Pituitary Adenylate Cyclase-Activating Polypeptide 38-Mediated Rin Activation Requires Src and Contributes to the Regulation of Hsp27 Signaling during Neuronal Differentiation

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Pituitary adenylate cyclase-activating polypeptide 38 (PACAP38) is a potent neuropeptide that acts through G-protein-coupled receptors. While it is well established that PACAP mediates both neurotrophic and neurodevelopmental effects, the signaling cascades that underlie these diverse actions remain incompletely characterized. Here we show that the Ras-related Rin GTP-binding protein, a GTPase that is expressed predominantly in neurons, is regulated by PACAP38 signaling, and loss-of-function analysis demonstrates that Rin makes an essential contribution to PACAP38-mediated pheochromocytoma cell differentiation. Rin is activated following stimulation of both Gαs and Gαi cascades but does not rely upon cyclic AMP (cAMP)-, Ca2+-, or Epac-dependent signaling pathways. Instead, Rin is activated in a Src kinase-dependent manner. Surprisingly, Rin knockdown significantly inhibits PACAP38-mediated neurite outgrowth, without affecting mitogen-activated protein kinase signaling cascades. Instead, Rin loss attenuates PACAP38-mediated HSP27 activation by disrupting a cAMP-protein kinase A cascade. RNA interference-mediated HSP27 silencing suppresses both PACAP38- and Rin-mediated neurite outgrowth, while expression of a constitutively active Rin mutant increases both HSP27 protein and phospho-HSP27 levels, supporting a role for Rin-HSP27 signaling in neuronal differentiation. Together, these observations identify an unsuspected role for Rin in neuronal PACAP signaling and establish a novel Gαs-Src-Rin-HSP27 signal transduction pathway as a critical element in PACAP38-mediated neuronal differentiation signaling.

The neuropeptide pituitary adenylate cyclase-activating polypeptide 38 (PACAP38) is widely expressed within the nervous system and is a member of the vasoactive intestinal peptide/secretin/glucacon polypeptide family (73, 77). PACAP38 binds and activates G-protein-coupled receptor (GPCR) family members to regulate a number of nerve cell functions, including differentiation, axonal and dendritic growth, and cell survival (79). In pheochromocytoma 12 (PC12) cells, PACAP38 exposure results in differentiation characterized by neurite elongation (13, 41, 63). Although a comprehensive understanding of the signaling network required to promote neuritogenesis following PACAP38 receptor stimulation is lacking, activation of adenylate cyclase and regulation of both extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein (MAP) kinase cascades have been shown to play critical roles (19, 35, 63, 72). While cyclic AMP (cAMP) signaling has classically been associated with activation of protein kinase A (PKA) and while cAMP analogues can induce PC12 cell differentiation (24), the neurotrophic effects of PACAP38 do not rely solely upon PKA signaling. Recent work has identified the cAMP-activated Epac guanine nucleotide exchange factors as crucial mediators of PKA-independent cAMP signaling (8, 67). Epac proteins are required for cAMP-mediated differentiation signaling (35), serving to link Gαs-coupled receptor signaling to both ERK and MAP kinase activation in neuronal cells (48), and for the activation of a second Rit-p38 MAP kinase signaling pathway (72). Thus, the cell-type-specific actions of cAMP are the result of both PKA-dependent and PKA-independent signaling cascades.

Recent studies have identified the Rin small GTP-binding protein as an important mediator of nerve growth factor (NGF)-dependent neuronal differentiation (30–32, 43, 68, 71, 74, 80). Rin is highly expressed in neurons and shares a conserved and unique effector domain with the closely related Rit and Drosophila Ric proteins (43, 68, 80). Rin fails to undergo posttranslational lipidation, a modification required for the association of the majority of Ras proteins with cellular membranes. Instead, Rin contains a conserved polybasic C-terminal domain that was recently shown to direct interactions with phosphatidylinositol lipids (27). We have shown that Rin is activated following NGF stimulation in pheochromocytoma cells and that Rin signaling plays a critical role in NGF-mediated neuronal differentiation (71, 74).

It is well established that neurotrophic factors act through cell surface receptors to activate often convergent signaling cascades to promote both neurite outgrowth in pheochromocytoma cells and elongation of axons and dendrites in neurons (64). Among the targets of these pathways are regulators of cytoskeleton dynamics, particularly Rho family GTPases (22). Another class of regulatory factors is the small heat shock proteins, including heat shock protein 27 (HSP27). HSP27 has reported roles in the regulation of apoptosis and in protein folding and interacts with both actin and tubulin (1, 2, 23, 39,
61). Phosphorylated HSP27 acts to promote actin polymerization and stress fiber formation and stabilizes the actin cytoskeleton (4, 23, 39, 40), while nonphosphorylated HSP27 inhibits in vitro actin polymerization (4, 56). HSP27 expression is developmentally regulated in dorsal root ganglion neurons, is up-regulated in response to nerve injury and hyperthermia (11, 29, 37, 46, 78), and contributes to axonal outgrowth (81, 82). Indeed, missense mutations of HSP27 are associated with peripheral neuropathies (17).

