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Fibroblast Growth Factors Regulate Prolactin Transcription via an Atypical Rac-Dependent Signaling Pathway

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Fibroblast growth factors (FGFs) play a critical role in pituitary development and in pituitary tumor formation and progression. We have previously characterized FGF signal transduction and regulation of the tissue-specific rat prolactin (rPRL) promoter in GH4 pituitary cells. FGF induction of rPRL transcription is independent of Ras, but mediated by a protein kinase C-δ (PKCδ)-dependent activation of MAPK (ERK). Here we demonstrate a functional role for the Rho family monomeric G protein, Rac1, in FGF regulation of PRL gene expression via an atypical signaling pathway. Expression of dominant negative Rac, but not RhoA or Cdc42, selectively inhibited FGF-induced rPRL promoter activity. Moreover, expression of dominant negative Rac also attenuated FGF-2 and FGF-4 stimulation of MAPK (ERK). However, in contrast to other Rac-dependent signaling pathways, FGF activation of rPRL promoter activity was independent of the c-Jun N-terminal kinase (JNK) and phosphoinositide 3-kinase/Akt cascades. FGFs failed to activate JNK1 or JNK2, and expression of dominant negative JNK or Akt constructs did not block FGF-induced PRL transcription. Consistent with the role of PKCδ in FGF regulation of PRL gene expression, activation of the rPRL promoter was blocked by an inhibitor of phospholipase Cγ (PLCγ) activity. FGF treatment also induced rapid tyrosine phosphorylation of PLCγ in a Rac-dependent manner. These results suggest that FGF-2 and FGF-4 activate PRL gene expression via a novel Rac1, PLCγ, PKCδ, and ERK cascade, independent of phosphoinositol-3-kinase and JNK. (*Molecular Endocrinology* 17: 1921–1930, 2003)

FIBROBLAST GROWTH FACTORS (FGFs) (1) comprise a family of polypeptides that are important regulators of cell growth, development, and differentiation (1, 2). Several FGFs function as oncogenes and have been implicated in the formation and progression of a variety of endocrine-related tumors of the breast, prostate, ovary, and pituitary (3–5). First characterized in the pituitary, FGFs regulate both lactotroph development and differentiation (6) and the synthesis and secretion of prolactin (PRL) (5, 7, 8). FGF-2 and FGF-4 have also been implicated in the development of pituitary adenomas (5, 9), which exhibit aberrant expression of FGF receptor isoforms (10–12). Thus, FGFs are critical factors in pituitary cell ontogeny and regulation of lactotroph function and oncogenesis.

We have previously used the rat PRL (rPRL) promoter in GH4 pituitary cells to define and characterize components of the FGF signal transduction pathway (7, 8). These differentiated neuroendocrine cells express the phenotypic markers PRL and GH, maintain normal hormonal and growth factor responses (13), and have been extensively used to define and characterize hormone and growth factor signal transduction pathways regulating the tissue-specific expression of the PRL and GH genes. Using this physiologically relevant model, we have identified FGF response elements in the rPRL promoter (7) and shown that, in contrast to other systems, FGF activation of PRL gene expression is independent of Ras, utilizing a specific protein kinase C isoform, PKCδ, to activate MAPK (8). In this report we investigated the role of the Rho family GTPases, phospholipase C (PLC) γ- and phosphoinositol-3-kinase (PI3K) in FGF induction of MAPK and rPRL transcription.

