Rin GTPase Couples Nerve Growth Factor Signaling to p38 and b-Raf/ERK Pathways to Promote Neuronal Differentiation*§

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In neuronal precursor cells, the magnitude and longevity of mitogen-activated protein (MAP) kinase cascade activation contribute to the nature of the cellular response, differentiation, or proliferation. However, the mechanisms by which neurotrophins promote prolonged MAP kinase signaling are not well understood. Here we defined the Rin GTPase as a novel component of the regulatory machinery contributing to the selective integration of MAP kinase signaling and neuronal development. Rin is expressed exclusively in neurons and is activated by neurotrophin signaling, and loss-of-function analysis demonstrates that Rin makes an essential contribution to nerve growth factor (NGF)-mediated neuronal differentiation. Most surprisingly, although Rin was unable to stimulate MAP kinase activity in NIH 3T3 cells, it potently activated isoform-specific p38 MAP kinase signaling and weakly stimulated ERK signaling in pheochromocytoma (PC6) cells. This cell-type specificity is explained in part by the finding that Rin binds and stimulates b-Raf but does not activate c-Raf. Accordingly, selective down-regulation of Rin in PC6 cells suppressed neurotrophin-elicted activation of b-Raf and p38, without obvious effects on NGF-induced ERK activation. Moreover, the ability of NGF to promote neurite outgrowth was inhibited by Rin knockdown. Together, these observations establish Rin as a neuronal specific regulator of neurotrophin signaling, required to couple NGF stimulation to sustain activation of p38 MAP kinase and b-Raf signaling cascades required for neuronal development.

Neurotrophin signaling is responsible for the regulation of a wide range of nerve cell functions, including differentiation, axonal and dendritic elongation, survival, and many aspects of neuronal activity (1). These diverse biological effects are mediated predominantly by the Trk family of receptor tyrosine kinases. Upon ligand binding, activated Trk receptors recruit adaptor proteins and activate Ras-related GTP-binding proteins and other effectors to modulate a diverse array of canonical signaling pathways (2). Among these cascades, both ERK2 and p38 MAP kinase pathways have been shown to be essential for cellular proliferation and the acquisition and maintenance of a differentiated phenotype. A distinguishing feature of neurotrophin signaling is the sustained activation of ERK kinase activity (3). Previous studies have suggested that the longevity of MEK/ERK pathway activation (4, 5) is critical for neuronal differentiation in many systems, and Ras-like GTPases have emerged as key elements in these pathways (1). The Raf-MEK-ERK kinase cascade is implicated in the regulation of diverse biological processes, including both cellular proliferation and differentiation, and an important remaining issue is how integration of MAP kinase cascade signaling promotes distinct biological responses. However, although an extensive literature supports a role for prolonged MEK/ERK signaling in neuronal differentiation, inhibition of the sustained phase of ERK activation does not block neurite outgrowth (6). In addition, Src and bone morphogenic factor-2 are unable to activate ERK but still induce neurite outgrowth in pheochromocytoma PC12 cells (7, 8), indicating the existence of additional signaling pathways important for neurite outgrowth.

Recent work has defined roles for p38 MAP kinase signaling in diverse neuronal functions (9), including a key role in neurite elongation. First, neurotrophin signaling induces sustained p38 activation in a variety of systems (9). Consistent with a critical role for the p38 pathway in neuronal differentiation, treatment with p38 inhibitors or dominant inhibitory p38 blocks neurite outgrowth (8, 10, 11), whereas activation of p38 by expressing constitutively active MKK3/6 induces neurite outgrowth in the absence of NGF stimulation (8). Moreover, neurite outgrowth induced by the expression of constitutively active MEK also depends upon p38 activity (10). How neurotrophin-mediated signals regulate the p38 MAP kinase cascade to induce neurotogenesis remains to be elucidated. A greater understanding of this signaling pathway would provide insight into neurotrophin-specific modulation of p38 MAP kinase cascade activity and the role for p38 kinase signaling in neurotogenesis.

Small molecular weight GTP-binding proteins serve as molecular switches to regulate a diverse array of cellular functions. These include control of cellular growth, differentiation, actin dynamics, protein trafficking, and nuclear transport (12, 13). Despite sequence differences between the members of this large family, all contain five conserved sequence domains and function as guanine nucleotide-dependent molecular switches. When in their active GTP-bound state, the GTPases interact through their effector domains with a variety of cellular targets to elicit their biological activity. We and others have described the cloning of Rin, a novel member of the Ras-related proteins (14–16). Although Rin shares more than 50% sequence identity with Ras, it is expressed solely in neurons and shares a conserved and unique effector domain with the closely related Rit and Drosophila Ric proteins. Moreover, Rin lacks a known recognition site for post-translational lipidation required for the association of many Ras proteins with cellular membranes, instead exhibiting calcium-dependent association with...
calmodulin via an extended C terminus (14, 16). Although our previous studies have demonstrated that the closely related Rit GTPase signals to a variety of Ras-responsive promoter elements, transforms NIH 3T3 cells to tumorigenicity, and activates the Raf exchange factor RGL3, Rin has little to no activity in these assays. Thus, Rin and Rit are likely to regulate distinct cellular functions, and characterization of Rin function remains incomplete (17–19). We have shown recently that Rin is activated following NGF stimulation of pheochromocytoma cells (19), and together with studies implicating Rin signaling in calcium-mediated neurite outgrowth (19, 20), these data have led to the proposal that Rin signaling may play a pivotal role in regulating neuronal signaling pathways.