In this report, we investigated the molecular events that mediate PACAP38-induced differentiation and the role of the Rin GTPase in this process. We demonstrate that Rin is activated by PACAP38 signaling, in a manner that depends upon a pertussis toxin (PTX)-sensitive Gα subunit signaling pathway but does not involve CaMP-, Ca2+, or Epac-dependent signaling pathways. Instead, Rin activation is dependent upon a Gso/Gio-mediated Src kinase signaling cascade. Importantly, Rin silencing blocked PACAP38-mediated neurite outgrowth, without altering either ERK or p38 MAP kinase signaling, but strongly inhibited HSP27 activation. Furthermore, expression of constitutively active Rin (RinQ78L) promoted increased phosphor-HSP27 levels and resulted in a threefold increase in endogenous HSP27 protein levels, while HSP27 silencing inhibited both PACAP- and Rin-mediated neuritogenesis. Moreover, Rin knockdown attenuated PACAP38-induced cAMP production, and both RinQ78L-mediated neurite outgrowth and HSP27 phosphorylation were found to be cAMP- and PKA-dependent processes. Together, these observations indicate that PACAP38-mediated neuronal differentiation relies upon a novel Rin-HSP27 signaling cascade.

MATERIALS AND METHODS

Plasmids and reagents. Epitope-tagged mouse and human Rin, Rap1A, and their mutants were genetically engineered as described previously (70–72). The expression vectors for Gαq2Q240L (QL), GqaQ2Q240L (QL), GsoQ229L (QL), dominant-negative e-Src (DN-Src) [pUcse-e-Src (K296R/Y258F)], and constitutively active e-Src (CA-Src) [pUcse-e-Src (Y529F)] were kindly provided by J. H. Kehrl (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) (69). Site-directed mutagenesis was used to generate Src mutants [pUcse-e-Src (S17A) and pUcse-CA-e-Src (S17A)]. The mutants were subsequently confirmed by sequencing. Constitutively active Epac2 (Epac2ACBR) was kindly provided by L. Quilliam (Indiana University School of Medicine, Indianapolis, IN) (47) and includes residues 430 to 994 of the full-length protein. PACAP38 was synthesized by Bachem California (Torrance, CA). Antibodies against the following were purchased: Flag (Sigma, St. Louis, MO); phospho-specific ERK1/2, phospho-specific p38, phospho-specific HSP27, p38 MAPK, and HSP27 (Cell Signaling, Beverly, MA); and ERK1/2 and actin (Santa Cruz Biotechnology, Santa Cruz, CA). The MEK1/2 inhibitor PD98059, the Src kinase inhibitor PP2 and its inactive analogue (PP3), 2',5'-dideoxyadenosine (ddA), H89, the inactive cAMP analog adenine 3',5'-cyclic monophosphorothioate, Rp isomer (Rp-cAMP), 8-bromo-adenosine-3',5'-cyclic monophosphate (8-Brop-cAMP), 8-(p-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8-CPPT-2-Me-cAMP), and cholera toxin (CTX) were purchased from Calbiochem, La Jolla, CA; the p38 MAP kinase inhibitor SB203580 was purchased from Tocris, Ellisville, MO; and PTX, A21387, potassium chloride (KCl), and forskolin were purchased from Sigma, St. Louis, MO. Gi1/2a Gq-switching phosphatases were prepared from bacteria expressing Gi1/2a-Raf-Ras interacting domain (GST-Raf-RID) and glutathione-agarose beads were prepared from bacteria expressing GST-Raf-RID as described previously (65).

Cell lines, cell cultures, and transfections. PC6 is a subline of PC12 cells that produces neurites in response to NGF but grows as well-isolated cells rather than in clumps (the generous gift of T. Vanaman, University of Kentucky, Lexington). The cells were maintained and transfected with Effectene (Qiagen) as described previously (71, 74).

