Adhesion-related Kinase Repression of Gonadotropin-releasing Hormone Gene Expression Requires Rac Activation of the Extracellular Signal-regulated Kinase Pathway*

Recent studies suggest that adhesion-related kinase (Ark) plays a role in gonadotropin-releasing hormone (GnRH) neuronal physiology. Ark promotes migration of GnRH neurons via Rac GTPase and concomitantly suppresses GnRH gene expression via homeodomain and myocyte enhancer factor-2 (MEF2) transcription factors. Here, we investigated the signaling cascade required for Ark inhibition of the GnRH promoter in GT1-7 GnRH neuronal cells. Ark repression was blocked by the MEK/ERK pathway inhibitor, PD98059, and dominant negative MEK1 but was unaffected by dominant negative Ras. Inhibitors of the Rho family GTPases, Clostridium difficile toxin B (Rho/Rac/Cdc42 inhibitor) and Clostridium sordellii lethal toxin (Rac/Cdc42 inhibitor), blocked Ark inhibition of GnRH transcription. Moreover, dominant negative Rac blunted both Ark activation of ERK and repression of the GnRH promoter, demonstrating an essential role for Rac in coupling Ark to ERK activation. Like Ark, a constitutively active mutant of Rac suppressed GnRH transcription in an ERK-dependent manner. Finally, Ark-mediated repression was significantly attenuated by a dominant negative MEF2C, whereas repression induced by constitutively active Rac was unaffected, indicating that MEF2 proteins are not targets of the Ark → Rac → MEK → ERK cascade. The data suggest that Ark suppresses GnRH gene expression via the coordinated activation of a Rac → ERK signaling pathway and a distinct MEF2-dependent mechanism.

The hypothalamic peptide gonadotropin-releasing hormone (GnRH) plays a role in normal reproductive function through its actions on the hypothalamic-pituitary-gonadal axis. During development, GnRH-producing neurons migrate from the olfactory placode, through the nasal cavity, and across the cribriform plate (nose-brain border) to their final destination in the hypothalamus (1–4). Although GnRH expression is tightly controlled during development, puberty, and in the adult, the underlying mechanisms involved in its regulation remain unknown.

We and others have used immortalized GnRH neuronal cell lines that retain properties of in vivo GnRH neurons to study GnRH neuronal physiology (5–10). Two types of GnRH neuronal cell line exist, migratory and postmigratory. The GT-1 cells (and subclones GT1-1, GT1-3, and GT1-7) were isolated from an SV40 T antigen-targeted hypothalamic tumor of postmigratory GnRH neurons, whereas the GN10, GN11, and NLT GnRH neuronal cells were derived from an olfactory tumor of migratory arrested GnRH neurons (11, 12). Like that observed in vivo, the GT cells express large amounts of GnRH and are nonmotile, whereas the GN/NLT cells express little GnRH and are intrinsically motile (6, 7).

Given the contrasting phenotypes of the GnRH neuronal cell lines, we proposed that factors expressed differentially between the cell lines may be important regulators of GnRH gene expression and GnRH neuronal migration. In a recent screen for differentially regulated genes, we identified the membrane receptor, adhesion related kinase (Ark), in migratory (GN10, GN11, NLT) but not postmigratory (GT1-7) GnRH neuronal cells (13). Ark expression was also detected along the GnRH neuron migratory route at embryonic day 13 of development, suggesting a potential role for Ark in migrating GnRH neurons (6). Ark (also known as Axl) (14–17) belongs to a unique receptor family that includes Tyro3 and Mer (18). Similar to growth factor receptors, members of this family contain an intracellular tyrosine kinase domain. However, Ark/Axl family members also contain extracellular fibronectin type III and immunoglobulin domains resembling cell adhesion molecules (19). Growth arrest-specific gene 6 (Gas6) functions as a common ligand for Ark/Axl receptors (19, 20) and has been shown to promote cell growth and survival (5, 21–27), adhesion (28, 29), and migration (7, 30) in a variety of cell types via multiple mechanisms.