In this report, we investigated the molecular events that mediate neurotrophin-induced differentiation and the role of the Rin GTAPase in neurotrophin signaling. We demonstrate that Rin associates with and activates the neuronal Raf isoform b-Raf, but not c-Raf, to promote modest ERK activation. More importantly, Rin promotes robust p38 MAP kinase activation and plays an essential role in regulating the magnitude and longevity of NGF receptor-mediated p38 activation, but it is not required for EGF receptor-induced p38 signaling in pheochromocytoma cells. Furthermore, Rin silencing blocks NGF-mediated neurite outgrowth, without altering ERK activation. These observations establish Rin as a critical component in the signaling machinery used to generate neurotrophin-mediated activation of b-Raf and p38 signaling pathways and highlight the essential role of p38 MAP kinase in maintaining normal nervous system function.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—Rin was amplified by PCR and subcloned into pEBOG (GST fusion) (kindly provided by Dr. J. H. Kehrl, NIAID, National Institutes of Health) or p3xFLAG-CMV-10 expression vectors (Sigma). Wild-type (WT) b-Raf, WT-c-Raf, HA-tagged MEK1, and kinase-dead Akt (Akt-KD) have been described previously (21). The construction of FLAG-tagged wild-type and dominant-negative p38 (α, β2, γ, and δ) expression vectors have been described (22). The cDNA clone for human Rin (RIT2) was obtained from the University of Missouri-Rolla CDNA Resource Center (www.cdna.org). Antibodies against the following were purchased: FLAG (Sigma); phospho-ERK1/2,-MEK1/2,-ERK5,-MKK3/6,-p38,-AKT,-HSP27, and p38, HSP27 (Cell Signaling, Beverly, MA); MEK1, ERK1, b-Raf, Raf-1 (c-Raf), GST, and actin (Santa Cruz Biotechnology); phospho-Raf (Ser183) and Ras (Upstate Biotechnology, Inc., Lake Placid, NY); and phospho-JNK1/2 (Thr183/Tyr185, BIOSOURCE). Biotinylated antibodies were generated using the protein biotinylation system (Amersham Biosciences). The anti-Rin and anti-Rit antibodies have been described (21). Our previous work (21) has found that immunoblotting with the anti-phospho-c-Raf (Ser183) antibody can be used to monitor the activation state of b-Raf in PC6 cells, and thus detection of phospho-b-Raf was accomplished by using this reagent. Recombinant human EGF and rat β-NGF were purchased from R&D Systems (Minneapolis, MN). The indicated reagents were purchased: JNK1/2 inhibitor SP600125, p38 MAPK inhibitor SB203580 (Tocris, Ellisville, MO), MEK1/2 inhibitor PD98059, PI 3-kinase inhibitor LY294002 (Calbiochem), PMA, and A23187 (Sigma).

Cell Lines and Transfections—PC6 cell is a subline of PC12 cells that produces neurites in response to NGF but grows as well isolated cells rather than in clumps and is suited to both biochemical and morphological analysis of signaling pathways (19, 21, 23). PC6 and COS cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 5% horse serum or 10% FBS, respectively. Transfections were performed using Effectene or SuperFect reagents (Qiagen).

GST-Pull Down Analysis and Immunoblotting—To examine the association of Rin and Raf proteins, cell lysates were prepared from COS cells transiently transfected with 3 μg of empty pEBOG (expressing unfused GST), pEBG-Rin-Q78L, or pEBG-H-Ras-Q61L and either 2 μg of pcDNA3-HA-b-Raf-WT or -c-Raf-WT-FLAG. Cell monolayers were cultured for 36 h after transfection and harvested following an additional 5 h of incubation in serum-free DMEM by scraping in phosphate-buffered saline (PBS) and resuspending in ice-cold kinase buffer (20 mM Hepes (pH 7.4), 50 mM KF, 50 mM β-glycerol phosphate, 150 mM NaCl, 2 mM EGTA, 1 mM sodium vanadate, 10% glycerol, 1% Triton X-100, and 1× protease inhibitor mixture (Calbiochem)). The cell suspension was lysed by sonication on ice. The homogenate was centrifuged at 4 °C for 10 min to eliminate the insoluble material. The protein concentration was determined by Bradford assay (Bio-Rad), and the supernatant was transferred to a fresh tube. Detergent-soluble lysates (400 μg) were incubated with 2 μl of a 50% slurry of glutathione-Sepharose 4B (Amersham Biosciences) in a total volume of 1 ml for 1–2 h at 4 °C with end-over-end rotation to allow isolation of the GST fusion proteins. The glutathione beads were then collected in a microcentrifuge for 5 min at 1 × 10^4 rpm at 4 °C, and the supernatant was discarded. The pellet glutathione beads were then washed two times with ice-cold kinase buffer, once with ice-cold 1 mM NaCl kinase wash buffer (kinase buffer containing 1 mM NaCl), and with two additional washes with ice-cold kinase buffer, after which the bound proteins were released by boiling in Laemmli sample loading buffer for 5 min and subjected to SDS-PAGE on 10% polyacrylamide gels. To examine the interaction of Rin and endogenous Raf kinase isoforms, cell lysates (10 mg of total cell lysate/reaction) prepared from COS cells transiently transfected with pEBOG, pEBG-Rin-Q78L, or pEBG-H-Ras-Q61L were subjected to GST-pull down analysis as described above.

To examine the interaction of Rin and Raf proteins by co-immunoprecipitation, cell lysates were prepared from COS cells transiently co-transfected with 3 μg of empty 3xFLAG vector, 3xFLAG-Rin-Q78L, or 3xFLAG-H-RasQ61L together with 2 μg of Myc-b-Raf-WT or Myc-c-Raf-WT expression vectors. Cell monolayers were allowed to recover for 36 h after transfection, serum-starved for 5 h of incubation in serum-free DMEM, resuspended in ice-cold kinase buffer, and lysed by sonication on ice. The homogenate was centrifuged at 4 °C for 10 min (1.4 × 10^4 rpm in a refrigerated microcentrifuge) to eliminate the insoluble material, and the cleared cell lysates (400 μg) were incubated with either 2 μg of anti-Myc monoclonal antibody or mouse IgG (Santa Cruz Biotechnology) (control) for 2 h with gentle rotation at 4 °C. Immunocomplexes were affinity absorbed onto 30 μl of protein G-Sepharose beads (Amersham Biosciences) for an additional 1 h at 4 °C with constant rotation. The Sepharose resin was collected by centrifugation (5 min at 1 × 10^4 rpm at 4 °C) and washed extensively (as described above), and bound proteins were eluted by incubation for 5 min at 100 °C in Laemmli buffer. Bound proteins and 10 μg of total cell lysate from each sample were resolved by using 10% SDS-polyacrylamide gels, transferred to nitrocellulose membrane (Protran, Schleicher & Schuell), and subjected to immunoblotting using biotin-labeled anti-FLAG or anti-Myc antibody.