Rho GTPases are members of the Ras superfamily of monomeric G-proteins that function as molecular switches, cycling between inactive GDP-bound and active GTP-bound forms to mediate signal transduction from growth factor receptors (14). The best characterized are RhoA, Rac1, and cdc42 in their role as regulators of cell cytoskeletal morphology, motility, and adhesion (15). However, more recently Rho GTPases have also been implicated in regulation of cell cycle, cell survival, and transcription (16–18). Here we present evidence that FGF activation of rPRL transcription is mediated by Rac1 and phospholipase Cγ. We also demonstrate that FGF activation of MAPKs (ERK1 and 2) is dependent on Rac1. In contrast to

Abbreviations: D609, Tricyclodecan-9-yl-xanthate, potassium salt; ET-18-OCH_3, 1-O-Octadecyl-2-O-methyl-*rac*-glycero-3-phosphorylcholine; FGF, fibroblast growth factor; JNK, c-jun NH_2-terminal protein kinase; PAI-1, plasminogen activator inhibitor; PI3K, phosphoinositol-3-kinase; PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C; PRL, prolactin; rPRL, rat prolactin; SRF, serum response factor.

other Rac signaling pathways, FGF activation of the rPRL promoter did not require activation of c-Jun N-terminal kinase (JNK) or phosphoinositol 3 kinase. FGF-2 and FGF-4 stimulation of rPRL expression by this atypical signaling pathway represents the first example of FGF-inducible, Rac-dependent transcriptional regulation of a tissue-specific gene.

RESULTS

Dominant Negative Rac Inhibits FGF Activation of the rPRL Promoter

We have previously shown that FGF stimulation of the rPRL promoter is mediated by protein kinase C δ (PKCδ) and MAPK but is independent of the Ras and Raf signaling pathway (7, 8). Members of the Rho family of G-proteins have been implicated in FGF regulation of cell cycle progression (19), DNA synthesis (20), cell morphology (21), cell survival (22), and transcription (23). They are also critical components of several PKC-dependent growth factor and cytokine signaling pathways (24–26). To determine the functional of role of Rho GTPases in FGF regulation of PRL transcription, GH4 cells were transiently transfected with a dominant negative form of Rac1, T17N Rac (27). As shown in Fig. 1, FGF-2 and FGF-4 activation of an rPRL luciferase reporter construct was inhibited by expression of T17N Rac in a dose-dependent fashion. The 3-fold FGF stimulation of rPRL promoter activity was essentially abrogated at the higher doses of T17N Rac. Dominant negative Rac expression did not significantly affect basal promoter activity (data not shown).

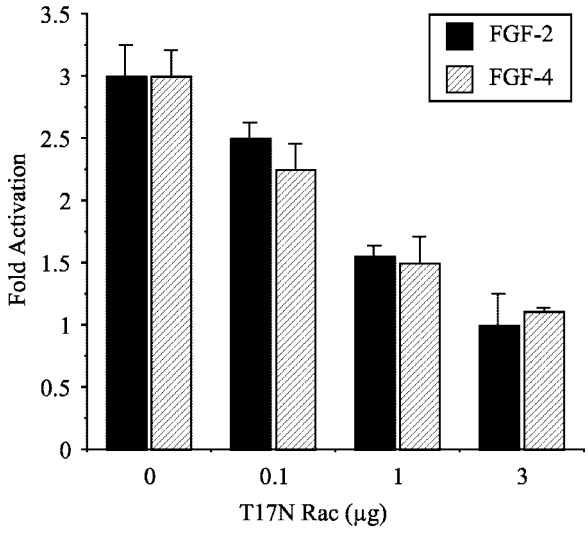

Fig. 1. Expression of Dominant Negative Rac Inhibits FGF Activation of the rPRL Promoter

GH4T2 cells were transfected with 3 μg of pA3rPRL-425luc, 3.0 μg of pSV40 βgal, and the indicated amount of pRK5T17Nrac. Total DNA was kept constant by addition of empty vector (pRK5). Cells were treated with FGF-2 or FGF-4 (10 ng/ml) 6 h before harvest. Cells were harvested 24 h post transfection. Luciferase activity was normalized to β-galactosidase units. FGF stimulation of rPRL promoter is expressed as fold increase over control at each dose of T17Nrac. Results are mean ± SEM of three independent experiments, each comprised of triplicate transfections.