RNA interference. The mammalian expression vector pSUPER-GFP/Neo (OligoEngine) was used for expression of small interfering RNA (siRNA) in PC6 cells. The vector allows direct synthesis of siRNA transcripts by use of the RNA polymerase H1 promoter and coexpresses green fluorescent protein (GFP) to allow detection of transfected cells. The gene-specific insert sequence of rat p38 MAPK (GACAAGAAGGCGCTGTGTGG [target sense]), which was separated by a 9-nucleotide noncomplementary spacer (TTCAGAAGA) from the reverse complement of the same HSP27-specific 19-nucleotide sequence, was synthesized and then subcloned into the BglII and HindIII sites of pSUPER-GFP/Neo to generate pSUPER-shHSP27-344 (shHSP27-344). Other rat target sense sequences used in this study included shHSP27-560 (TTTCAAGGCCCCGTGCGCC AA), shPACR1-384 (TCATCTCGAGTTGTTGGG), and shRin99 (AGGCC AGTGCATACATGACGT) (71). The resulting constructs were verified by DNA sequencing. A siRNA with no predicted target site in the rat genome (Scramble) was inserted into the same sites in pSUPER-GFP/Neo to generate pSUPER-Scramble (shScramble) and served as a negative control (71). To reconstitute the Rin deficiency in PC6 cells, PC6 cells expressing shRin99 or shCTR (1.5 μg) were cotransfected with 0.2 μg of pCDNA1.1-hrin-WT. To determine the effects of shHSP27 on the expression of endogenous HSP27 proteins, PC6 cells were transfected with 1.5 μg of shHSP27-344, shHSP27-560, or shCTR as a control. siRNA-transfected cells were enriched by G418 selection (400 μg/ml) for 60 h, and total cell lysates were prepared with kinase lysis buffer and subjected to immunoblotting with either anti-Rin or anti-HSP27 antibodies to determine the expression levels of overexpressed human Rin (hrin) or endogenous HSP27. Levels of actin in the lysates were monitored to demonstrate equal loading. To determine the efficacy of shPACR1-384 treatment, total RNA was isolated, using an RNasey Mini kit (Qiagen), from PC6 cells transfected with shCTR or shPACR1-384 (1.5 μg) for 60 h. Total RNA (2 μg) was subjected to reverse transcription by use of an Omniscript reverse transcription kit (Qiagen) and the following primers used for PCR analysis: for rat PACR1, GCTGGCCCGCTCCACCTACT and TCAGGTTGG CCAAGGTGCGGGCC (358 bp); and for rat β-actin, GTTGAGACCTTCA CACCC and ATACCTCTGGTGGTACCTE (642 bp).

Neurite outgrowth. To determine the requirement of Rin for PACAP38-mediated neurite outgrowth, PC6 cells were transfected with shRin99 or shCTR after pretreatment with or without SB203580 (10 μM) or PD98059 (10 μM), using Effectene (Qiagen), and then replated at a 1:4 dilution and exposed simultaneously to PACAP38 (5 nM) to initiate differentiation and to G418 selection to eliminate untransfected cells. On days 3 and 7 after PACAP exposure, cells were fixed with methanol-acetone (3:1), and images of random fields were captured on an Axiosvert 200 M phase-contrast microscope (Zeiss), using a ×20 objective lens and OpenLab 5.1.4 imaging software. We analyzed the percentage of neurite-bearing cells, neurite number per cell body, neurite length, and number of branch points per neurite in three separate experiments, as described previously (70–72). To determine whether the shRin99-mediated differentiation block could be recovered by the reintroduction of exogenous Rin, PC6 cells were transfected with 0.2 μg of empty pcDNA3.1 vector or pcDNA3.1-hrin-WT in the presence of 1.5 μg of shRin99 (see below). Twenty-four hours after transfection, total RNA (2 μg) was subjected to reverse transcription by use of an Omniscript reverse transcription kit (Qiagen) and the following primers used for PCR analysis: for rat RinG, GCTGGCCCGCTCCACCTACT and TCAGGTTGG CCAAGGTGCGGGCC (358 bp); and for rat β-actin, GTTGAGACCTTCA CACCC and ATACCTCTGGTGGTACCTE (642 bp).

To examine the requirement for Src and PKA signaling for PACAP38-mediated neuronal differentiation, PC6 cells seeded at a low density (1 × 105/cm2) were pretreated with P2P (10 μM), PP3 (10 μM), H89 (10 μM), Rp-cAMP (50 μM), or dimethyl sulfoxide (DMSO) before initiation of neurite outgrowth by PACAP38 (10 nM), and the percentages of neurite-bearing cells were analyzed on days 3 and 7 as described above. To determine the requirement for HSP27 in Rin-mediated neurite outgrowth, PC6 cells were cotransfected with either 3×Flag-Rin-Q78L or empty 3×Flag vector and either shCTR, shHSP27-344, or shHSP27-560 (all vectors were used at 1.0 μg). To examine the role of HSP27 in PACAP38-mediated neurite outgrowth, PC6 cells expressing either shCTR, shHSP27-344, or shHSP27-560 were exposed to PACAP38 (10 nM) as indicated, while untransfected treated cells were used as a negative control for these studies. To explore the signaling pathways in Rin-mediated neurite outgrowth, PC6 cells were transfected with 3×Flag-Rin Q78L (1.5 μg) after pretreatment with H89 (10 μM), Rp-cAMP (50 μM), P2P (10 μM), PP3 (10 μM), or DMSO vehicle and then replated at a 1:4 dilution with complete Dulbecco’s modified Eagle’s medium (DMEM) containing G418 (400 mg/ml) in the presence of the appropriate inhibitors or vehicle as a control.