Immunoblots were blocked in 1% casein (Sigma) in PBS plus 0.1% Tween 20 (PBST) for 1 h at 25 °C and incubated with an appropriate dilution of the primary antibody in 1% casein or 5% BSA in PBST for 1–2 h. The immunoblots were washed three times with PBST before the addition of horseradish peroxidase-conjugated secondary antibodies or horseradish peroxidase-conjugated streptavidin (Zymed Laboratories).
In Vitro Kinase Assays—To perform b-Raf kinase assays, COS or PC6 cells were either co-transfected with expression vectors for GST-tagged Raf kinases and different Ras family GTPases or with shRNA expression vectors, cultured for 36–60 h, and subjected to serum starvation for 5 h prior to growth factor addition (where indicated) and the preparation of whole cell lysates. Immune complex kinase assays using either GST-bound protein or b-Raf immunoprecipitates were performed by using a Raf kinase cascade assay kit (Upstate Biotechnology, Inc.) essentially as described previously (21). For p38 kinase assays, HA-RinQ78L and FLAG-tagged p38 isoform co-transfected PC6 cells were lysed in breaking buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na3VO4, 1 μM leupeptin), and p38 kinase activity was monitored with a nonradioactive assay kit (Cell Signaling Technology) by anti-FLAG immunoprecipitation. The immune complexes were washed twice with breaking buffer and twice in kinase buffer (5 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2) and were incubated in kinase buffer containing 200 μM ATP and 2 μg of ATF-2 as substrate. After incubation for 30 min at 30 °C, the assays were terminated by the addition of Laemmli buffer. The samples were resolved by SDS-PAGE, and p38 activity was determined by immunoblotting with anti-phospho-specific ATF-2 antibody.

For the studies on ERK and p38 MAP kinase activation, PC6 cells were transfected as described above with either 1.0 μg of empty 3xFLAG vector or 3xFLAG-Rin-Q78L after pretreatment with PD98059 (10 μM) or SB203580 (10 μM) for 30 min or not. Cell lysates were harvested after starvation, and the phosphorylation levels of ERK or p38 MAP kinase were analyzed by immunoblotting with the appropriate phospho-specific antibodies. The activation of ERK in PC6 cells was also analyzed by immunoprecipitation using 500 μg of total lysate proteins with 2 μg of anti-ERK1 monoclonal antibody performed in a similar immunoprecipitation procedure as described above. The phosphorylation of MKK3/6, ERK5, PKB/AKT, or JNK1/2 was analyzed by immunoblotting lysates with phospho-specific antibodies as indicated in Fig. 1.

To study the role of inhibitors and b-Raf on Rin signaling, PC6 cells were transfected with 1 μg of empty 3xFLAG vector or 3xFLAG-Rin-Q78L in the absence or presence of Myc-b-Raf-WT (0.5 μg) after pretreatment with SB203580 (10 μM), PD98059 (10 μM), or not. The cells were then starved for 5 h in the presence or absence of inhibitors and harvested with kinase buffer. The extracted detergent-soluble lysates were fractionated by SDS-PAGE. The phosphorylation status of ERK, p38 MAPK, and HSP27 was detected by immunoblotting with phospho-specific antibodies. The levels of protein expression were blotted with the appropriate antibodies.

Neurite Outgrowth Studies—PC6 cells were exposed to DNA-Effecten complexes for 9–16 h, re-plated after dilution (1:4 dilution), and subjected to 400 μg/ml G418 selection for 3–7 days. The percentage of neurite-bearing cells, neurite number per cell body, neurite length, and the number of branch points per neurite were determined in two to four separate experiments. At least 200 cells were counted per experiment (in 9–12 random fields) with each experiment performed in triplicate as described previously (21). The cells were then fixed with methanol/acetone (3:1) at 4 °C for 45 min, and the images were captured on an Axiovert 200 m phase-contrast microscope (Zeiss). To examine the effect of Rin on neurite outgrowth, PC6 cells were transfected with 1 μg of empty 3xFLAG vector, 3xFLAG-Rin-Q78L, or 3xFLAG-Rit-Q79L as control, and recombinant proteins were visualized using an immunofluorescent microscope (Zeiss) with OpenLab 3.1.4 imaging software (Improvision Inc., Lexington, MA). Immunostaining was performed by incubation with anti-FLAG monoclonal antibody (1:200; diluted in 0.5% BSA/PBS) at 37 °C for 1 h after blocking with 0.5% BSA/PBS for 30 min, then washed with PBS three times before incubation with FITC-conjugated anti-mouse IgG (Zymed Laboratories Inc., 1:200, diluted in 0.5% BSA/PBS), and terminated by extensive washing with PBS. To investigate the involvement of a variety of signaling pathways on Rin-mediated neurite outgrowth, PC6 cells were pretreated with SB203580 (10 μM), PD98059 (10 μM), SP600125 (25 μM), or LY294002 (10 μM) for 30 min or co-transfected with 1 μg of AKT-KD. To explore the possible mechanisms of Raf on Rin-mediated neurite outgrowth, PC6 cells were transfected with 1 μg of empty 3xFLAG vector or 3xFLAG-Rin-Q78L in the absence or presence of empty Myc vector or Myc-b-Raf-WT (1 μg) after pretreatment with PD98059 (10 μM), SB203580 (10 μM), or without drug addition. To explore the contribution of specific p38 kinase isoforms on Rin-mediated neurite outgrowth, PC6 cells were co-transfected with 1 μg of 3xFLAG-Rin-Q78L or empty 3xFLAG vector and 1 μg of FLAG-tagged dominant-negative-p38α/β2, -γ, or -δ.

