml chloramphenicol and 50 μg/ml ampicillin at 22 °C (220 rpm). At OD_{600} of 0.8, IPTG was added to a final concentration of 0.1 mM and cells were harvested after 6 hours of induction. Soluble GST-tagged PKCι (1-113) protein was isolated using B-PER GST Fusion Protein Purification Kit (Pierce, Rockford, IL) and dialyzed against Tris buffer (50 mM Tris (pH 8.0), 135 mM NaCl, 10% glycerol). Expression and purification of GST-Rit, as well as guanine nucleotide exchange, has been described previously [13]. GDP- and GTPγS-bound GST-Rit were aliquoted into 50 μg samples and frozen at −80°C prior to use.

Cell lines, cell culture, and transfection

PC6 is a sub-line of PC12 that produce neurites in response to NGF, but grow as well isolated cells in culture (the generous gift of Dr. T. C. Vanaman, University of Kentucky, Lexington, KY). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT), 5% (v/v) heat-inactivated horse donor serum (HS; Life Technologies), 100 μg/ml streptomycin and 100 U/ml penicillin at 37°C in a humidified atmosphere of 5% CO_2. PC6 cells were transfected using Effectene (Qiagen, Valencia, CA) as described previously [30,41]. COS cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% (v/v) FBS, 100 μg/ml streptomycin and 100 U/ml penicillin at 37 °C in a humidified atmosphere of 5% CO_2. Transfection of COS cells was performed using Superfect (Qiagen) as described previously [29].

Generation of Rit effector loop mutants

Nine effector loop mutants, each with one residue changed to alanine, were produced in the constitutively active Rit^{L79} background using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Successful mutation was verified by DNA sequence analysis. The generation of Rit^{L79S53} was described previously [13].

Neurite Induction

PC6 cells were exposed to the indicated Effectene-DNA complexes for 12–16 hours [41]. The cells were then diluted (1:4), re-plated, and subjected to G418 selection (Life Technologies, San Diego, CA). At day 3 after neurite induction, cells were examined with a Zeiss Axiovert25 inverted phase-contrast microscope using a 20x objective. The percentage of cells bearing neurites greater than one cell body diameter was determined in three separate experiments. At

least 150 cells in 10–15 random fields were counted for each condition. For representative images of the mutants, cells were fixed at day 3 after neurite induction for 15 minutes in fresh 4% paraformaldehyde, rinsed three times with phosphate buffered saline (PBS), and permeabilized for 5 minutes in 0.1% Triton X-100 [44]. Cells were then incubated for 1 hour at room temperature with shaking in 1:1000 FITC-phalloidin (Sigma, Saint Louis, MO) in 5% bovine serum albumin, washed three times in PBS, and images captured with an Orca ER camera attached to a Zeiss Axiovert 200M fluorescence microscope using a 32x objective.

MAP kinase immunoblotting

To examine ERK and p38 MAP kinase activation, PC6 cells seeded in 6-well plates were transfected with p3xFlag-Rit^{L79}, a p3xFlag-tagged effector loop mutant, or empty p3xFlag vector as control. 48 h after transfection, cells were starved for serum 5 h prior to the preparation of whole cell lysates. The phosphorylation status of ERK1/2 and p38 MAP kinases was determined by immunoblotting with phospho-specific antibodies as described previously [29].

Immunoprecipitation, Glutathione-S-Transferase (GST) pull-down analysis, and Par6 binding assay

To examine the ability of the Rit effector loop mutants to bind to a known downstream target of Rit, the Ral GEF, RGL3, GST fusion proteins containing the Rit binding domain of RGL3 (residues 610-709) were expressed and purified as described previously [16,18,29,41]. PC6 cells seeded in 6-well plates were transiently transfected with p3xFlag-Rit^{WT}, p3xFlag-Rit^{L79}, p3xFlag-tagged Rit effector loop mutant, or empty p3xFlag vector and incubated for 36 h to allow maximal expression. Cell monolayers were lysed in GST pull-down assay buffer (20 mM HEPES [pH 7.4], 250 mM NaCl, 50 mM KF, 50 mM β-glycerol phosphate, 1% Triton X-100, 10% glycerol, and 1x protease inhibitor cocktail) with sonication. Insoluble material was pelleted by centrifugation. GST resin (20 µgGST-RGL3-RBD/20 µL glutathione beads) was added to 200 µg lysate, incubated for 1 h with end-over-end rotation at 4°C, and the resin collected by brief centrifugation. The pelleted beads were washed once with ice-cold GST pull-down buffer, twice with ice-cold GST pull-down buffer supplemented with 500 mM NaCl, and twice with ice-cold GST pull-down buffer. Bound Rit proteins were released by boiling in Laemmli sample buffer, separated by sodium dodecylsulfate (SDS)-10% polyacrylamide gels, transferred to nitrocellulose (Protran; Schleicher & Schuell Bioscience Dassel, Germany), and detected by immunoblotting with Flag antibody.