Recent work from our laboratory suggests that Ark may have several roles in developing GnRH neurons. First, Gas6 promotes survival of Ark-expressing GnRH neuronal cells (5). Second, Gas6/Ark signaling stimulates GnRH neuronal chemotaxis via activation of the Rho family GTPase, Rac (7). And third, our studies indicate that while promoting GnRH neuronal survival and migration, Ark concomitantly acts to suppress expression of GnRH. The latter occurs via the actions of myo-
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cyte enhancer factor 2 (MEF2) proteins and a putative homeodomain transcription factor within the proximal GnRH promoter (6). In the current study, we further investigated Ark regulation of the GnRH gene by defining the signaling cascade(s) involved. The results illustrate that Ark-mediated suppression of GnRH gene expression requires Rac GTPase and the downstream activation of the extracellular signal-regulated kinase (ERK) pathway. Although MEF2 proteins are required for Ark repression of the GnRH promoter, our studies indicate that they are not effectors of the Ark → Rac → ERK cascade and therefore may be engaged via a parallel but essential pathway. Given that Rac GTPase is required for Ark regulation of both migration and GnRH gene expression, these studies further suggest that Rac is a proximal and fundamental coordinator of Ark signaling in GnRH neuronal cells.

MATERIALS AND METHODS

Reagents—Wortmannin, SB203580, PD98059, rapamycin, bisindolylmaleimide, KN93, FK506, and cyclosporin A were purchased from Calbiochem (San Diego, CA). The rabbit polyclonal antibody that recognizes the Tyr/Thr dually phosphorylated forms of ERK1 and 2 was purchased from Cell Signaling Technology (Madison, WI). The rabbit polyclonal anti-ERK2 antibody was obtained from Santa Cruz (Santa Cruz, CA). The mouse monoclonal HA antibody was purchased from Roche Molecular Biochemicals (Germany). The Rac activation kit, pUSE-RasS17N (31), and the mouse monoclonal Ras and Myc antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). The BCA protein assay kit was from Pierce (Rockford, IL). The complete protease inhibitor mixture was from Roche Molecular Biochemicals. Hybrid polyvinylidene difluoride, enhanced chemiluminescence (ECL) reagents, and horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Biosciences (Piscataway, NJ). Dulbecco’s modified Eagle’s medium and antibiotics were from Invitrogen, and the fetal calf serum was from Gemini Bio-Products (Calabasas, CA). Clostridium sordellii toxin B was generously provided by Klaus Aktories (Universität Freiburg, Germany).

Cell Culture—GT1-7 GnRH neuronal cells (11) were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37 °C in humidified 5% CO2 and 95% air.

Plasmids—The pα2LUC plasmid contains a trimered SV40 polyadenylation signal located upstream of inserted promoter sequences, resulting in minimal background luciferase (LUC) activity in the promoterless vector (32). A HindIII fragment containing nucleotides −3026 to +116 of the rat GnRH promoter was ligated into the HindIII site of pα2LUC (rGnRH-LUC), placing the GnRH promoter upstream of the LUC coding region (10). PRK5-Ark contains the mouse Ark cDNA and was provided by Paola Bellotta (New York University) (17). Plasmids encoding constitutively active and dominant negative human MEK1 were generously provided by Natalie Ahn (University of Colorado). The constitutively active pCEP4-MEK1-R4F contains ΔN3 (deleted residues 32–51)/S218E/S222D, and the dominant negative pCEP4-MEK1-S5E contains the mutation K97M (33). The MEK5 mutants were generously provided by Jiing-Dwan Lee (Scripps Research Institute). The constitutively active pCEP4-MEK5(D) contains (ΔIII) fragment containing nucleotides −116 of the rat GnRH promoter.