Rho family members have been shown to act in hierarchical cascades, such that Rac may activate Rho and Cdc42 may activate Rac. Rho G-proteins may also act downstream of Ras (28). However, Rho family members can also induce isoform-specific responses that may be competitive or functionally antagonistic (29, 30). To further investigate the role of Ras and Rho GTPases in regulation of PRL transcription by FGFs, we used analogous dominant negative constructs of RhoA and Cdc42 (31) (Fig. 2). Whereas transfection of T17NRac essentially abrogated FGF-2 and FGF-4 activation of the rPRL promoter (Fig. 2A), expression of N19Rho A or N17Cdc42 constructs had no significant effect on FGF-induced PRL transcription (Fig. 2B), indicating a specific Rac-dependent pathway. No inhibition of the rPRL promoter was observed in dose response experiments using up to 10 μg of the N19Rho A or N17Cdc42 constructs (data not shown). Expression of RhoA and Cdc42 constructs was verified by Western blotting with anti-FLAG antibody (Fig. 2C). Consistent with a role for Rac1 in the FGF-mediated regulation of PRL transcription, expression of a constitutively GTP-bound active Rac 1 construct (Q61L) (32) resulted in a 3-fold increase in basal promoter activity, mimicking the effects of FGFs (Fig. 2A).

We have previously shown that FGFs activate the rPRL via a Ras- and Raf-independent pathway, unaffected by dominant negative Ras or Raf constructs (7). In agreement with these results, activation of the rPRL promoter by expression of V12 Ras or a constitutively active Raf kinase construct (BXBRaf) (7, 33) was not affected by cotransfection with dominant negative Rac (Fig. 3), signifying that the Ras and Rac pathways are independent. Thus, dominant negative Rac, but not RhoA or Cdc42, selectively inhibited FGF-induced rPRL promoter activity.

Rac-Dependent FGF Activation of MAPK

We have documented that FGF activation of the rPRL promoter is dependent on MAP (ERK) kinase activity and that FGF-2 or FGF-4 rapidly induce phosphorylation and activation of MAPK in GH4 cells (7, 8). To investigate the role of Rac1 in FGF stimulation of MAPK, cells were transfected with dominant negative Rac1, treated with FGF-2 or FGF-4, and harvested for Western blotting using antibodies to phosphorylated MAPK or total MAPK as indicated in Fig. 4 (the two observed bands correspond to p42 and p44 MAPK). Expression of dominant negative Rac 1 significantly inhibited FGF-2- or FGF-4-induced MAPK phosphorylation (Fig. 4A, lanes 7 and 8 and 11 and 12). Total MAPK levels were not affected (Fig. 4, A and B, total MapK panels), and dominant negative Rac1 had no