To examine the Par6 isoform Rit binds, COS cells were transiently transfected with pKH3-Par6A, pKH3-Par6B, or p3xFlag-Par6C and empty GST vector or GST-Rit^{L79}. Cells were allowed to recover for 36 h after transfection. Cells were harvested by scraping in ice-cold GST pull-down assay buffer and lysed with sonication on ice. Insoluble material was pelleted by centrifugation. Pre-washed glutathione-sepharose resin (GE Healthcare Bio-sciences Corp., Piscataway, NJ) was added to 500 µg lysate in a total volume of 1 ml and incubated with end-over-end rotation for 2 h at 4 °C. The resin was collected by brief centrifugation and the supernatant discarded. The resin was extensively washed, twice with ice-cold GST pull-down buffer, once with ice-cold GST pull-down buffer supplemented with 1 M NaCl, and twice with ice-cold GST pull-down buffer. Bound proteins were released by boiling in Laemmli sample loading buffer. Bound proteins and 10 µg total cell lysate from each sample were resolved using SDS-10% polyacrylamide gels, transferred to nitrocellulose, and detected by immunoblotting with hemagglutinin (HA), Flag, or GST antibody.

To examine the ability of the Rit effector loop mutants, wild type, activated, and dominant negative Rit to bind Par6C, cell lysates were generated from COS cells transiently transfected with empty p3xFlag vector, p3xFlag-Rit^{WT}, p3xFlag-Rit^{L79}, p3xFlag-Rit^{N35}, or p3xFlag-

tagged effector loop mutant along with empty GST vector, GST-Par6C, GST-Par6CΔPDZ, or GST-Par6CΔN. GST pulldowns were performed with whole-cell lysates as described above.

To assess Rit binding to Par6 *in vitro*, recombinant biotinylated human Par6 protein was covalently bound to streptavidin-coated 96-well microtiter plates (Nunc, Roskilde, Denmark) as described previously [42]. Purified GST-tagged PKCɩ (1-113), GST-tagged GTPγS-bound Rit, GST-tagged GDP-bound Rit or active unfused GST (Biovision, Mountain View, CA) was added to the wells at the concentrations indicated and allowed to bind to Par6 overnight at 4° C. Wells were then washed twice with PBS-Tween 20 to remove unbound protein and incubated with rabbit anti-GST polyclonal antibody (1:2500) (Chemicon International) at room temperature for 2 h. After two washes with PBS-Tween 20, Alexa Fluor® 532 goat anti-rabbit IgG (1:250, Invitrogen) was added to each well and incubated at room temperature for 3 h. The wells were washed twice with PBS-Tween 20, once with PBS, and binding determined by measuring fluorescence intensity using a Typhoon 9410 imager (GE Healthcare Bio-sciences Corp., Piscataway, NJ) and ImageQuant software (Amersham). Specific binding was calculated as total binding minus binding of GST at each concentration. Data were analyzed using SigmaPlot (Systat Software, Inc).

Results and Discussion

Mutations within the Rit effector loop alter biological activity

Effector domain mutants have proven to be useful reagents for defining the contribution of specific effector pathways to the actions of Ras and Rho family proteins, including their actions in PC12 cell differentiation [7,45,46]. To examine the contribution of known signaling pathways to Rit-mediated neurite outgrowth, single amino acids within the effector domain (residues 48-59) were mutated to alanine in the context of constitutively active Rit^{L79} (Fig. 1A). These double mutants were tested for their ability to induce PC6 cell differentiation as measured by the extension of neurites. Another Rit mutant, Rit^{L79S53}, previously shown to allow preferential activation of Raf/MEK/ERK signaling but not of RalGEF, was also examined [13]. Expression of activated Rit^{L79} in PC6 cells induced 10-fold higher neurite formation than cells transfected with empty vector alone and caused 1.5 times as many cells to exhibit neurites as wild-type Rit (Fig. 1B, C). The neurite induction of the various effector loop mutations could be classified into those with activity equal to that of Rit^{L79} (Rit^{L79A48}, Rit^{L79A49}), those with a significantly reduced ability to induce neurite formation compared to wild-type Rit (Rit^{L79A54}, Rit^{L79A55}, and Rit^{L79A56}), and those with an intermediate phenotype with induction slightly greater than wild-type Rit (Rit^{L79A50}, Rit^{L79A51}, Rit^{L79S53}, Rit^{L79A58}, and Rit^{L79A59}). Since no single effector domain mutation was found to completely disrupt Rit-dependent neuritogenesis, these data suggest that activation of more than one effector pathway is likely necessary for full neurite induction.