RESULTS

Ark Represses the GnRH Promoter via the ERK Pathway—The ability of Ark to repress GnRH gene expression was originally shown by expressing Ark in GT1-7 (Ark-negative) GnRH neuronal cells using transient transfection and assessing its effects on the activity of a GnRH-LUC reporter (6). Ark couples to many signaling effectors including phosphoinositide 3-kinase, Src, and S6 kinase, as well as the mitogen-activated protein kinases p38 and ERK1/2, in a variety of cell types (5, 7, 22, 23, 25, 26, 39–41). To identify signaling pathways required for Ark inhibition of the GnRH promoter, pharmacological inhibitors of a variety of signaling pathways were included in the transfection assay (Fig. 1). Ark and the full-length rat GnRH promoter fused to luciferase (rGnRH-LUC) were transfected into GT1-7 GnRH neuronal cells in the presence of inhibitor or vehicle (Me2SO), and transcriptional activity was assessed using the LUC reporter. Similar to our previous studies, Ark

![Image](http://www.jbc.org/)

FIG. 1. Effect of pharmacological inhibitors on Ark repression of the rat GnRH promoter. GT1-7 cells were electroporated with 10 μg of rGnRH-LUC, 0.5 μg of RSV-β-gal, and 5 μg of pRK5 or pRK5-Ark. Immediately after transfection, the cells were treated with vehicle (Me2SO) or inhibitor for 16–18 h. Cells were harvested and assayed for LUC and β-gal activities. The LUC activity of cells that received empty vector (pRK5), RSV-β-gal, and rGnRH-LUC without inhibitor (Me2SO) was set at 100% (Control, light gray bar). Concentrations of inhibitors are given under “Results.” The effect of PD98059 on Ark repression was statistically significant by Student’s paired t test. (Ark versus Ark + PD98059, n = 6, p < 0.005).

Western Blot Analysis—GT1-7 neuronal cells were washed once in phosphate-buffered saline at 4 °C and lysed in 0.1 ml of cell lysis buffer containing 20 mM HEPES, 1% Triton X-100, 50 mM NaCl, 1 mM EGTA, 20 mM sodium orthovanadate, and 50 mM sodium fluoride supplemented with complete protease inhibitors. Cell debris was removed by centrifugation at 14,000 × g for 15 min at 4 °C. The protein concentration of the supernatant was determined using the Bio-Rad Fluor-S Multi Imager and Quantity One software.

Statistical Analysis—Statistical analysis was performed using the one-way analysis of variance followed by the Tukey Kramer multiple comparisons test unless otherwise indicated.
repressed the GnRH promoter by 60% (black bar), and repression was not augmented in the presence of Gas6 (data not shown) (6). Inhibitors of p38 mitogen-activated protein kinase (10 μM SB203580), phosphoinositide 3-kinase (1 μM wortmannin), protein kinase C (100 nM bisindolylmaleimide), calmodulin kinase (5 μM KN93), or calcineurin (1 μM FK506 or 1 μM cyclosporin A) had no effect on Ark repression of the GnRH promoter (data not shown). In contrast, Ark repression decreased from 62% ± 2.6% to only 4% ± 1.4% in the presence of the MEK1/2 inhibitor PD98059 at 30 μM (*p < 0.005). Together, these data suggested that Ark inhibits GnRH gene transcription via activation of the ERK pathway.

Recent studies by Kamakura et al. (42) demonstrated that PD98059 inhibits the activity of MEK5, the upstream ERK5 kinase, in addition to MEK1 and 2. To determine which mitogen-activated protein kinase pathway was required for Ark repression of the GnRH promoter, constitutively active and dominant negative MEK1 and MEK5 mutants were tested in the transfection assay (33, 34). As shown in Fig. 2A, left, the constitutively active MEK1 (MEK1-R4F) (open bar) mimicked Ark repression of GnRH (black bar) (MEK1-R4F, 47% inhibition compared with Ark, 48% inhibition) (the effect of MEK1-R4F was significantly different from control, **p = 4, p < 0.01). In the presence of both Ark and MEK1-R4F, promoter inhibition was not augmented further. Basal GnRH promoter activity was inhibited modestly upon expression of dominant negative MEK1 (MEK1-8E, dark gray bar) (15% inhibition). However, in the presence of MEK1-8E, Ark repression was abrogated markedly (Ark, 48% ± 3% inhibition versus Ark + MEK1-8E, 24% ± 2% inhibition (**p = 4, p < 0.01). To confirm protein expression after transfection, anti-HA immunoblotting was performed to detect the HA-tagged MEK1 mutants (Fig. 2A, right). Although immunoblotting confirmed that the MEK5 mutant proteins were also expressed after transfection (Fig. 2B, right), constitutively active (MEK5(D)) (open bar) and dominant negative MEK5 (MEK5(A)) (dark gray bar) had no effect on basal GnRH promoter activity or Ark repression (Fig. 2B, left). Collectively, the data demonstrate that Ark repression requires the MEK1/2 → ERK1/2 (ERK pathway) signaling pathway but not the MEK5 → ERK5 pathway.