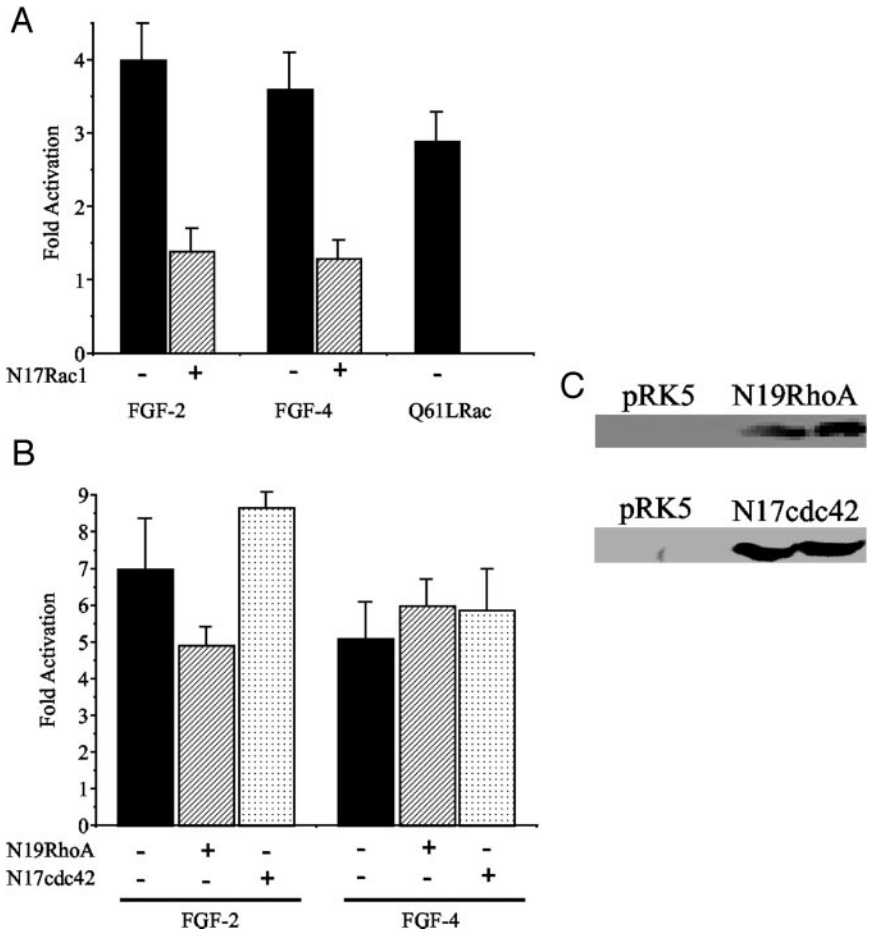

Fig. 2. Dominant Negative Rac, But Not RhoA or CdC42, Specifically Inhibits FGF Activation of the rPRL Promoter

A, GH4T2 pituitary cells were cotransfected with 3 μg of pA3rPRL-425luc, 3.0 μg of pSV40 βgal, and 2 μg of either T17N Rac 1 (dominant negative Rac 1), Q61LRac1 (constitutively active), or vector and serum starved for 16 h. The cells were then treated $\pm$ 10 ng/ml FGF-2 or FGF-4 for 6 h before harvest. Luciferase activity was normalized to βgal activity, and FGF-induced PRL promoter activity was expressed as fold increase over the appropriate control (vector, T17Nrac or Q61Lrac). Results are the means $\pm$ SEM of eight experiments, each consisting of triplicate transfections. B, GH4T2 cells were cotransfected with 2 μg of pA3rPRL-425luc, 3.0 μg of pSV40 βgal, and 2 μg of either vector, N17Cdc42 or N19RhoA. FGF activation of the rPRL promoter was determined as described. Results are the mean $\pm$ SD (n = 6). C, Cell pellets from transfection harvest described in panel B were analyzed by Western blot (to demonstrate functionality of expression vectors).

detectable effects on basal MAPK activity (Fig. 4, A and B, lanes 1 and 2 *vs*. lanes 3 and 4). Serum-induced phosphorylation of MAPK was not blocked by T17N Rac (Fig. 4B, lanes 5 and 6 *vs*. 7 and 8), indicating that the effects of dominant negative Rac 1 were specific for FGF-dependent inducible MAPK activity and not a result of nonspecific cellular toxicity. Consistent with a role for Rac in activation of MAPK, transient transfection of the constitutively active Rac construct (Q61L) significantly increased phosphorylation of MAPK in GH4 cells in the absence of serum or exogenous growth factors (Fig. 4C). Figure 4D shows the effects of dominant negative Rac expression on MAPK activation based upon densitometric analysis of Western blots derived from four independent experiments. These data indicate that FGFs activate MAPK via a Rac-dependent pathway in GH4 cells.

Rac-dependent signals typically activate the c-Jun NH_2-terminal kinase (JNK) pathway (16, 34). Moreover, JNK has been implicated in activation of the rPRL promoter by PRL-releasing peptide (35). Thus, we examined the role of JNKs in FGF stimulation of the rPRL promoter. As shown in Fig. 5, transient transfection of GH4 cells with dominant negative constructs of JNK1 and JNK2 (36) had no effect on basal or FGF-2- or FGF-4-induced rPRL promoter activity. Expression of JNK constructs was confirmed by Western blotting with anti-hemagglutinin antibody (not shown). Furthermore, treatment of GH4 cells with FGF-2 or FGF-4 did not result in phosphorylation and activation of JNK 1 or JNK 2 or changes in JNK protein levels (Fig. 6). An extended time course from 5 min to 8 h failed to show any activation of JNKs in response to FGFs (not shown).