Short Hairpin-RNA Interference—The mamalian expression vector, pSUPER.gfp/neo (OligoEngine), was used for expression of shRNA in PC6 cells as described previously (21). The vector allows direct synthesis of shRNA transcripts using the polymerase-H1-RNA gene promoter and co-expresses GFP to allow detection of transfected cells. The shRNA specific insert sequence AGCGCAGTCACAATGCAGT (target sequence sense (TS)) was subcloned into pSUPER.gfp.neo (OligoEngine) to generate shRin99. Rat target sequences are as follows: shRin448-TS, CTCCTGGTCCAGACTACAA, and shCTR-TS, TTCTCCGACGTTCAGCT. Expression constructs were introduced to PC6 cells by transfection using Effectene reagents, and the cells were subjected to G418 (200 μg/ml) selection. The expression of Rin, Ras, or Rit GTPases was analyzed by immunoblotting with anti-Rin, anti-Ras, or anti-Rit monoclonal antibody. To determine the effects of shRNA-mediated knock-down of endogenous Rin on signaling, PC6 cells seeded in 6-well plates were transfected with either shRin99 or shCTR. Cell monolayers were subjected to G418 (400 μg/ml) selection for 2 days, serum-starved for 5 h, stimulated as indicated, and then harvested in kinase lysis buffer. The phosphorylation levels of ERK, p38 MAP kinase, and HSP27 were detected by immunoblotting with phospho-specific antibodies. The p38 MAP kinase activities were also determined by p38 MAP kinase assay following immunoprecipitation with immobilized anti-phospho-p38 MAP kinase antibody. To explore the requirement for Rin function in NGF-mediated neurite outgrowth, PC6 cells were transfected with the appropriate shRNA construct and then reseeded onto 35-mm dishes (1:4 dilution) in complete DMEM containing 400 μg/ml G418 with or without NGF (100 ng/ml) stimulation to initiate differentiation. The neurite outgrowth was analyzed, and the cells were photographed as described above at days 3 and 7 following NGF exposure.

RESULTS

Rin Activates Both ERK and p38 MAP Kinase Signaling to Induce Differentiation—The Rin gene was originally identified as a calmodulin-binding GTPase expressed exclusively in neurons, and we have...
shown that Rin is rapidly activated following NGF stimulation of PC6 cells (19). Propagation of TrkA signaling is associated with activation of a variety of small GTP-binding proteins, suggesting that Rin might be directly involved in neurotrophin signaling. However, in our earlier analysis the expression of GFP-tagged activated Rin failed to induce neurite outgrowth (14, 16, 19). A recent report by Hoshino and Nakamura (20) implicated Rin in calcium/calmodulin-dependent neurite outgrowth and suggested that addition of a large GFP fusion tag may interfere with Rin function. To explore this possibility, we first determined whether expression of an activated Rin mutant, RinQ78L, induced neurite outgrowth in pheochromocytoma cells when bearing a smaller N-terminal FLAG epitope tag. As seen in Fig. 1A, a majority (>60%) of PC6 cells expressing FLAG-RinQ78L demonstrated modest neurite outgrowth, whereas wild-type FLAG-Rin also induced differentiation (>25%), although at a lower rate (data not shown), consistent with a role for activated Rin in neural differentiation. Rin-induced neurites showed linear extensions and few branches (Fig. 1A), whereas PC6 cells transfected with activated Rit developed multiple neurites and profuse branching, suggesting that these highly related GTPase may use distinct effector pathways to regulate neuritogenesis (21, 23, 24).

Neuronal differentiation of PC12 cells is associated with prolonged ERK kinase signaling, which appears to require the integration of both Ras- and Rap1-dependent pathways (3, 6), but has been shown recently to involve a more complex signaling program, including contributions from PI 3-kinase, JNK, and p38 kinase signaling cascades (2, 25, 26). To assess if Rin modulates similar signaling events, we determined whether expression of an activated Rin mutant, RinQ78L, influenced neurite outgrowth from PI 3-kinase, JNK, and p38 kinase signaling cascades (2, 25, 26). To assess if Rin modulates similar signaling events, we determined whether expression of an activated Rin mutant, RinQ78L, influenced neurite outgrowth. As shown in Fig. 1B, blockade of either p38 (10 μM SB203580) or MEK/ERK (10 μM PD98059) signaling attenuated Rin-mediated neurite outgrowth. In contrast, inhibiting either JNK (25 μM, SP600125) or PI 3-kinase (10 μM, LY294002; co-expression of AKT-KD) had no effect on RinQ78L-induced morphological differentiation (Fig. 1B). Consistent with these results, RinQ78L potently stimulated the phosphorylation of both MKK3/6 and p38 kinases in transfected PC6 cells, very modestly stimulated JNK activation, and failed to stimulate ERK5 or AKT phosphorylation (Fig. 1, C and D). Indeed, RinQ78L generated levels of phospho-p38 that were as great as those induced by either activated Ras or following NGF stimulation (data not shown). Thus, Rin-mediated neurite elongation appears to be mediated in part through activation of a MKK3/6-p38 signaling cascade.

Although previous studies have failed to demonstrate Rin-induced ERK activation (18–20), RinQ78L-mediated neurite outgrowth was sensitive to MEK inhibition (Fig. 1B), and so we next examined the activity of ERK in RinQ78L-transfected cells by Western blot analysis using phospho-specific ERK antibody. As shown in Fig. 1D, immunoblot analysis of total cell lysates from RinQ78L expressing cells did not show obvious ERK activation, although both NGF stimulation and transfection with activated H-Ras resulted in robust ERK activation (Fig. 1C, D, and E). Because Rin-mediated neurite outgrowth was sensitive to inhibition of MEK activity (Fig. 1B), we increased the sensitivity of the analysis by first immunoprecipitating total cellular ERK, followed by Western blot analysis using anti-phospho-ERK antibody. As seen in Fig. 1E, by using this enhanced assay RinQ78L was found to stimulate modest ERK activation that was clearly above that seen in vector control lysates (compare lanes 1 and 2). Moreover, pretreatment with PD98059 (a MEK inhibitor) blocked RinQ78L-induced ERK activation (Fig. 1E). Taken together, these data suggest that both p38 and ERK signaling pathways are involved in Rin-mediated neurite elongation in PC6 cells.