Previous studies from our laboratory demonstrated that the Ark ligand, Gas6, promotes activation of the ERK pathway in the Ark-expressing GnRH neuronal cell lines GN10 (5) and NLT (7). In addition, Ark overexpression in GT1-7 cells results in Gas6-independent Ark activation (6). To determine whether Ark activated the ERK pathway when transfected into GT1-7 neuronal cells, immunoblotting was performed with a phospho-specific ERK1/2 antibody that recognizes the dually phospho-
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Ark Repression of the GnRH Promoter Is Ras-independent. Left, GT1-7 cells were electroporated with 10 μg of rGnRH-LUC, 0.5 μg of RSV-β-gal, and 5 μg of pRK5 or pRK5-Ark. The effects of Ark and RasS17N alone were calculated relative to the LUC activity in the presence of rGnRH-LUC, RSV-β-gal, and empty vector. The effect of RasS17N on Ark repression was calculated relative to the LUC activity in the presence of rGnRH-LUC, RSV-β-gal, empty vector, and RasS17N. The data are presented as percent LUC activity of the respective control, which was set at 100% (Control, light gray bar). The Ark value was not statistically different from Ark + RasS17N (n = 3, p > 0.05). Right, transfected cells were analyzed by Western blot with anti-Ras antibody to confirm expression of dominant negative Ras.

Ark Repression Involves Rho Family GTPases. GT1-7 cells were electroporated with 10 μg of rGnRH-LUC, 0.5 μg of RSV-β-gal, and 5 μg of pRK5 or pRK5-Ark. Immediately after transfection, the cells were treated with 5 ng/ml C. difficile toxin B or 200 ng/ml C. sordellii lethal toxin for 16–18 h and assayed for LUC and β-gal activities. The effects of Ark, lethal toxin, or toxin B alone were calculated relative to the LUC activity of rGnRH-LUC, RSV-β-gal, and empty vector. The effects of the toxins on Ark repression were calculated relative to the LUC activity of rGnRH-LUC, RSV-β-gal, and empty vector in the presence of inhibitor. Data are presented as percent LUC activity of the respective control, which was set at 100% (Control, light gray bar). The effects of toxin B and lethal toxin on Ark repression were statistically significant by Student’s paired t test (*n = 3, p < 0.05).

Ark Repression of GnRH Gene Expression Is Ras-independent—Typically, ERK1 and 2 are activated via the upstream signaling cascade that includes Ras → Raf-1 → MEK1/2 (43). To determine whether Ras activity was required for Ark-mediated GnRH promoter inhibition, a dominant negative Ras mutant (RasS17N) (31) was tested in the reporter assay (Fig. 4). Ark repressed the GnRH promoter by 49% (black bar) (Fig. 4, left). Although dominant negative Ras was clearly expressed after transfection (Fig. 4, right), it had little effect on basal GnRH promoter activity and also had no effect on Ark inhibition (left panel, dark gray bars) (n = 3; Ark, 49% ± 3.1% inhibition versus Ark + RasS17N, 33% ± 2.2% inhibition, p > 0.05). In contrast, RasS17N completely abrogated insulin-like growth factor I-mediated activation of ERK2 via the insulin-like growth factor I receptor (data not shown). Together, these results indicate that Ark repression of the GnRH gene is Ras-independent.