ERK activation correlates with neurite formation

Several pathways are known to be required for NGF-mediated PC12 cell differentiation including the Raf/MEK/ERK and p38 MAPK cascades [23,27,47,48]. Constitutively activated mutants of Ras, Raf, and MEK are all capable of inducing neuronal differentiation in PC12 cells highlighting the importance of this MAP kinase signaling cascade in cell fate [17,27,49, 50]. Expression of Rit^{L79}, but not the empty Flag vector, increased the cellular levels of both phospho-ERK1/2 and phospho-p38 (Fig. 2) in PC6 cells, consistent with earlier studies [29, 30,51]. All of the Rit effector loop mutants appear to be expressed to approximately equal levels (see Figs. 2 and 3) suggesting that the alanine substitutions did not result in global destabilization. Surprisingly, each of the Rit mutant proteins was found to promote p38 MAP kinase activation to levels seen here with Rit^{L79} (Fig. 2, *top panel*) and with previous studies using wild-type Rit [29]. These data indicate that none of these substitutions disrupt the

interaction(s) required for activation of the p38 MAP kinase signaling cascade. The organization of molecular complexes by protein scaffolds is one mechanism known to confer specificity to MAP kinase cascades and may contribute to Rit-dependent p38 signaling [52]. Thus, the nature of the molecular machinery that links Rit and p38 remains to be characterized and is a goal of ongoing studies.

Although each of the Rit mutants were expressed at comparable levels in PC6 cells (Fig. 2, *lower panel*), their ability to stimulate ERK MAP kinase activity could be divided into three groups: mutants that induce phospho-ERK1/2 to the same level as Rit^{L79} (Rit^{L79A48}, Rit^{L79A49}), mutations that impair Rit-mediated ERK activation similar to the level of Rit^{WT} in previous studies (Rit^{L79A54}, Rit^{L79A55}, Rit^{L79A56}), and those with a slight reduction in the ability to activate ERK (Rit^{L79A50}, Rit^{L79A51}, Rit^{L79S53}, Rit^{L79A58}, Rit^{L79A59}) [29]. Importantly, ERK activation mirrored the ability of individual effector loop mutants to induce neuronal differentiation, consistent with earlier studies suggesting a critical role for MEK-ERK signaling in Rit-mediated neuritogenesis [17,29].

RGL3 binding is not required for Rit-mediated neurite induction

Ral GEF proteins are downstream targets of both Ras and Rit signaling [16,53]. The importance of Ral GTPase function to neurite induction is currently unclear, with some studies suggesting that Ral-mediated activation of the exocyst complex is necessary for membrane fusion to drive protrusion of neurites, while others suggest that Ral signaling impedes neuritogenesis by inhibiting Rho family GTPase-regulated cytoskeletal reorganization [54,55]. Since the Ras/Rit Binding Domain (RBD) of RGL3 is both necessary and sufficient for GTP-dependent Rit binding, we examined the ability of Rit effector loop mutants to associate with GST-RGL3-RBD by glutathione bead precipitation [16]. As seen previously, both wild-type Rit and Rit^{L79} were found to potently bind RGL3-RBD [16]. This is explained by the observation that overexpressed wild-type Rit is predominantly GTP-bound and is thus able to both bind RGL3-RBD and promote neurite outgrowth [17]. Unlike p38 activation, RGL3 association was particularly sensitive to effector domain mutation. Only Rit^{L79A49} and Rit^{L79A55} displayed robust RGL3-RBD association (see Fig. 3 *lanes 4 and 9*), while both Rit^{L79A48} and Rit^{L79A59} exhibited weak but detectable binding (Fig. 3 *lanes 3 and 12*). Interestingly, both Rit^{L79A48} and Rit^{L79A49} induce neurite formation to the same extent (Fig 1C), which is significantly higher than that for Rit^{L79A55}, suggesting that interaction with RalGEFs and activation of Ral signaling are not required for Rit-mediated neurite induction.