Rin Is Necessary for NGF-induced PC6 Cell Differentiation—It has been demonstrated in a variety of systems that sustained activation of ERK plays an essential role in neurite outgrowth (1), although recent studies indicate that p38 signaling is also necessary for neurite elongation (9). Because both NGF and activated Rin can stimulate neuritogenesis, and NGF can activate Rin (19), we next exploited the highly specific method of RNA interference (27) to examine the contribution of Rin to NGF-induced neurite outgrowth. Two different small hairpin interfering RNAs (shRNAs) (shRin99 and shRin448) for rat Rin were con-
Rin Is Required for Neuritogenesis and p38 Activation

FIGURE 2. Rin GTPase is required for NGF-induced neurite outgrowth. A, shRNA-mediated silencing of Rin GTPase expression. PC6 cells were transfected with either a control hairpin shRNA vector (shCTR), shRNA-Rin99 (shRin99), or shRNA-Rin448 (shRin448) and subjected to G418 (200 μg/ml) selection for 48 h. The lysates were then harvested and the expression levels of endogenous Rin, Rit, and Ras were analyzed by immunoblotting with anti-Rin, Rit, or Ras monoclonal antibodies. B and C. Rin is required for NGF-mediated neuritogenesis. PC6 cells were transfected with shRin99 or shCTR; neurite formation was induced with NGF (100 ng/ml), and transfected cells were enriched by drug selection (G418). At day 3 and 7 after NGF stimulation, the percentage of neurite-bearing cells, neurite numbers, length of neurite, and neurite branches were counted and calculated. The results were presented as mean ± S.D. of four experiments in triplicate. Representative micrographs from 7-day cultures are shown. D, human Rin expression is not subject to shRin99-mediated silencing. PC6 cells were transfected with 0.5 μg of pcDNA3-hRin-WT in the presence of 1.5 μg of either shCTR or shRin99, and the transfected cells were enriched by drug selection (G418). The expression levels of Rin were analyzed by immunoblotting with anti-Rin monoclonal antibody, and the actin expression served as a loading control. E, human Rin restores NGF-induced neurite outgrowth to shRin99-expressing PC6 cells. PC6 cells were transfected, and differentiation was stimulated with NGF as in D. Neurite outgrowth was analyzed on day 3 after NGF stimulation, and the percentage of neurite-bearing cells and the neurite numbers were counted. The results were presented as mean ± S.D. from two individual experiments in triplicate. EV, empty pcDNA3 vector.

Rin Is Required for NGF-induced p38, but Not ERK, Activation—NGF-mediated suppression of neurite outgrowth resulted from a blockade in NGF-mediated signal transduction. MAP kinase activation in shRNA transfected PC6 cells was monitored by immunoblotting with phosphospecific MAP kinase antibodies following ligand stimulation. As opposed to cells expressing control shRNAs, in which NGF-induced ERK and p38 activation was readily detected within 10 min and remained elevated for ~1 h following stimulation (Fig. 3A, lanes 2, 5, and 8), Rin silencing potently inhibited p38 activation in response to NGF treatment, inhibiting both the strength and duration of the kinase response. By contrast, ERK activation was not affected by Rin depletion at any time point (Fig. 3A), consistent with the pronounced stimulation of p38 by RinQ78L but only modest ERK activation (Fig. 1, C and E). Rin knockdown also resulted in a potent blockade of NGF-mediated activation of heat shock protein 27 (HSP27), which serves both a neuroprotective function and directs cytoskeletal remodeling in neurons, in a manner that is dependent on both MEK and p38 kinase activity (28). These alterations were not a consequence of changes in p38 protein expression but were as profound as those caused by treatment with the p38 inhibitor SB203580 (Fig. 3A). Thus, Rin is required for TrkA-mediated p38 signaling.

Rin Selectively Mediates NGF-dependent p38 Activation—To explore the broad contribution of Rin to neuronal signaling, we next examined the activation of endogenous MAP kinases in response to a range of cellular stimuli. Most surprisingly, although Rin has been reported to be activated by a variety of extracellular stimuli (19, 29), the effects of Rin knockdown on MAP kinase stimulation were restricted to NGF-mediated signaling. EGF-, PMA-, and Ca²⁺-mediated activation of ERK and p38 were unaffected by Rin silencing under conditions in which shRin99 potently suppressed NGF-mediated p38 activation (Fig. 3A and data not structed, whereas a shRNA with no predicted target site in the rat genome (shCTR) served as a negative control for these studies. The results of immunoblot analysis revealed that endogenous Rin expression was specifically reduced by at least 80% in cells transfected with shRin99, whereas expression of H-Ras and the highly related Rit GTPase remained elevated for ~1 h following stimulation (Fig. 3A). We first tested the effect of Rin loss on NGF-induced neurite outgrowth. As shown in Fig. 2, B and C, shRin99-mediated silencing significantly inhibited formation of NGF-dependent neurite extensions. Quantitation of transfected cells showed that Rin depletion resulted in greater than 70% inhibition of NGF-induced neurite outgrowth, inhibiting the percentage of neurite-bearing cells, neurite length, neurite branching, and neurite number per cell body (Fig. 2, B and C). This inhibition was equivalent to that induced by pharmacological blockade of p38 activity and exceeded that resulting from inhibition of MEK activity (data not shown) (21). Neither shRin448 nor shCTR disrupted neurite outgrowth, consistent with their inability to knock down Rin expression (Fig. 2A and data not shown). Because shRNAs used in these experiments are specific for the rat, we reconstituted the Rin deficiency by co-transfecting PC6 cells with wild-type human Rin and shRNA expression vectors. As expected, human Rin escaped shRin99-mediated gene silencing (Fig. 2D) and fully restored NGF-induced PC6 cell differentiation (Fig. 2E), demonstrating the specificity of the knockdown experiment. Thus, Rin silencing resulted in a profound blockade in NGF-dependent neurite outgrowth and indicated that Rin is required for neuronal differentiation.
shown). To confirm the role of Rin in NGF-induced p38 activation, shRNA-transfected PC6 cells were stimulated with NGF, EGF, PMA, or elevated Ca\(^{2+}\), and p38 kinase activity was examined by in vitro kinase assay using ATF2 as substrate (Fig. 3B). Consistent with our earlier studies, Rin knockdown inhibited p38 activity exclusively in response to NGF treatment but did not reduce p38 activation in response to these other stimuli. Taken together, these data indicate that Rin selectively facilitates NGF-dependent signal transmission to p38 MAP kinase signaling, making an essential contribution to neurotrophin-mediated signal transduction but not serving as a general regulator of p38 activity.

Isoform-specific p38 Regulation by Rin—We next examined the role of individual p38 kinase isoforms in Rin-mediated neurite outgrowth. As shown in Fig. 4A, the co-expression of a dominant-negative form of p38\(\alpha\), and more modestly p38\(\beta\) or \(\delta\), inhibited Rin\(\alpha\)-mediated neurite outgrowth. The specificity of Rin-mediated p38 kinase cascade activation in PC6 cells was confirmed by using in vitro kinase.