Ark Repression of the GnRH Promoter Requires Rac Activation of the ERK Signaling Cascade—Recent studies have shown that the ERK pathway can also be activated by members...
Ark repression of GnRH gene expression required Rac activation of the ERK pathway but is independent of Pak1. A, left, GT1-7 cells were electroporated with 10 μg of rGnRH-LUC, 0.5 μg of RSV-β-gal, and 5 μg each of pRK5, pRK5-Ark, pRK5-RacQ61L (constitutively active), pRK5-RacT17N (dominant negative), pRK5-Pak1T423E (constitutively active), or pRK5-Ark + pRK5-RacT17N. Data are presented as percent LUC activity of the respective control (see legends to Figs. 2 and 4), which was set at 100% (light gray bar). The value for Ark versus Ark + RacT17N was statistically different (**n = 4, p < 0.05). The value for RacQ61L was significantly decreased compared with the control (**n = 3, p < 0.05). A, right, cell lysates were analyzed by anti-Myc immunoblotting to confirm expression of the mutant proteins. B, GT1-7 cells were electroporated with 10 μg of rGnRH-LUC, 0.5 μg of RSV-β-gal, and 5 μg of pRK5 or pRK5-RacQ61L in the presence of 30 μM Me₆SO or PD98059. Data are presented relative to the LUC activity of rGnRH-LUC, RSV-β-gal, and empty vector in the presence of Me₆SO. The RacQ61L value was statistically different from RacQ61L + PD98059 (**n = 3, p < 0.05).

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of the Rho family of monomeric GTPases, Rho, Rac, and Cdc42 (44–48). Moreover, our previous studies have shown that Gas6/Ark signaling promotes actin cytoskeletal remodeling and migration of GnRH neuronal cells via the Rac GTPase (7). Cisloidal toxins that specifically inactivate members of the Rho family GTPases have recently become useful tools for dissecting Rho GTPase involvement in a given signaling pathway (49, 50). C. difficile toxin B, for instance, specifically glucosylates and inactivates Rho, Rac, and Cdc42 but does not affect other small GTPases such as Ras, Rab, or Arf (51). Toxin B-mediated covalent modification of these proteins blocks their ability to interact with downstream effectors. Similarly, lethal toxin, isolated from C. sordellii, primarily glucosylates and inactivates Rac, and to a much lesser extent Cdc42, as well as other members of the Ras family (Ral, Ras, and Rap) (50, 52, 53). Addition of either toxin B or lethal toxin to the LUC reporter assay in GT1-7 cells resulted in a significant decrease in Ark repression of the GnRH promoter from 62% ± 9.1% to only 32% ± 13 and 31% ± 12.7%, respectively (**n = 3, p < 0.01) (Fig. 5). Given that Rac is the major overlap in specificity between the two toxins, these data suggested that Rac GTPase activity may be necessary for Ark inhibition of the GnRH promoter.

To determine whether Ark promoted Rac activation, the amount of active (GTP-bound) Rac was measured after expression of Ark in GT1-7 cells (Fig. 6A). Briefly, Rac-GTP was precipitated from lysates transfected with pRK5 or Ark using the Rac binding domain of the effector protein Pak. Subsequently, precipitated Rac was detected by immunoblot analysis. Because Pak interacts exclusively with active (GTP-bound) Rac, the Rac immunoblot represents the amount of active Rac in each sample (54). GT1-7 cells expressing Ark displayed a 2.9-fold increase in active Rac (Rac-GTP, upper panel) compared with cells transfected with vector alone (pRK5), whereas Ark expression had no effect on the amount of total Rac present in the cell lysates (Rac blot, lower panel, Fig. 6A). Thus, Ark stimulates Rac GTPase activity in GT1-7 GnRH neuronal cells.

Given that Ark repression of GnRH gene expression required activation of the ERK pathway (see Fig. 2), we determined whether Rac activity was required for Ark stimulation of ERK2. GT1-7 cells were transfected with Ark in the presence or absence of dominant negative Rac (RacT17N) (35) followed by immunoblotting for phospho-ERK1/2 and total ERK2 (Fig. 6B). Similar to previous results (see Fig. 3), Ark stimulated a 2.75-fold increase in the amount of phospho-ERK2 present in GT1-7 cells compared with control (pRK5) (first two lanes). Cells expressing dominant negative Rac alone contained a similar amount of active ERK2 compared with control cells transfected with empty vector (pRK5) (first and third lanes). However, in the presence of dominant negative Rac, Ark failed to increase phospho-ERK2 levels above base line (first and fourth lanes). Together, these data demonstrate that Ark activation of the ERK pathway requires the upstream activation of Rac.