Interaction of effector mutants with Par6 correlates with neurite induction

Directing polarization of the actin cytoskeleton is critical for initiating neurite formation and is controlled, in part, by the Par3-Par6-aPKC complex, which localizes to the tips of growing neurites [36,56–58]. Hoshino and colleagues first described the interaction between GTP-Rit and the PDZ domain of Par6, and found that Par6 synergistically enhanced Rit^{L79}-dependent NIH 3T3 cell transformation [34]. Because of the critical role for the Par complex in axonal growth, we next examined whether Par6 contributes to Rit-mediated neuritogenesis [34,36]. There are three mammalian members of the Par6 family (Par6A-C), and while Hoshino and colleagues described a Rit-Par6 interaction, it was unclear which isoform was used in their studies [34,59]. To address this issue, we co-expressed GST or $GST-Rit^{L79}$ and the three Par6 isoforms in COS cells and assessed Par6 binding following glutathione resin precipitation. $GST-Rit^{L79}$, but not unfused GST, interacted with all three Par6 isoforms (Fig. 4A). Because Par6C is preferentially expressed in the brain, this isoform was used in the remaining GST pull-down assays [59].

To examine the effector-domain dependence of Par6C binding, cell lysates co-expressing either Rit^{L79} or Rit effector loop mutants and GST-Par6C or unfused GST were generated and

subjected to glutathione-sepharose bead pull-down analysis. As expected, Flag-Rit^{L79} bound to full length Par6C, but not unfused GST (Fig. 4B). Unlike the strong correlation found between ERK1/2 activation and PC6 cell differentiation, binding of the Rit effector mutants to Par6C did not clearly mirror neurite induction. Those Rit effector mutants that promoted robust neurite induction, Rit^{L79A48} and Rit^{L79A49}, bound Par6C, while mutants with significantly impaired neurite initiation (alanine substitutions at residues 54-56) failed to bind Par6C, suggesting that interaction with Par6C might contribute to Rit-mediated neurite induction. However, Par6 association is not essential since Rit^{L79S53} does not display appreciable Par6 binding (Fig. 4B) but promotes neurite formation (Fig. 1C). In addition, except for Rit^{L79S53}, all the Rit effector mutants that fail to bind Par6C also fail to activate ERK (Fig. 2). Thus, it not possible to separate the relative contributions of these two effector pathways to Rit function. Additional studies will be necessary to more fully characterize the role of Par6 in Rit-mediated neuronal signaling.

Characterization of an indirect Rit-GDP-Par6 interaction

Because this is the first reported interaction of Rit and Par6C, we next examined the nucleotide dependence of binding. Lysates expressing Flag-tagged wild-type Rit, Rit^{L79}, or Rit^{N35} with GST-Par6C or unfused GST were generated and GST pull-down assays performed. As expected, none of the Rit constructs interacted with unfused GST while both wild-type Rit and Rit^{L79} displayed modest Par6C binding (Fig. 5A). Surprisingly, dominant negative Rit, Rit^{N35}, a mutant which is predominantly GDP-bound, does not promote neurite outgrowth, and fails to interact with all previously identified Rit effectors, displayed robust association with Par6C (Fig. 5A) [13,17,29]. Thus, in addition to the previously characterized GTP-dependent interaction of Rit with the PDZ domain of Par6, these data suggest that Rit associates with Par6C in a GTP-independent manner [34].

To further characterize the GTP-independent interaction of Rit with Par6, recombinant wild-type GST-Rit protein was pre-loaded with either GDP or the non-hydrolyzable GTP analog, GTPγS [13]. We took advantage of a well established *in vitro* human Par6C binding assay [42]. As seen in Fig. 5B, binding studies were first performed with unfused GST or GST-PKCι, an established binding partner of Par6 [43]. As expected, no association between GST and Par6 was observed whereas GST-PKCι demonstrated strong binding with an apparent K_D of 24.6 nM. Interestingly, both GDP- and GTPγS-Rit bound Par6 with approximately equal affinity (K_D=5.9 μM and KD=7.3 μM, respectively) (Fig. 5C). These data support the observations of Hoshino and colleagues that Rit can directly bind Par6, albeit with a much lower binding affinity when compared with GST-PKCι, a known component of the Par3-Par6 complex [34,35]. Additionally, these data indicate that the direct *in vitro* interaction between Rit and Par6 is GTP-independent, since GDP- and GTPγS-bound Rit display approximately equal binding (Fig. 5C). Moreover, they suggest that the direct binding of GDP-Rit to Par6 does not account for the robust binding of Rit^{N35} to Par6 seen in whole cell lysates. Indeed, the very low affinity observed for the *in vitro* binding of Rit to Par6 suggests that the observed intracellular association is likely not due to direct interaction, but instead is mediated by Rit association to the larger Par6 scaffolding complex.