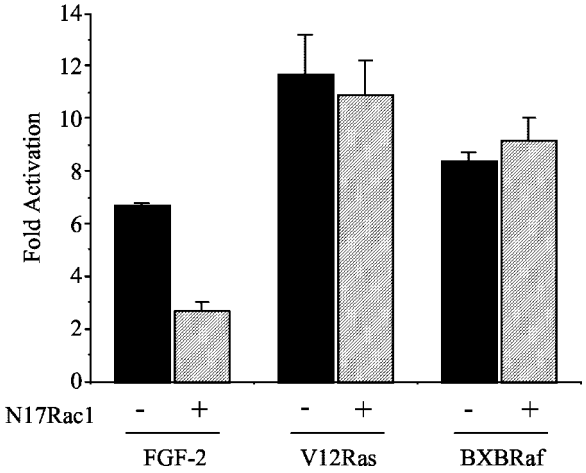

Fig. 3. Dominant Negative Rac1 Selectively Inhibits FGF, But Not V12Ras, Stimulation of the rPRL Promoter

GH4T2 pituitary cells were cotransfected with 3 μg pA3rPRL-425luc, 3 μg pSV40 βgal, and 2 μg pSVRas or 5 μg pBXBRaf in the presence or absence of 2 μg of N17Rac as indicated. Total DNA was kept constant by addition of empty vectors. Cells were treated with FGFs as indicated, and PRL promoter activity was determined as in Fig. 2 and expressed as fold increase over control. Results are mean fold $\pm$ SEM of three experiments consisting of triplicate transfections.

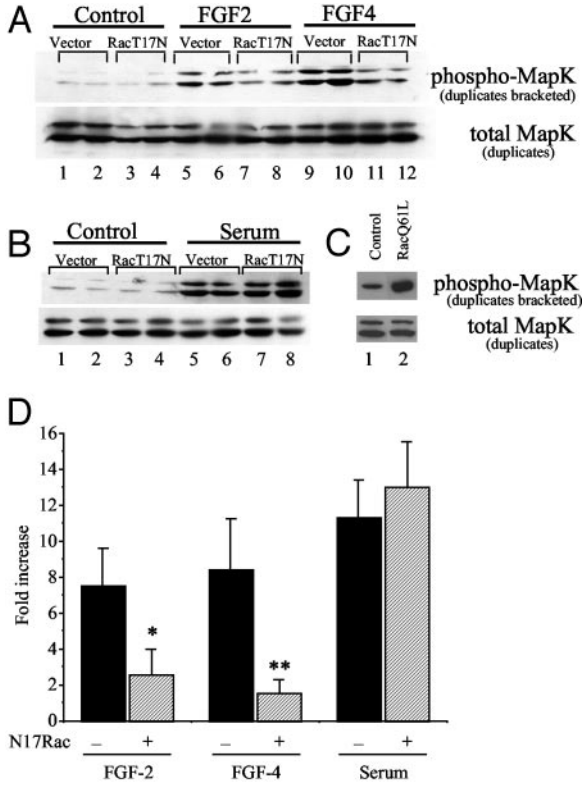

Fig. 4. Dominant Negative Rac Inhibits FGF Activation of MAPK (ERK)