To determine whether Rac activity was required for Ark repression of the GnRH promoter, constitutively active and dominant negative Rac mutants (35, 36) were tested in the LUC reporter assay (Fig. 7A). Constitutively active Rac (RacQ61L) (open bar) repressed GnRH promoter activity similarly to Ark (black bar) but did not augment Ark repression (data not shown) (RacQ61L, 58% ± 3% inhibition; Ark, 54% ± 0.6%, and the effect of RacQ61L was decreased significantly compared with control, **n = 3, p < 0.05). Dominant negative Rac (RacT17N) had no effect on basal GnRH promoter activity. However, in the presence of RacT17N, Ark inhibition of the GnRH promoter was abrogated markedly (dark gray bar) (Ark, 54% ± 0.6% inhibition; Ark + RacT17N, 10% ± 13%; *n = 4, p < 0.05). In addition, Pak1, a well established downstream...
The effector of Rac (55–57), was also tested for its ability to regulate the GnRH promoter (Fig. 7A). Surprisingly, although constitutively active Pak1 (Pak1T423E) (37) was expressed in the GT1-7 cells after transfection (Fig. 7A, right), it had no effect on GnRH promoter activity (open bar). Thus, the data indicate that Ark-mediated GnRH promoter inhibition requires the activity of Rac GTPase and can be mimicked by constitutive activation of Rac but not by constitutive activation of the Rac effector, Pak1.

The phospho-ERK2 immunoblot shown in Fig. 6B demonstrated that Ark-mediated ERK2 activation required the upstream activation of Rac GTPase. In addition, constitutively active Rac (RacQ61L) repressed GnRH promoter activity to the same extent as Ark (see Fig. 7A). To evaluate whether RacQ61L-mediated GnRH promoter inhibition also occurred via the ERK pathway, the MEK inhibitor PD98059 was included in the transfection assay. As shown in Fig. 7B, constitutively active Rac (RacQ61L) inhibited GnRH promoter activity by 63%, similar to that observed for Ark (see Fig. 7A). PD98059 stimulated the GnRH promoter modestly (1.6 ± 0.96-fold). However, in the presence of PD98059, Rac-mediated GnRH promoter inhibition was abrogated significantly from 63% ± 4.3% to only 20% ± 7.5% (*n = 3, p < 0.05). Thus, the inhibitory effect of constitutively active Rac on the GnRH promoter occurs primarily via activation of the ERK pathway.

**MEF2 Proteins Are Not Targets of Ark → Rac → ERK Signaling**—Our previous studies demonstrated that Ark inhibition of GnRH gene expression is conferred by a combination of MEF2B/C and a putative homeodomain protein within the proximal GnRH promoter (6). Given the current data implicating Rac GTPase upstream from the ERK pathway in Ark repression, we postulated that MEF2 family proteins were the nuclear targets of ERK. To test whether MEF2C was downstream from Rac and ERK, a dominant negative form of MEF2C (MEF2C-S387A) was tested in the reporter assay (38) (Fig. 8A). MEF2C-S387A encodes a mutation from serine to alanine within the MEF2C transactivation domain. This mutant is transcriptionally inactive and is thought to act as a dominant negative by interacting with endogenous MEF2 proteins resulting in nonproductive heterodimers. Similar to our previous studies (6), dominant negative MEF2C significantly attenuated Ark repression of the GnRH promoter (Ark, 57% ± 4.8% repression versus Ark + MEF2C-S387A, 26% ± 7.5%) (*n = 3, p < 0.05). In contrast, RacQ61L-mediated promoter blockade was unaffected by dominant negative MEF2C (RacQ61L, 56% ± 5.6% repression versus RacQ61L + MEF2C-S387A 55% ± 5.2%). These data demonstrate that although MEF2 proteins are required for Ark repression of GnRH gene expression, they are not direct downstream targets of Rac → ERK signaling. Given that Ark repression requires the coordinated binding of both MEF2 and homeodomain proteins to the GnRH promoter, the data suggest that the homeoprotein may be the target of the Ark → Rac → MEK → ERK signaling cascade in GT1-7 GnRH neuronal cells and that MEF2s may be recruited to the promoter via a separate but essential mechanism (Fig. 8B).