To begin to explore the nature of the Rit^{N35}-Par6C interaction, we examined which region of Par6 was bound by Rit^{N35}. Lysates expressing Flag-tagged Rit^{N35} and either GST-Par6C (full-length: residues 1-345), GST-Par6CΔPDZ (residues 1-139), or GST-Par6CΔN (Residues 154-345) were generated and subjected to GST pull-down analysis. Surprisingly, Rit^{N35} was found to bind all three Par6C constructs tested (Fig. 4D), suggesting that the GTP-independent association of Rit with Par6C does not require an intact PDZ domain [34].

Recently, the Rac1 GTPase has been shown to interact in a complex fashion with the Par3/6 polarity complex. In this case, active GTP-bound Rac1 binds to the semi-CRIB domain of Par6

while GDP-Rac1 interacts with the Rho family GEF, Tiam1, which associates with components of the larger Par3-Par6-aPKC complex [39,40]. The Par/Tiam1 complex allows for rapid and localized Rac1 activation following chemokine stimulated-induction of Rap1 in T lymphocytes, providing a mechanism by which Rap1 activation can control chemotaxis [60]. Thus, it is tempting to speculate that dominant negative Rit (Rit^{N35}) can associate with the Par3/6 complex in a similar fashion, involving a scaffolded RitGEF protein. This interaction would not have been detected in the study by Hoshino and colleagues, since they examined the nucleotide-dependence of the Rit-Par6 interaction with recombinant proteins and additional cellular co-factor(s) would be absent in their assays [34]. The possibility that the Par6 complex contains a RitGEF is supported by the robust association seen with Rit^{N35}, which bound more tightly than either wild-type or activated Rit (Fig. 5A). Dominant-negative Ras GTPases are preferentially bound by cellular GEFs but not by other effectors which favor a GTP-bound conformational state [4]. Taken together, these data suggest that Rit-Par6 association is unexpectedly complex, and may provide a novel mechanism for regulating Rit activity. Indeed, we have recently found that Rit is controlled by cross-talk between the NGF and BMP signaling pathways and suggest a role for Rit in regulating axonal and dendritic growth via activation of the ERK MAP kinase signaling pathway [44]. Since the Par3/6 complex is involved in axon-dendrite specification in hippocampal neurons [36], studies are currently underway to further investigate the role of Par3/6 binding in Rit-mediated control of axonal and dendritic growth.