**FIGURE 3.** Rin is required for NGF-mediated p38 activation. A, Rin GTPase is required for NGF-mediated p38 MAPK and HSP27 activation, but it is not essential for p38 MAPK activation by other stimuli. PC6 cells were transfected with shRin99 or shCTR and enriched with G418 (400 \(\mu\)g/ml) selection for 3 days. Cells were serum-starved for 5 h prior to NGF (100 ng/ml, lanes 2–10), EGF (100 ng/ml, lanes 12–20), PMA (100 ng/ml, lanes 22–25), or A23187 (2.5 \(\mu\)M, lanes 26–29) stimulation for the indicated duration. Lysates were prepared, and the phosphorylation levels of indicated proteins were examined by immunoblot analysis with appropriate phospho-specific antibodies. Pretreatment of shCTR expressing cells with either MEK/ERK (10 \(\mu\)M, PD98059; phospho-ERK panel, lanes 4, 7, 10, 14, 17, and 20), or p38 (10 \(\mu\)M, SB203580; phospho-p38 MAPK and phospho-HSP27 panel, lanes 4, 7, 10, 14, 17, and 20) inhibitors were used as controls. B, Rin is required for NGF-induced p38 activation. PC6 cells were transfected with shCTR or shRin99 and enriched with G418 selection. Serum-starved monolayers were stimulated with NGF (100 ng/ml, 10 min), EGF (100 ng/ml, 5 min), PMA (100 ng/ml, 30 min), or A23187 (2.5 \(\mu\)M, 30 min), and kinase activity was assayed following anti-phospho-p38 immunoprecipitation using ATF2 as substrate. The level of phosphorylated ATF2 was analyzed as an indicator for p38 MAP kinase activity by immunoblotting with phospho-specific ATF2 antibody.

**FIGURE 4.** Inhibition of Rin-induced neuronal differentiation by dominant-negative p38\(\alpha\). A, p38\(\alpha\) MAP kinase activity is required for Rin-induced neurite outgrowth. PC6 cells were transfected with 1 \(\mu\)g of 3xFLAG-Rin-Q78L in the absence or presence of 1 \(\mu\)g of FLAG-tagged dominant-negative p38\(\alpha\), -\(\beta\), -\(\gamma\), or -\(\delta\) and then replated and enriched by drug selection (G418). The percentage of neurite-bearing cells and the neurite number per cell were determined at day 3 after transfection. The data were shown as mean \(\pm\) S.D. of three experiments. B, Rin GTPase activates p38\(\alpha\) MAP kinase. PC6 cells were co-transfected with 3xHA-Rin-Q78L and wild-type FLAG-p38\(\alpha\), -\(\gamma\), or -\(\delta\). 2 days after transfection, the transfected p38 isoforms were immunoprecipitated (IP) with anti-FLAG monoclonal antibody from 400 \(\mu\)g of total lysate proteins, and the anti-FLAG immunoprecipitates were subjected to in vitro p38 kinase assays as described under “Experimental Procedures.” The data shown are representative of three independent experiments. I.B., immunoblot.
assays from PC6 cells co-expressing FLAG-tagged p38 kinase isoforms and RinQ78L (Fig. 4B). As expected, RinQ78L activated p38\(_{19251}\) but did not noticeably stimulate p38\(_{19253}\) or p38\(_{19254}\) kinase activity. As a general control, we used NGF stimulation, which resulted in the activation of both p38\(_{19251}\) and p38\(_{19253}\). Taken together, these studies suggest that isoform-specific p38 MAP kinase activation is required for Rin-mediated neuronal differentiation.

**Rin Activates b-Raf to Promote Neurite Outgrowth**—Because the Raf kinases are major activators of the MEK/ERK cascade, Rin-mediated differentiation was sensitive to MEK inhibition (Fig. 1B), and the closely related Rlt GTPase demonstrates b-Raf-selective regulation (21), we next determined whether Rin associated with and activated Raf kinases. Because a vast literature has demonstrated that Raf kinases are genuine Ras effectors (30), the ability of Rin and Ras to bind and activate Raf proteins was compared. For these studies, COS cells were transiently co-transfected with plasmids expressing unfused GST, GST-RinQ78L, GST-RasQ61L, or empty Myc vector (lanes 1 and 5) in the presence of 3xFLAG-RinQ78L (lanes 3 and 7), 3xFLAG-H-RasQ61L (lanes 4 and 8), or empty 3xFLAG vector (lanes 1, 2, 5, and 6), and protein association was examined by co-immunoprecipitation (IP) using anti-Myc antibody as described under “Experimental Procedures.” The precipitated Rin or Ras was detected by immunoblotting with biotinylated anti-FLAG monoclonal antibodies, whereas the efficiency of co-immunoprecipitation and the expression of transfected Rin and Ras proteins were determined by immunoblotting with isoform-specific anti-Myc or anti-FLAG antibodies. The results are representative of three individual experiments.

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![FIGURE 5. Rin binds and activates b-Raf.](image)

**A.** Association of b-Raf and c-Raf with Rin GTPase. COS cells were co-transfected with empty GST vector (lanes 1, 2, 5, and 6), GST-RinQ78L (lanes 3 and 7), or GST-H-RasQ61L (lanes 4 and 8) plus either HA-b-Raf-WT (lanes 2–4), c-Raf-WT-FLAG (lanes 6–8), or empty Myc vector (lanes 1 and 5), and protein association was examined by GST-pull down as described under “Experimental Procedures.” The presence of Raf protein in the GST-pull down complex was detected by immunoblotting with biotinylated anti-HA or anti-FLAG monoclonal antibodies. The efficiency of GST-pull down and expression of transfected b-Raf and c-Raf were shown. The results are representative of three independent experiments.

**B.** Association of b-Raf and c-Raf. COS cells were transfected with Myc-b-Raf-WT (lanes 2–4), Myc-c-Raf-WT-FLAG (lanes 6–8), or empty Myc vector (lanes 1 and 5) in the presence of 3xFLAG-RinQ78L (lanes 3 and 7), 3xFLAG-H-RasQ61L (lanes 4 and 8), or empty 3xFLAG vector (lanes 1, 2, 5, and 6), and protein association was examined by co-immunoprecipitation (IP) using anti-Myc antibody as described under “Experimental Procedures.” The precipitated Rin or Ras was detected by immunoblotting with biotinylated anti-FLAG monoclonal antibody, whereas the efficiency of co-immunoprecipitation and the expression of transfected Rin and Ras proteins were determined using biotinylated anti-Myc or anti-FLAG antibodies. The results are representative of three individual experiments.