**DISCUSSION**

Gas6/Ark signaling influences a variety of cellular functions including cell growth, survival, adhesion, and migration. We and others have recently begun to explore the nuclear targets of Ark/Axl receptor signaling. Gas6 induces expression of the class A scavenger receptor, an important mediator in atherogenesis, via a phosphoinositide 3-kinase/Akt pathway (39). The antiapoptotic effects of Gas6 in NIH3T3 cells are mediated by the transcription factor NFkB and also through a phosphoinositide 3-kinase/Akt dependent mechanism (40). Furthermore, Gas6 stimulates mesangial cell proliferation via activation of the transcription factor, STAT3 (58). Our previous work demonstrated that Ark suppression of neuronal GnRH gene expression requires the coordinated activities of both MEF2 and homeodomain proteins within the proximal rat GnRH promoter (6). The focus of the current study was to identify the signaling pathway(s) engaged by Ark to repress expression of GnRH. The results illustrate that the ERK pathway is required for Ark-mediated GnRH promoter inhibition. Of interest, Ras, a common activator of the ERK pathway, is not required for the Ark effect on the GnRH gene. This latter finding was somewhat surprising given that the mitogenic effects of Gas6/Axl signaling in NIH3T3 cells require Ras activity (26). Furthermore, Gas6-mediated proliferation of C57 mammary cells involves activation of the ERK pathway, presumably via upstream sig-
nals emerging from Ras (23). Consistent with our previous studies showing Gas6-mediated GnRH neuronal survival in the absence of proliferative signals (5), the current data may reflect cell type-specific differences in coupling of Ark/Axl family receptors to Ras.

Members of the Rho family of monomeric GTPases, Rho, Rac, and Cdc42, are best recognized for their effects on the actin cytoskeleton. Via the activation of downstream effectors, Rho GTPases control cell shape and movement (59). In addition, however, these proteins regulate the cell cycle, gene transcription, and cell survival (60–62). Given recent studies implicating Rho GTPases in ERK pathway activation (44–48), we examined a potential role for Rac in Ark-mediated GnRH promoter suppression. The Rho family selective inhibitors, toxin B and lethal toxin, both blunted Ark repression of the GnRH promoter, thus implicating Rac in the signaling pathway. Moreover, Ark stimulated Rac activation, and dominant negative Rac attenuated both Ark activation of ERK and Ark repression of the GnRH promoter, demonstrating a critical role for Rac in coupling Ark to ERK activation. Finally, constitutively active Rac mimicked Ark repression of GnRH and was also dependent on ERK. Hence, Ark repression of the GnRH promoter occurs via a Rac → MEK → ERK pathway.

Both receptor tyrosine kinase and G protein-coupled receptors have been shown to activate ERK downstream of Rac (44, 45, 48). However, in both cases, Rac merely synergized with Raf-1 (downstream of Ras) to activate ERK. Our studies indicate that Ark/Axl family receptors are capable of activating the Rac → ERK pathway in the absence of additional signals from the Ras → Raf-1 pathway. Coupled with our recent studies showing the requirement of Rac in Gas6/Ark-induced GnRH neuronal migration (7), the data suggest that Rac coordinates a variety of Ark signaling pathways in GnRH neurons.

The downstream effects of Rac on gene transcription have only recently been explored. For instance, Rac activates transcription factors including NFκB, cyclin D1, SRF, and Nfat, resulting in increased gene transcription (63–66). Regarding transcriptional repression, lipopolysaccharide-mediated downregulation of the scavenger receptor class BI gene is mediated via a Rac-dependent mechanism (67). To our knowledge, other than GnRH, scavenger receptor class BI is the only other gene known to be negatively regulated by Rac GTPase.