In summary, using a series of Rit effector loop mutants we have found that Rit-mediated neuronal differentiation depends upon activation of the ERK MAP kinase cascade, but does not require activation of RalGEF/Ral signaling. In addition, our data demonstrates an unexpectedly complex association of Rit with Par6 and suggests that the larger Par3/6/aPKC polarity complex may contain a scaffolded RitGEF. Since Rit has recently been shown to regulate axonal-dendritic growth via activation of ERK MAP kinase signaling, it will be important to examine the contribution of Par3/6 complex to the regulation of this process.
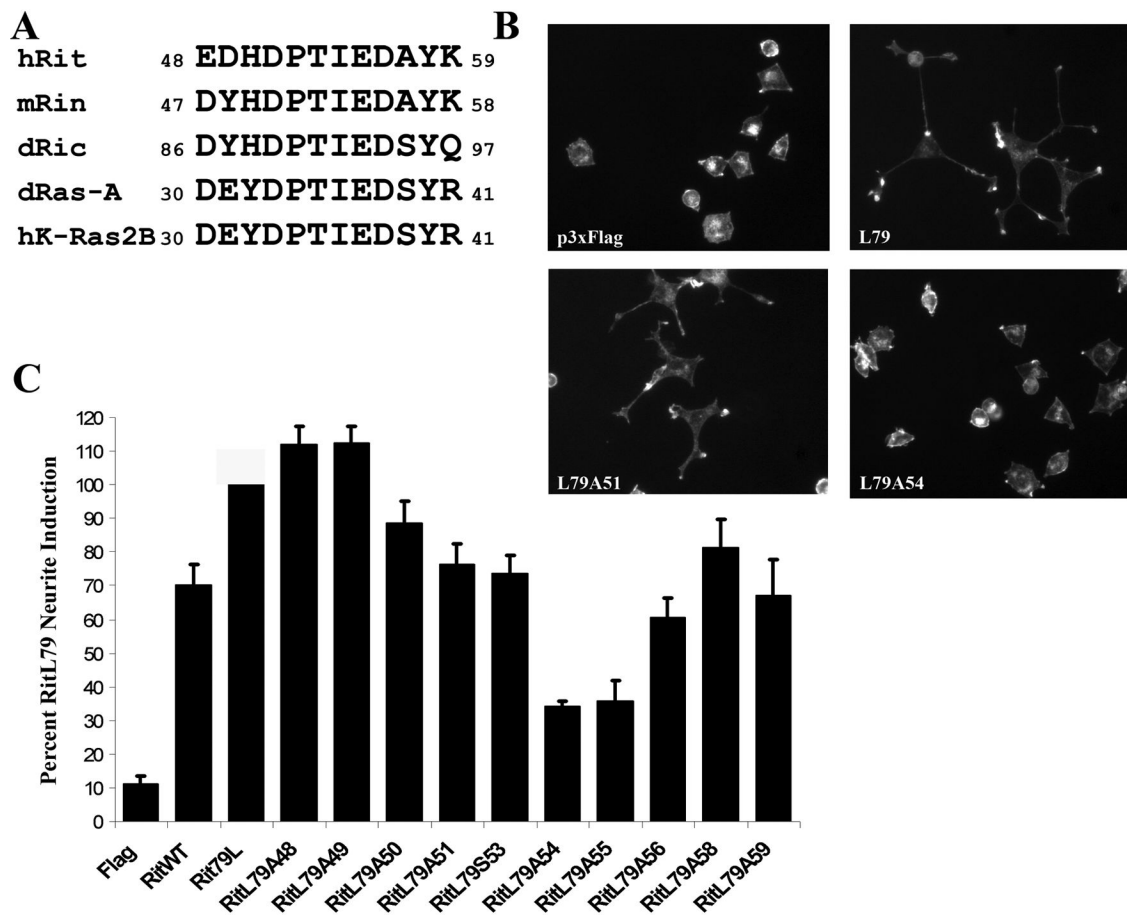
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A

| hRit | 48 EDHDPTIEDAYK 59 |
mRin	47 DYHDPTIEDAYK 58
dRic	86 DYHDPTIEDSYQ 97
dRas-A	30 DEYDPTIEDSYR 41
hK-Ras2B	30 DEYDPTIEDSYR 41