MAP kinase assay and immunoblotting. PC6 cells seeded in six-well plates were transfected with shRin99 or shCTR as a control and then subjected to G418 selection for 48 h to remove untransfected cells. The remainder of the data was generated from RNA (shRNA)-expressing cells that were starved for 5 h before stimulation with PACAP38 (5 nM) for the indicated duration. The phosphorylation levels of ERK1/2, p38 MAPK, and HSP27 (Ser 82) were determined by immunoblotting with phosphorys-specific antibodies. To determine the signaling pathways involved...
in Rin signaling, PC6 cells were pretreated with H89 (10 μM), Rp-cAMP (50 μM), P2P (10 μM), PP3 (10 μM), or DMSO vehicle for 30 min before transfection with 3×Flag-Rin-Q76L and then were subjected to G418 (400 mg/mL) selection for 48 h. Cells were then starved for 5 h and analyzed by immunoblotting. To determine the signaling pathways that contribute to PACAP38 signaling, PC6 cells seeded at a density of 5 × 10^5/cm² were allowed to grow for 48 h, subjected to serum starvation for 5 h, and subsequently treated with P2P (10 μM), PP3 (10 μM), PTX (100 ng/mL), ddA (50 μM), Rp-cAMP (50 μM), or DMSO vehicle for 30 min prior to stimulation with PACAP38 (10 nM) for 20 min. NGF stimulation (100 ng/mL for 15 min) was used as a positive control.

Immunoblots were blocked in 1% casein (Sigma) in phosphate-buffered saline (PBS) supplemented with 0.1% Tween 20 (PBST) for 1 h at 25°C and then incubated with an appropriate dilution of the primary antibody in 1% casein or 5% bovine serum albumin in PBST for 1 h. The immunoblots were washed three times with PBST before the addition of a horseradish peroxidase (HRP)-conjugated secondary antibody (Zymed Laboratories Inc., San Francisco, CA) diluted 1:20,000 in 1% casein in PBST. The signal was detected by chemiluminescence (SuperSignal West Pico system; Pierce, Rockford, IL) as described previously (70–72). Stripping and reprobing of immunoblots to ensure equal expression of recombinant proteins were performed as described previously (71, 72).

cAMP assay. cAMP assays were performed using a cAMP direct immunoassay kit (BioVision, Mountain View, CA) following the manufacturer’s instructions. In brief, transfected PC6 cells were washed once with 1× PBS, incubated with 500 μL of 0.1 N HCl for 20 min at room temperature, and removed from the plate by repeated pipetting to achieve a uniform suspension. The collected fractions were centrifuged (13,000 rpm) at room temperature for 10 min, and the supernatants were transferred to fresh tubes. The resulting cell supernatants were neutralized with neutralizing buffer (50 μL) and acetylated at room temperature for 10 min, and assay buffer (845 μL) was added to each sample. Standard cAMP provided by the manufacturer was serially diluted with 0.1 N HCl to concentrations from 100 to 1.5625 fmol/50 μL and then neutralized and acetylated as described above to generate a standard curve. Acetylated standard (50 μL) or test samples (50 μL) were added to a Protein A-coated assay plate, assay buffer (10 μL) containing anti-cAMP polyclonal antibody was added to each well (except for the 0-fmol standard), and the mixtures were incubated for 1 h at room temperature with agitation. Diluted cAMP-HRP (10 μL) was then added to each well, and the mixtures were incubated for a second time for 1 h at room temperature with agitation. Wells were washed five times with assay buffer (200 μL) before the addition of HRP developer (100 μL). Reaction mixtures were finally incubated for 1 h at room temperature with agitation, and reactions were stopped by the addition of 1 N HCl (100 μL) and read immediately at 450 nm, using a microplate reader (Bio-Rad). The cAMP levels were calculated using the cAMP standard curve after subtraction of the background reading (0-fmol cAMP well result). Results are represented as changes in induction, calculated by dividing the cAMP amount in experimental wells by that in the negative control wells. To determine the signaling pathways that contribute to PACAP38-mediated cAMP activation, PC6 cells expressing 3×Flag-Rin-WT, with or without DN-Src (0.5 μg), were stimulated with PACAP38 (10 nM), cotransfected with CA-Src (20, 50, or 100 ng), and the amount of GTP-bound Rin was determined. To determine the requirement for Src in PACAP38-mediated Rin activation, PC6 cells expressing 3×Flag-Rin-WT were cotransfected with either shCTR or shPACR1-384 (1.5 μg) and then stimulated with PACAP38 (10 nM) for 10, 30, or 60 min before GTP-bound Rin levels were determined by pull-down assay. All experiments were repeated three to six times.