Rac-mediated potentiation of the ERK pathway has been proposed to occur via two potential mechanisms. Rac may activate ERK by increasing the phosphorylation of Raf-1 on serine 338, a residue important for full Raf-1 activation, and/or by increasing the association of MEK1 with Raf-1, via phosphorylation of serine 298 on MEK1 (44, 45, 48). Interestingly, both serine phosphorylations are mediated by members of the Pak family, which are well characterized Rac-specific effector proteins (55–57). With respect to Ark/Axl signaling, Rac has recently been implicated upstream of Pak1 in Gas6-induced survival of NIH3T3 fibroblast cells (26). Although GnRH promoter blockade was recapitulated by a constitutively active Rac mutant (RacQ61L), a constitutively active form of Pak1 (Pak1T423E) was not sufficient to inhibit GnRH promoter activity in GT1-7 cells. These data are consistent with a mechanism whereby Ark inhibits GnRH gene expression via Rac activation of the ERK pathway but may not require the effector protein Pak1. Further studies are necessary to identify the complement of effectors both upstream and downstream of Rac involved in Ark regulation of the GnRH promoter.

Activated ERKs translocate to the nucleus and phosphorylate transcription factor substrates resulting in transcriptional induction or suppression (69–73). Because our previous studies implicated MEF2B/2C in Ark repression of the GnRH promoter, we postulated that MEF2 proteins were the nuclear targets of activated ERK. Although a constitutively active mutant of Rac (RacQ61L) blunted GnRH promoter activity via an ERK-dependent mechanism, a dominant interfering MEF2C mutant (MEF2C-S387A) had no effect on Rac inhibition of the promoter, although it blunted Ark repression substantially. These data suggested that MEF2 proteins are not targets of the Ark → Rac → ERK signaling pathway in GnRH neuronal cells and furthermore that Rac-independent pathways may be involved in Ark signaling.

Given that Ark suppression of GnRH gene transcription requires a complement of MEF2 and homeodomain proteins (6), our studies point to the homeoprotein as the potential ERK substrate in the nucleus. Consistent with this hypothesis, Miserro et al. (74) showed that the transcriptional activity of the homeodomain protein TTF-1, a thyroid-specific transcription factor, is inhibited via Rac activation of the ERK pathway. Activation of the Ras → Raf-1 → MEK → ERK pathway in thyroid cells results in phosphorylation of TTF-1 on three serine residues following by a striking decrease in TTF-1-mediated transcription. Interestingly, Rac suppresses TTF-1 transcriptional activity without altering its ability to bind DNA (75). Similarly, in Ark-expressing GnRH neuronal cells, inhibition of ERK activity with the MEK inhibitor, PD98059, does not alter DNA binding of the putative homeoprotein. Therefore, ERK phosphorylation of the homeoprotein may alter its ability to interact with accessory proteins such as the MEF2s or perhaps the basal transcriptional machinery. Thus, the focus of future studies lies in the identification of the homeoprotein(s) required for the effect of Ark on GnRH gene transcription and whether it is a direct substrate for the Rac → MEK → ERK pathway identified in this report. In addition, further studies are necessary to determine whether MEF2s are recruited to the promoter by a parallel Ark pathway or perhaps via the target of the Rac → ERK cascade (see Fig. 8B).

In summary, Ark repression of GnRH gene transcription occurs through two coordinated events, one involving activation of a Rac → MEK → ERK signal and the other involving a MEF2 pathway. Collectively, our studies suggest that in addition to promoting GnRH neuronal migration and survival, one function of Ark in the developing GnRH neuron may be to repress the differentiated phenotype (i.e. level of GnRH expression) until the neurons reach their final destination in the hypothalamus. Given the critical role of GnRH in establishing reproductive competence, elucidation of mechanisms that impinge upon GnRH expression, such as that induced by Ark, is of great importance to our understanding of reproductive physiology.

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