B

C

Figure 1. Mutation of the Rit effector loop alters neurite outgrowth ability
(A) Sequence alignment of the effector domains from several Ras family GTPases. (B) PC6 cells were transiently transfected, subjected to G418 (400 μg/ml) selection for 3 days, fixed and stained with FITC-phalloidin to label actin filaments. Representative fluorescent micrographs from random fields are presented. (C) The percentage of neurite-bearing cells was calculated at day 3 and is presented relative to RitL79 (100%) as means ± standard error from three independent experiments.
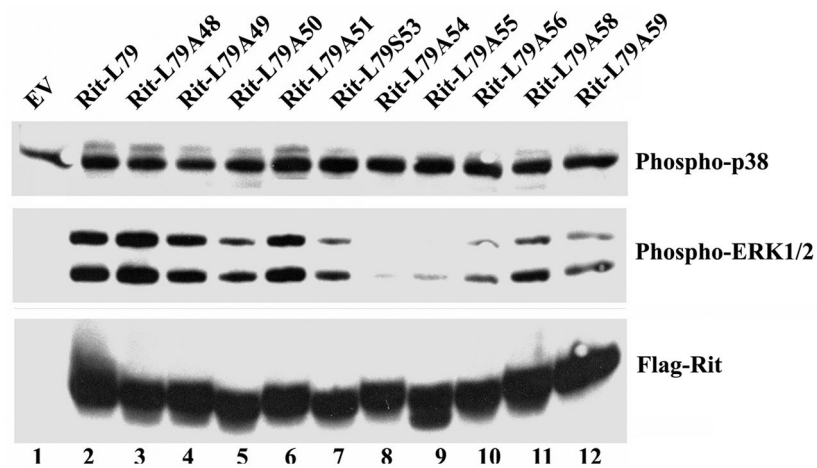
Figure 2. Mutation of the Rit effector loop alters activation of downstream MAPK pathways
PC6 cells were transiently transfected with expression vectors for the indicated Rit mutant, subjected to serum starvation, and harvested as described under "Materials and Methods". 50 μg of pre-cleared lysate was resolved on SDS 10%-polyacrylamide gels and the levels of activated ERK1/2 and p38 MAPK were determined by immunoblotting with the appropriate phospho-specific antibodies. Blots are representative of three independent trials.
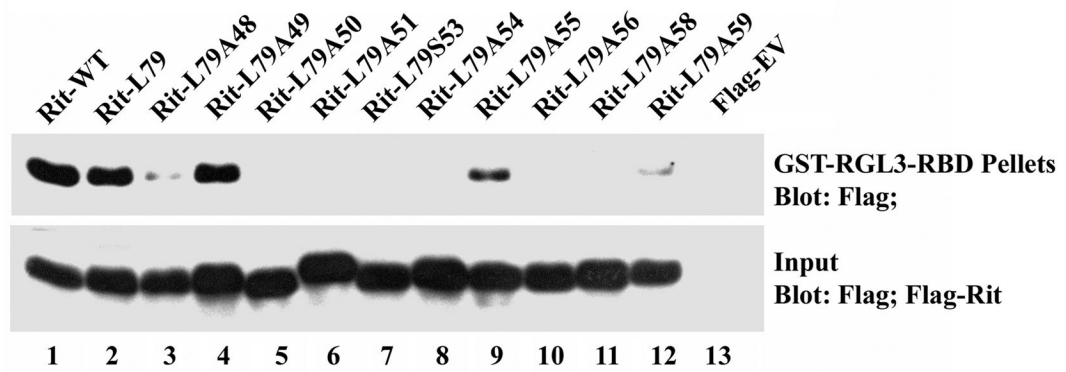
Figure 3. Rit effector loop mutants exhibit differential binding to RGL3-RBD
PC6 cells were transiently transfected with the indicated expression vectors, and the cells starved with serum-free DMEM for 5 h prior to the preparation of whole cell lysates. Cell lysates (200 µg) were incubated at 4 °C for 1 h with GST-RGL3-RBD pre-bound to glutathione beads. The RGL3-RBD complexed affinity beads were pelleted and washed extensively, and the amount of bound Rit protein in the pellet fraction was determined by anti-Flag immunoblotting. The expression of Rit present in each lysate was also determined by immunoblot analysis. Blots are representative of three independent trials.
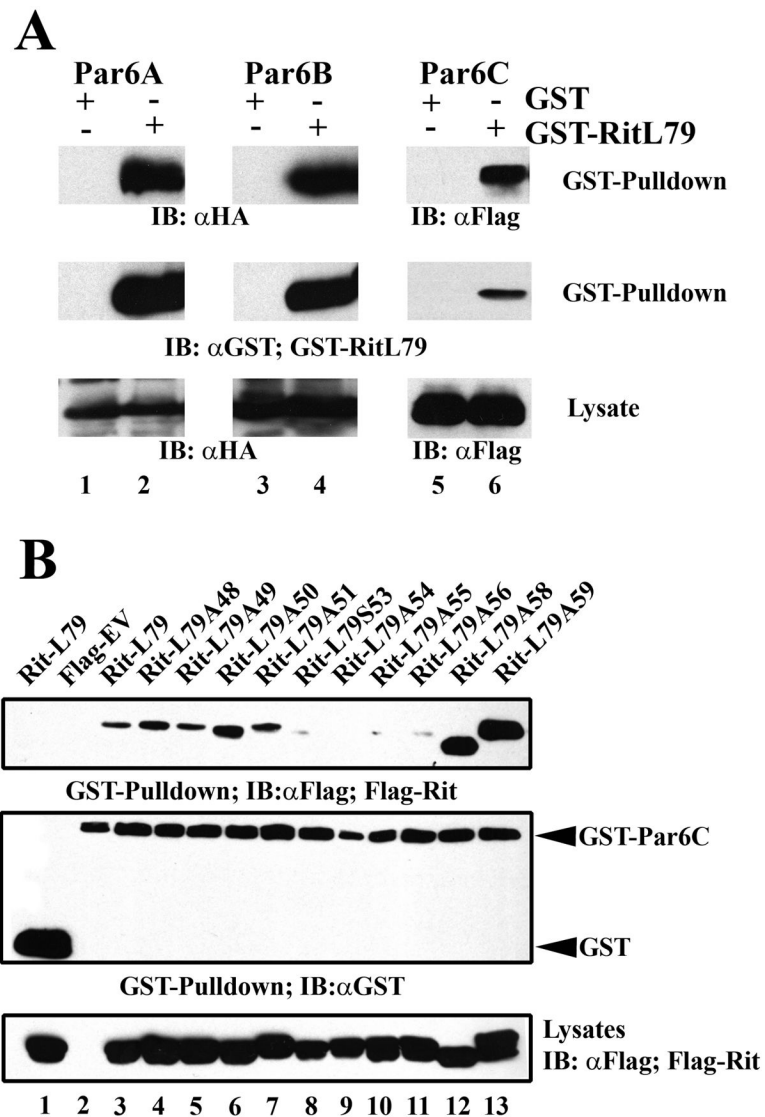
Figure 4. Rit interacts with Par6C in an effector loop dependent manner