RESULTS

Role for Rin in PACAP38-mediated neurite outgrowth. PACAP38 activates PACR1 to regulate a complex signaling network to promote neurite outgrowth in pheochromocytoma cells (19, 28, 41, 63), including activation of the Rit GTPase (72). Since we have shown that Rin is rapidly activated following NGF stimulation of PC6 cells (a PC12 subline) and that Rin silencing inhibits NGF-induced neurite elongation by attenuating p38 MAP kinase activation (71), we wished to examine the requirement for Rin signaling in PACAP38-mediated differentiation. To directly assess whether Rin signaling is involved in PACAP38-mediated neurite outgrowth, we used siRNA-mediated RNA interference (16) to selectively inhibit the expression of endogenous Rin. We previously developed the short interfering RNA (siRNA) shRNA shRin99 and demonstrated that it potently and specifically reduces rat Rin protein levels >80% when transfected into PC6 cells, whereas a control shRNA with no predicted target in the rat genome (shCTR) has no effect on Rin expression (71). As shown in Fig. 1, shRin99-induced silencing potently inhibited PACAP38-dependent neurite outgrowth, resulting in a >75% reduction in the percentage of PACAP38-stimulated cells displaying neurites. Rin silencing also resulted in decreased neurite length (64%), neurite branching (58%), and neurite number per cell body (63%) (Fig. 1A). Surprisingly, this inhibition was even greater than that induced by pharmacological blockade of either MEK/ERK (10 μM PD98059) (22.8% reduction of neurite-bearing cells at day 7) or p38 (10 μM SB203580) MAP kinase (20.9% reduction of neurite-bearing cells at day 7) signaling (Fig. 1A). Treatment of shRin99-transfected cells with either PD98059 or SB203580 had no significant additional inhibitory effect on neuritogenesis.
(Fig. 1A). However, the same inhibitors resulted in a significant decrease in neurite outgrowth in PACAP38-stimulated shCTR-transfected PC6 cells, indicating that both MEK/ERK and p38 MAP kinase cascades are required for PACAP38-mediated neurite outgrowth. To control against nonspecific cellular effects, we reconstituted the Rin deficiency by cotransfecting PC6 cells with wild-type (WT) hRin and shRNA expression vectors. As expected, hRin escaped shRin99-mediated gene silencing (Fig. 1C) and restored PACAP38-mediated PC6 cell differentiation (Fig. 1D). Taken together, these data suggest that Rin plays a central role in PACAP38-dependent neuronal differentiation.

PACAP38 activates Rin in a Gs/β3-mediated but cAMP-independent manner. We reasoned that if Rin serves as an important mediator of PACAP38-mediated signaling, it must be activated following PACAP38 stimulation. To examine Rin activation, we utilized a GST fusion protein containing the RID of the Raf kinase in a pull-down assay to monitor the cellular GTP-binding status of Rin following PACAP38 stimulation (74). PC6 cells transiently transfected with Flag-tagged Rin were incubated in serum-deficient medium for 5 h, and pull-down experiments were performed on cell lysates prepared at various times following PACAP38 stimulation. Although Rin protein levels were constant (Fig. 2A) and serum-starved PC6 cells contain barely detectable levels of GTP-Rin, PACAP38 stimulation led to a rapid increase in the level of GTP-Rin. Activation of Rin was detected within 2 min following PACAP38 stimulation and had begun to return to basal levels within 60 min (Fig. 2A).

In PC12 cells, PACAP38 has been shown to activate PACR1 and to promote neuronal differentiation (63). While PACR1 activates a number of signaling pathways, including the phospholipase C and phosphatidylinositol 3-kinase pathways, and stimulates L-type Ca2+/H11001 channel activation (77), it predominantly activates the Gs/β3-adenylate cyclase-cAMP signaling pathway. To confirm that the effects of PACAP38 were PACR1 dependent, we developed an shRNA vector (shPACR1-384) which resulted in a partial knockdown of endogenous PACR1 in transiently transfected PC6 cells (Fig. 2B) and impaired PACAP38-mediated Rin activation (Fig. 2C). Consistent with a role for Gs/β3 signaling in PACAP38-mediated Rin activation, cells expressing a GTPase-deficient activated mutant of Gs/β3 (Gs/β3Q227L) resulted in dose-dependent Rin activation (Fig. 2D and E). Furthermore, treatment of PC6 cells with CTX, which is a known activator of Gs/β3 proteins (10), increased GTP-Rin in a time-dependent fashion (Fig. 2E). Surprisingly, CTX-mediated Rin activation was not inhibited by the direct adenylate cyclase inhibitor ddA (50 μM).
Moreover, addition of 8-Br-cAMP, a nonhydrolyzable cAMP analog, did not result in Rin activation (Fig. 2D). We have recently shown that PACAP38-mediated Rit activation involves a cAMP-Epac signaling pathway (72). To confirm that a similar pathway was not involved in Rin activation, we examined the ability of the membrane-permeating Epac-selective cAMP analog 8-CPT-2'Me-cAMP, expression of CA-Epac2, and either activated Rap1A (a known cellular target of the Epac guanine nucleotide exchange factors) (7) or coexpressed CA-Epac2 and Rap1A to activate Rin. As illustrated in Fig. 3A, elevated Epac-Rap1 signaling did not promote Rin activation. Taken together, these data suggest that Gs-mediated Rin activation occurs in a cAMP/Epac-independent manner.

Both Gsα and Gsα signaling pathways contribute to PACAP38-mediated Rin activation. Because PACR1 signaling has been shown to stimulate Ca2+-dependent events (63, 77) and previous work has suggested a role for Ca2+-calmodulin in Rin regulation (31), we next examined the contribution of Ca2+ to PACAP38-dependent Rin activation. PC6 cells transiently transfected with Flag-tagged WT Rin were incubated in (Fig. 2E). Moreover, addition of 8-Br-cAMP, a nonhydrolyzable cAMP analog, did not result in Rin activation (Fig. 2D).