**C.** Rin GTPase binds endogenous b-Raf. Cell lysates (10 mg) prepared from COS cells expressing GST-RinQ78L, GST-H-RasQ61L, or GST alone were subjected to GST-pull down analysis. Pelleted c-Raf and b-Raf proteins were identified by immunoblotting with isoform-specific antibodies.

**D.** Activation of b-Raf by Rin GTPase. COS cells were co-transfected with the indicated plasmids and cell lysates (1 mg) subjected to GST-pull down. The precipitated fractions were divided equally, and Raf activity was examined by immune complex kinase assay and immunoblotting with phospho-specific Raf antibody. The efficiency of GST-pull down and equal loading of Raf to the activity assay were determined by immunoblotting with isoform-specific b- or c-Raf. The expression levels of transfected Rin or Ras GTase were analyzed by immunoblotting with anti-Rin or -Ras monoclonal antibodies.
expression vectors for FLAG-tagged activated Rin or activated Ras and Myc epitope-tagged c-Raf or b-Raf, and Raf kinase association was analyzed by anti-Myc immunoprecipitation. As seen in Fig. 5B, activated Rin and activated Ras were each co-precipitated in a Raf protein-dependent manner but were not precipitated using mouse IgG in the reaction or in the absence of co-expressed Raf protein (data not shown). Although RinQ78L consistently bound both c-Raf and b-Raf, Rin demonstrated stronger binding with b-Raf in these studies (Fig. 5A, compare lanes 3 and 7).

To confirm these interactions, and to examine further the relative binding affinity of Rin for the Raf kinases, we expressed GST-RinQ78L and GST-RasQ61L proteins in COS cells, precipitated the GST fusion proteins, and assayed for co-precipitation of endogenous Raf proteins by immunoblotting with isomor-specific anti-Raf antibodies (Fig. 5C).

More importantly, GST-RinQ78L robustly associated with endogenous b-Raf when expressed in COS cells, but was only weakly associated with endogenous c-Raf, although c-Raf is the predominant Raf isoform expressed in these cells (21). Thus, Rin preferentially associates with b-Raf.

We next examined the nucleotide dependence of Rin association and activation of b-Raf. Confirmation of the ability of Rin to selectively activate b-Raf was obtained in COS cells transiently transfected with expression vectors for the Raf proteins and wild-type, dominant-negative (RinS34N), constitutively active Rin (RinQ78L) or an empty control vector (Fig. 5D). Activated H-Ras, which is known to activate both Raf isoforms, was used as a positive control for these studies. Lysates normalized for levels of exogenously expressed GST-Raf proteins were isolated on glutathione affinity resin and either assayed using a phospho-
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**DISCUSSION**

Rin is well suited to serve as a regulator of neurotrophin-mediated signaling. The Rin GTPase is expressed exclusively in the nervous system, is activated by neurotrophic signaling, and remains GTP-bound for an extended period of time (19), consistent with a role in controlling a prolonged neurotrophin-mediated response. To clarify the role of Rin in neuronal signaling, we initiated experiments to determine whether Rin was necessary for NGF-dependent neuronal differentiation. In earlier studies we had shown that a dominant-negative mutant of Rin blocks NGF-mediated neurite outgrowth in PC6 cells (19), but we found that the expression of constitutively active GFP-Rin fusion protein failed to promote neurite outgrowth. Recently, Hoshino and Nakamura (20) reported that N-terminal GFP fusions disrupt Rin function. In agreement with these studies, we show here that expression of activated FLAG-tagged Rin induces ligand-independent neurite outgrowth in PC6 cells (1) and, more importantly, that shRNA-mediated Rin silencing potently inhibits NGF-stimulated neuronal differentiation (Fig. 2). These data strongly argue that Rin is necessary for NGF-mediated differentiation. Thus, Rin joins a select group of small molecular weight GTP-binding proteins with a demonstrated role in neuronal differentiation (1, 32). Moreover, our data suggest that Rin plays a distinct signaling function. Although regulation of sustained ERK signaling appears central to the contribution of previously identified Ras subfamily GTPases, including Ras, R-Ras3, and Rit (21, 33, 34), Rin predominately regulates p38 MAP kinase signaling. These results indicate that sustained p38 activation plays an essential role in NGF-dependent neuronal differentiation, in agreement with a variety of recent studies (9).

ERK is regarded as the major cellular target of Raf kinases, and the...
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strength and duration of ERK activity can determine the specificity of biological responses, including neuronal differentiation (1, 31). Earlier analysis of Rin function in NIH 3T3 (18), PC12 (20), and PC6 cells (19, 23) has failed to demonstrate Rin-mediated MAP kinase activation. In part, this is because of our use of GFP-tagged Rin proteins, in which the GFP fusion appears to disrupt Rin-mediated signaling (data not shown and see Ref. 20). In this study, we find that FLAG-tagged Rin utilizes the neuronal specific Raf isoform, b-Raf, to promote modest ERK stimulation and that Rin-induced p38 activation relies, at least in part, upon b-Raf-MEK signaling (Figs. 1 and 4–6, and data not shown). Thus, Rin may activate ERK, and perhaps p38, only in cell types that express b-Raf, such as neurons. Recently Hoshino and Nakamura (20) reported that RinQ78L fails to activate ERK in PC12 cells using phospho-specific ERK immunoblotting. This discrepancy may be because of the difference in the cell lines. However, Rin promotes only modest ERK activation in PC6 cells, and to circumvent this problem, we found that precipitation of total ERK protein was needed to increase the sensitivity of the immunoblotting method (Fig. 1F). More importantly, Hoshino and Nakamura (20) did not examine the ability of Rin to regulate p38 signaling in PC12 cells.