(A) COS cells were transfected with GST or GST-Rit[L79] and 3xHA-Par6A, 3xHA-Par6B, or 3xFlag-Par6C, whole-cell lysates were generated, and GST pull-down assays performed as described under "Materials and Methods". Precipitated Par6 proteins were detected by immunoblot analysis using anti-HA, or anti-Flag antibodies. Equal expression of Par6 proteins was detected from total lysates by immunobloting. Blots are representative of three independent trials. (B) COS cells were co-transfected with either unfused GST or GST-Par6C expression vectors and the indicated Flag-tagged Rit mutants. Whole-cell lysates were generated and GST pull-down assays performed as described under "Materials and Methods". Precipitated Rit and Par6 protein was detected by immunoblot analysis using anti-Flag and anti-GST antibodies, respectively. Expression of Rit protein was examined by anti-Flag immunoblotting of total lysate. Blots are representative of three independent trials.
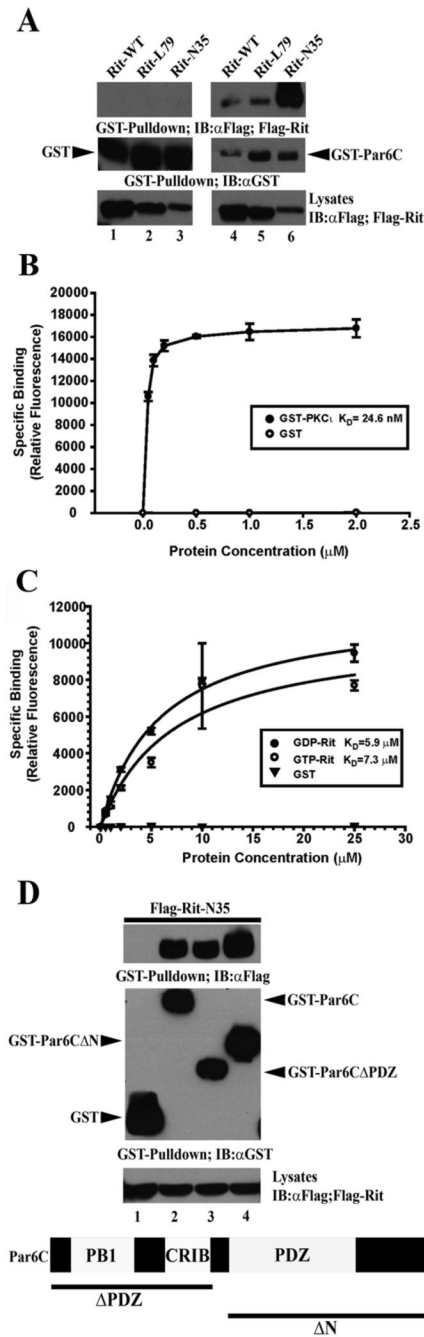
Figure 5. Dominant negative Rit strongly interacts with Par6 independent of its PDZ domain
(A) COS cells were transfected with GST or GST-Par6C and Flag-tagged Rit[WT], Rit[L79] or
Rit[N35]. GST precipitations were performed as in Figure 4B. Note that Rit[N35] displays stronger
association with Par6 than either Rit[WT] or Rit[L79]. Blots are representative of three independent
trials. (B,C) Biotinylated Par6C was bound to streptavidin-coated plates and incubated with
the indicated concentrations of recombinant GST, GST-PCKι, or GST-Rit pre-loaded with
non-hydrolyzable GTPγS (GTP) or GDP. Binding isotherms for PKCι (B) and GTPγS-Rit and
GDP-Rit (C) to Par6 indicate that PKCι binds Par6 with much higher apparent K_D than Rit
and that GTP- and GDP-Rit bind to Par6 with similar affinities. (D) COS cells were co-
transfected with GST, GST-Par6C full length, GST-Par6CΔPDZ, or GST-Par6CΔN and Flag-

Rit[N35] and whole cell lysates were generated and subjected to GST pull-down analysis as described under "Materials and Methods". Blots are representative of three independent trials.