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pressed PACAP38-mediated Rin activation, while expression of an activated Src (CA-Src [Src(Y529F)]) mutant alone resulted in strongly elevated Rin-GTP levels in PC6 cells (Fig. 5A). Furthermore, treatment with the Src inhibitor PP2 (10 μM), but not with the inactive PP3 isomer (10 μM), potently inhibited PACAP38-mediated Rin activation (Fig. 5B). Src inhibition also disrupted PACAP38-dependent neurite elongation (Fig. 5C). However, PP2 treatment did not inhibit RinQ78L-mediated (Fig. 5D) neurite outgrowth, suggesting that Src functions as an upstream regulator of Rin.

Recent studies suggest that PKA-dependent phosphorylation of Src at residue serine 17 (S17) plays a critical role in Rap1 GTPase activation in response to both elevated cAMP in a wide range of cells and NGF stimulation of PC12 cells (60). To determine whether a similar mechanism might contribute to PACAP-mediated Rin activation, we generated a Src mutant incapable of being phosphorylated by PKA (SrcS17A). As shown in Fig. 5E, expression of SrcS17A had no effect on PACAP38-dependent Rin activation. Furthermore, expression of an activated SrcS17A mutant (CA-Src[Y529F/S17A]) resulted in strongly elevated Rin-GTP levels (Fig. 5E). Taken together, these data indicate that PKA-mediated Src phosphorylation is not required for PACAP38-dependent Rin activation.

Loss of Rin does not alter MAP kinase signaling but downregulates HSP27 phosphorylation. Since PACAP38-mediated neurite outgrowth is associated with sustained activation of MAP kinase cascades (19, 63), we next examined whether siRNA-mediated Rin knockdown would alter PACAP38-induced MAP kinase signaling. Kinase activation in shRin99- or shCTR-transfected PC6 cells was monitored by immunoblotting with phospho-specific antibodies following PACAP38 stimulation. Surprisingly, Rin silencing had no obvious effect on PACAP38-mediated ERK or p38 MAP kinase activation (Fig. 6A), and a coupled kinase assay was used to confirm that p38 signaling did not rely upon Rin (Fig. 6B). However, Rin silencing inhibited activation of HSP27, a protein with known roles in both neurite outgrowth and survival (3, 81), in response to PACAP38 treatment (Fig. 6A). This alteration was not a consequence of reduced HSP27 levels, since protein levels remained constant during these studies (Fig. 6A). These data suggest that Rin silencing may contribute to PACAP38-mediated neurite outgrowth, in part through regulation of HSP27 activation.

Src is required for PACAP38-mediated Rin activation and neurite outgrowth. Recent studies indicate that Gia and Gso activate a Src-dependent signaling pathway to regulate both neurite outgrowth and Rap1 activation (60), and they are known to be stimulated in PACAP38-treated PC12 cells (41, 44). To determine whether PACAP38-mediated Rin activation involves a similar pathway, we next examined whether Rin was regulated in a Src-dependent manner. Consistent with a role for Src signaling in PACAP38-mediated Rin activation, expression of a dominant inhibitory Src mutant (DN-Src [SrcK296R/Y528F]) sup-

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The ability of Rin to modulate cellular cAMP levels that Rin contributes to PACAP38-mediated cAMP signaling. Production (Fig. 8A). Taken together, these results suggest that regulation of a cAMP-PKA-HSP27 signaling cascade might contribute to neuritogenesis. Consistent with this proposed signaling pathway, treatment with the known PKA inhibitors H89 (10 μM) and Rp-cAMP (50 μM), but not inhibition of Src signaling using PP2 (10 μM), decreased RinQ78L-mediated activation of HSP27, as measured by anti-phospho-specific antibody immunoblotting (Fig. 8C). Inhibition of Gαi (PTX at 100 ng/ml), Src (PP2 at 10 μM), adenylyl cyclase (ddA at 50 μM), or PKA (Rp-cAMP at 50 μM) also inhibited PACAP38-mediated HSP27 activation (Fig. 8D and E). Importantly, PKA signaling was also necessary for RinQ78L- and PACAP38-mediated PC6 cell neurite outgrowth. As shown in Fig. 8F and G, addition of H89 or Rp-cAMP attenuated both Rin- and PACAP38-mediated neuritogenesis, supporting the notion that a novel Rin-PKA-HSP27 signaling pathway contributes to PACAP38-dependent neuronal differentiation (Fig. 8H).

**DISCUSSION**

Numerous studies have characterized trophic factors, including growth factors, cytokines, and hormones, that are capable of regulating both axonal and dendritic growth and the differentiation of neural tumor cells. Among these many factors, PACAP38 has been shown to have particularly prominent and widespread effects within the nervous system (25, 73, 77). For example, PACAP38 treatment increases cell survival and neurite elongation in both primary neurons (21, 49) and PC12 cells (13, 63) and regulates dendritic growth in cultured sympathetic neurons (15). Recent studies have demonstrated a role for Rin in Fig. 7, expression of activated Rin also results in increased HSP27 protein expression in PC6 cells (2.73-fold induction at day 7), supporting the notion that Src-Rin-HSP27 signaling contributes to PACAP38-mediated differentiation.