Our findings support a model in which Rin participates in a neurotrophin-regulated signalosome, the integrity of which is required for efficient NGF-mediated b-Raf phosphorylation and activation of the p38 signaling cascade. The association of Rin with NGF-mediated b-Raf and p38 activity (Figs. 5 and 6) suggests that Rin could either directly aid in kinase activation, modulate cellular localization, or serve to recruit needed regulatory factors. The organization of higher order molecular complexes by scaffolding proteins is one mechanism known to confer specificity to MAP kinase signaling (35). Whether Rin signaling relies upon such proteins remains an important challenge for the future. The Rin–b-Raf signaling module might also provide for efficient activation of a p38 regulator, such as MKK3/6, to couple NGF stimulation to prolonged p38 MAP kinase activation (Fig. 6). More importantly, as seen with Rin knockdown in PC6 cells (Fig. 3), b-Raf-deficient neurons demonstrate normal ERK activation following neurotrophin exposure but do not respond to neurotrophic factors for their survival (36). Thus, ERK signaling alone appears insufficient to mediate the survival effect of neurotrophins in primary neurons or neurite outgrowth of PC12 cells (9). Although p38 has been conventionally thought of as a mediator of neuronal cell death, recent studies suggest that p38 signaling may also play a neuroprotective role (9). In addition, disruption of p38 kinase signaling inhibits neurite outgrowth in several systems (8, 11, 37, 38), indicating that p38 signaling plays a central role in both neural differentiation and survival. We speculate that b-Raf may serve a dual signaling function in neurons, activating the MEK–ERK cascade via regulation by Ras, Rit, and potentially Rap1 GTPases (21, 33, 34), although also collaborating with Rin to activate p38 signaling. Because these MAP kinase pathways share common downstream targets but also diverge markedly via distinct cellular targets (39), these data indicate that NGF-mediated b-Raf activation generates integrated and cooperative signaling involving both ERK and p38 MAP kinase cascades to exert its cellular effects. Interaction would allow functional cross-talk between these distinct MAP kinase pathways, which may be critical for both signal integration and to modulate the biological outcome. The ability to serve as a dual modulator of both ERK and p38 kinase signaling may allow Rin to control various aspects of neuronal function while also monitoring the extent of extracellular stress for maintenance of the nervous system. Thus, a well balanced activation of the ERK and p38 pathways may be necessary for neuronal differentiation and may explain why sustained ERK and p38 MAP kinase responses are associated with neurotrophic signaling cascades (1).

Rin initiates cytoskeletal changes, neurite elongation, and differentiation when expressed in PC6 cells, utilizing biochemical signaling routes distinct from those activated by other Ras-related GTPases. Specifically, available evidence suggests that the p38α but not JNK or PI 3-kinase/Akt pathways are required for Rin-dependent neuronal differentiation (Figs. 1, 4, and 6). Why Rin activates only p38α is not known, as MKK3 and MKK6 have been reported to act upstream of all p38 MAP kinase family members (40). Recently, Rit has been shown to promote isoform-specific p38γ activation and that MKK3/6-p38γ signaling contributes to Rit-mediated NIH 3T3 transformation (41). Moreover, NGF stimulation of PC6 cells results in activation of p38α but not p38β (Fig. 4A). Thus, both Rit and Rin appear to demonstrate isoform-specific p38 MAP kinase regulation and together would be expected to recapitulate NGF-mediated p38 kinase activation. It will be important to examine the contribution of individual p38 kinase isoforms to neurotrophin-mediated differentiation. Additional regulatory factors, such as the expression of p38-specific phosphatases or the existence of scaffolding molecules directing the activation of specific kinase modules, may help explain the specific activation of p38α by Rin. In addition, although we present evidence to support a role for b-Raf and MEK in Rin-mediated p38 activation, the molecular machinery that links Rin and p38 in neurons remains largely uncharacterized. Recently, RinQ78L has been shown to stimulate Rac/Cdc42 activation (20). This is of interest because of the known roles for these Rho family GTPases in regulating both p38 and ERK signaling through a Rac/Cdc42-PAK signaling module (42–45). The possibility that these proteins contribute to Rin-mediated signaling is currently under investigation.

There are multiple downstream targets for p38α with known roles in differentiation and survival signaling in the central nervous system (9). Potential p38 signaling effectors include transcription factors, such as cAMP-responsive element-binding protein and MEF2, cytoskeleton modulators (46), and a number of protein kinases (9). On the other hand, expression of activated p38 alone does not result in neurite outgrowth (10). Thus, we can speculate that neurite elongation may require co-stimulation of p38 and ERK signaling. The effect of converging MAP kinase pathways on neuronal differentiation remains to be addressed. Whether such signaling results in the coordinated activation of ERK and p38-specific targets or the actions of effector proteins that are regulated downstream of both kinase cascades and therefore only activated during co-stimulation remains to be addressed. It is possible that factors such as MNK1/2, MSK1/2, and cAMP-response element-binding protein, which are co-regulated by ERK/p38 signaling and participate in neurotrophin-regulated differentiation, survival, and synaptic plasticity in the central nervous system, may serve as critical intermediates in the integration of neurotrophic signaling required for generating an appropriate biological response (9, 47). In this regard, the ability of activated Rin to stimulate HSP27 is intriguing (Fig. 6F). HSP27 directs both cytoskeletal remodeling and neuronal survival (48). Further work will be needed to fully elucidate the nature of the signaling events regulated by Rin, as well as to unravel the complexity of the molecular mechanisms whereby this GTPase may promote neuronal differentiation.

In summary, the loss-of-function analysis presented here directly implicates Rin as a key participant in NGF receptor–specific signal propagation. The marked effect of Rin knockdown on NGF-induced p38 activation, but not on activation of other MAP kinases, or disruption of MAP kinase signaling mediated by EGF, calcium, or protein kinase C suggests that Rin provides a function to neurotrophic signaling that cannot be compensated for by other Ras family GTPases. Rin directly
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interacts with and activates b-Raf and might therefore function as an essential component of a neuronally specific b-Raf signaling complex, in which Rin provides spatial and/or substrate specificity to the b-Raf-MEK kinase cascade to direct stimulation of p38 signaling. Sustained p38 MAP kinase signaling appears crucial for diverse neuronal function, including induction of long term depression of synaptic transmission, differentiation, and survival (1, 9, 47, 49–53). Thus, the Rin signaling module may be an important general regulator of neurotrophic dependent cellular processes. How prolonged p38 activation contributes to differentiation is an important issue that remains to be addressed. The identification of p38 kinase target molecules in Rin/b-Raf-signaling should provide insight into neuronal regulation and development.

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