**Rin signaling contributes to PACAP38-mediated cAMP production.** HSP27 is subject to complex phosphorylation, and p38 MAP kinase signaling has been reported to play a central role in this process (18, 66, 75). However, recent reports indicate that both PKC and PKA contribute to HSP27 phosphorylation (6, 33, 55). Since Rin signaling is required for PACAP38-mediated HSP27 phosphorylation (Fig. 6A) but does not alter p38 MAP kinase activity (Fig. 6A and B), we predicted that Rin might control a cAMP-PKA signaling pathway to regulate HSP27 activation. To address this issue, we examined whether Rin could modulate PACAP38-dependent cAMP production. As shown in Fig. 8A, PACAP38 stimulation of empty vector-transfected PC6 cells resulted in a potent increase in cellular cAMP levels within 10 min (~15-fold induction), with cAMP levels beginning to return to basal levels by 30 min. Expression of dominant-negative Rin or Ras mutants significantly inhibited PACAP38-mediated cAMP production (Fig. 8A). In contrast, expression of either a constitutively active Rin (RinQ78L) or Ras (RasQ61L) mutant in PC6 cells resulted in potent stimulation of cAMP levels (fivefold and eightfold, respectively) (Fig. 8B). Importantly, shRin99-mediated Rin knockdown also resulted in a potent inhibition of PACAP38-mediated cAMP production (Fig. 8A). Taken together, these results suggest that Rin contributes to PACAP38-mediated cAMP signaling.

**Rin-induced neurite outgrowth requires PKA-HSP27 signaling.** The ability of Rin to modulate cellular cAMP levels...
signaling in PC12 cell differentiation (71, 74), motivating studies to explore the role of Rin signaling in PACAP38-dependent neuronal differentiation. In this report, we describe a novel pathway in which Gs-dependent activation of a Src family kinase leads to stimulation of Rin signaling to promote neurite elongation via PKA-dependent HSP27 activation.

To determine whether Rin signaling was involved in transducing PACAP38-mediated neuronal differentiation signals, we investigated in vivo Rin regulation. When PC6 cells are stimulated with PACAP38, Rin is rapidly activated (Fig. 2A). These studies provide the first demonstration that Rin can be activated by G-protein-coupled receptor signaling pathways and establish Rin as a direct downstream target of PACAP38 signaling. More importantly, loss-of-function analysis directly implicates Rin signaling in PACAP38-induced differentiation (Fig. 1). Thus, Rin plays a critical role in the process of neurite outgrowth, acting downstream of both GPCR and NGF-TrkA (71)-dependent signaling cascades. The molecular mechanisms that regulate neuritogenesis remain incompletely characterized, although members of both Ras and Rho GTPase subfamilies have established roles in neuronal development and regeneration (22), and studies presented here suggest that HSP27 activation may underlie the contribution of Rin signaling to neurite outgrowth (Fig. 6 and 7). We recently completed studies exploring the role of Rit signaling in the regulation of axonal and dendritic growth in primary neurons (45). To more firmly define a role for Rin in PACAP38-mediated differentiation signaling, it will be necessary to undertake a similarly detailed analysis of Rin function in primary neurons.

Surprisingly, while GTP loading of Rin is stimulated by expression of activated Gsα (Fig. 2D and E), Rin activation did not rely upon Epac-cAMP signaling (Fig. 3A), the pathway that regulates the closely related Rit GTPase following PACAP exposure (72). Neither stimulation with 8-bromo-cAMP (Fig. 2D), a membrane-permeating cAMP analog, nor that with forskolin (Fig. 3B), a direct adenylyl cyclase activator, resulted in Rin activation. Furthermore, cAMP-bound Rin levels were not elevated following exposure to the GPCR-specific cAMP analog 8-CPT-2-Me-cAMP or by constitutively activated Epac2 (Fig. 3A). The inability of cAMP signaling to activate Rin prompted us to explore other potential signaling pathways. These studies found that Gαq, but not G12α or Gqα, signaling was also capable of inducing Rin activation (Fig. 4). While neither cAMP nor calcium alone stimulated Rin activation (Fig. 2 and 3), previous studies had identified Src family tyrosine kinases as a common downstream target of Gαi/Gqα signaling (26, 44, 60). Consistent with a role for Src in PACAP38-mediated Rin signaling, expression of activated Src was sufficient to induce Rin activation (Fig. 5A), while a dominant-negative Src mutant or pharmacological blockade of Src family kinase signaling inhibited PACAP38-mediated Rin stimulation (Fig. 5A and B). In addition, treatment with the Src kinase inhibitor PP2 potently blocked PACAP38-mediated neuritogenesis but not RinQ78L-mediated neurite outgrowth (Fig. 5C and D). These data indicate that Rin is activated downstream of PACAP in a Gαi/Gqα-Src-dependent fashion. While the molecular mechanisms by which GPCR signaling promotes Src activation remain relatively poorly understood, Huang and colleagues (50) found that both Gαi and Gqα directly bind the catalytic domain to stimulate Src kinase activity. Interestingly, Kim et al. recently identified a direct interaction between the closely related Rit GTPase and both Gαi and Gqα (36). While the ability of Rin to serve as a Gαi binding partner remains to be proven and is the focus of ongoing studies, these data suggest that a higher-order signaling complex containing Gαi, Src, and Rin proteins might contribute to PACAP-mediated Rin activation. In addition, because of the prominent role of Src kinase signaling in a variety of neuronal differentiation cascades, including a critical role in the cross talk between GPCR and TrkA signaling pathways (44), further studies are required to determine whether Src-mediated Rin signaling contributes to these pathways.

How Src signaling results in Rin activation remains uncharacterized and is the target of ongoing studies. There is a large body of literature reporting Src family kinase-mediated regulation of Ras family GTPases. Several common molecular mechanisms emerge from these studies, including direct phosphorylation and activation of either individual guanine nucleotide exchange factors or their adaptor proteins, modulation of
GAP protein activity, and Src-mediated activation of receptor tyrosine kinases downstream of GPCRs to allow cross talk (5, 20, 26, 34, 44, 57, 83). However, authentic Rin regulatory proteins have yet to be defined. Thus, an important goal of ongoing studies is to identify these regulatory proteins and examine their ability to be modulated by Src signaling. Previous work has demonstrated that Ras family GTPases contribute to PACAP38-mediated neuronal differentiation, including roles for both Ras and Rap in the activation of ERK MAP kinase signaling and, more recently, our studies demonstrating a central role for the Rit GTPase in PACAP-mediated p38 MAP kinase activation (19, 63, 72, 76). Indeed, our previous analysis of Rin signaling in NGF-mediated neurite outgrowth found that Rin was required for p38 signaling but only modestly contributed to ERK MAP kinase signaling downstream of the TrkA receptor (71). Thus, the finding that Rin knockdown had no effect on either ERK or p38 MAP kinase signaling was unexpected. Instead, these studies found that Rin function was necessary for HSP27 activation in response to PACAP38 (Fig. 6A) in a cAMP-PKA-dependent manner (Fig. 8C). Rin signaling is also critical for NGF-dependent HSP27 activation (71), suggesting that Rin-mediated HSP27 activation might be central to its role in both NGF- and PACAP-driven neuronal differentiation.

HSP27 phosphorylation has been reported to be controlled predominantly by the p38 MAP kinase cascade (18, 66, 75), although PKC, PKG, and PKA kinase pathways have all been reported to phosphorylate HSP27 (9, 33, 52). While activation of the p38 MAP kinase cascade likely regulates HSP27 after heat shock and other stresses, it appears that activation of cAMP-PKA signaling downstream of Rin is involved in PACAP38-dependent HSP27 activation. Rin signaling contributes to PACAP38-mediated cAMP production in PC6 cells (Fig. 8A), and expression of activated Rin alone is sufficient to promote elevated cAMP levels (Fig. 8B), perhaps by regulating the activity of cellular adenylyl cyclases. Additionally, Rin signaling may control the activity of phosphodiesterase enzymes, thus modulating the concentration and kinetics of cAMP.
cAMP production following PACAP38 stimulation. Consistent with an important role for cAMP in Rin signaling, inhibition of PKA blocked both RinQ78L- and PACAP38-mediated HSP27 phosphorylation (Fig. 8C and E) and neurite outgrowth (Fig. 8F and G). Recent studies have found that PKA phosphorylation of Src mediates Rap1 activation in response to cAMP signaling in PC12 cells (60), suggesting that PKA signaling might be required for Rin activation. However, expression of a Src mutant incapable of PKA phosphorylation did not disrupt PACAP-dependent Rin activation (Fig. 5E), supporting a role for PKA solely as a downstream component of PACAP-Src-Rin signaling.

In summary, the loss-of-function analysis presented here indicates that Rin is a central participant in PACAP38-mediated signal transduction. The marked effects of Rin loss on PACAP38-mediated HSP27 activation, cAMP-PKA signaling, and neuritogenesis suggest that a novel Src-Rin-HSP27 pathway contributes to PACAP38-dependent neuronal differenti-
atation. Studies are ongoing to examine the effects of selective Rin knockdown in primary neurons to further explore the importance of this pathway. Previous work indicating a prominent role for HSP27 in antipoptotic signaling in neurons suggests that future studies must assess the potential role of Rin-HSP27 signaling in neuronal survival. Targeted inactivation of the PACAP and PACR1 genes in mice results in complex behavioral and neurological changes involving alterations in learning and memory and dysregulation of cellular stress responses, among others (25). These defects likely result from the inactivation of canonical PACAP38-PACR1 signaling pathways, but also from the loss of secondary cascades. For example, PACAP38-Src signaling has been implicated in the modulation of NMDA receptor function (51), PACAP signaling is required for injured sensory and motor neuron survival (52), and it will be necessary to consider the potential contribution of Rin signaling to these physiological processes.

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putritory adenylate cyclase-activating polypeptide induces neurite outgrowth in PC12 cells that is dependent on protein kinase C and extracellular signal-regulated kinase but not on protein kinase A, nerve growth factor receptor tyrosine kinase, p21(ras) G protein, and p60(c-src) cytoplasmic tyrosine kinase.