A, GH4T2 cells were transfected with 3 μg pRK5 or pRK5T17Nrac vector and serum starved 16 h. Cells were then treated with FGF-2 or FGF-4 (10 ng/ml) for 15 min before harvesting for Western blot analysis as described in *Materials and Methods*. MAPK activation was assessed by using a phosphospecific MAPK antibody. Blots were stripped and reprobed with total MAPK antibody as a loading control. B, GH4T2 cells were transfected and starved as described above. Cells were then treated with full serum for 15 min and harvested for Western blotting. MAPK activation was assessed by using a phosphospecific MAPK antibody. Blots were stripped and reprobed with total MAPK antibody as a loading control. C, GH4T2 cells were transfected with 3 μg pRK5 or pRK5Q61Lrac vector (constitutively active Rac1) and serum starved 16 h. Cells were harvested for Western blotting as described above. D, Four independent experiments (as described above) were quantitated using a Kodak EDAS digital imaging system and Kodak 1D image analysis software. Phospho-MAPK was normalized to total MAPK, and fold changes relative to control (set at 1.0) were calculated. Data were analyzed using a one-tailed pairwise *t* test (*, $P < 0.005$; and **, $P < 0.006$).

As a control, exposure of GH4 cells to UV-C light resulted in rapid phosphorylation of both JNK1 and JNK2 without affecting total JNK protein levels (Fig. 6), demonstrating an intact JNK signaling pathway in these cells. These results indicate that, in contrast to typical Rac signal transduction pathways, the Rac-dependent FGF activation of the rPRL promoter is not mediated via JNKs.

FGF Activation of the rPRL Promoter Is Independent of PI3K

PI3K and its downstream kinase effector protein kinase B (PKB/Akt) are components of multiple Rac-dependent signaling pathways regulating cytoskeletal rearrangements, apoptosis, and transcription and may function either up or downstream from this G-protein (30, 37, 38). Activated Rac and cdc42 also bind to PI3K in response to platelet-derived growth factor treatment (39). Furthermore, PI3K and Akt are required to mediate activation of the rPRL promoter by PRL-releasing peptide and insulin, the former acting via a calcium and PKC-dependent pathway (40). To determine the role of PI3K in FGF activation of the PRL promoter, GH4 cells were preincubated with PI3K inhibitor wortmannin (38). As shown in Fig. 7, wortmannin did not affect FGF-2 or FGF 4 stimulation of rPRL promoter activity. In contrast, IGF-I activation of the rPRL promoter, which is reportedly mediated via PI3K (41), was blocked by wortmannin (Fig. 7), demonstrating its efficacy in this system. Treatment with a second PI3K antagonist, LY294002 (38), also failed to block FGF induction of the rPRL promoter (not shown).

The functional role of Akt in FGF activation of the rPRL promoter was investigated using dominant negative Akt constructs mutated in the phosphorylation sites necessary for activation of the kinase (threonine 308 and serine 473) (42, 43). As shown in Fig. 8, transfection of the single mAktA (T308 to A) or double mAktAA (T308 to A and S473 to A) mutants

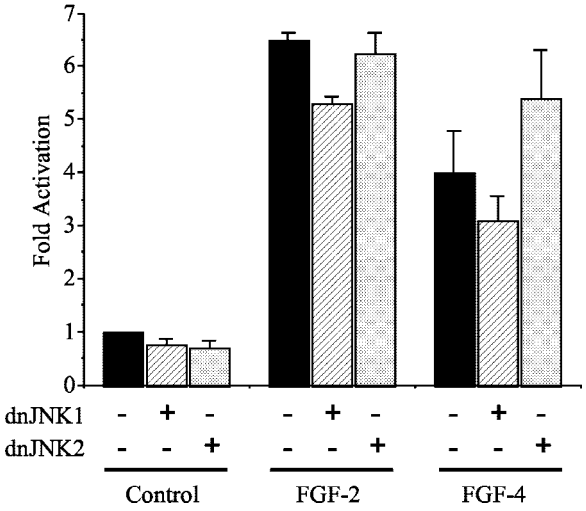

Fig. 5. FGF Activation of the rPRL Promoter Is Not Dependent on JNK

GH4T2 pituitary cells were cotransfected with 3 μg pA3rPRL-425luc and 0.33 μg pCMV βgal in the presence or absence of 10 μg pSRαJNK1APF or pSRαJNK2APF (dominant negative JNKs) as indicated. Cells were treated with FGFs and assayed for PRL promoter activation as in Fig. 2. Results are mean ± SD of six to nine transfections.

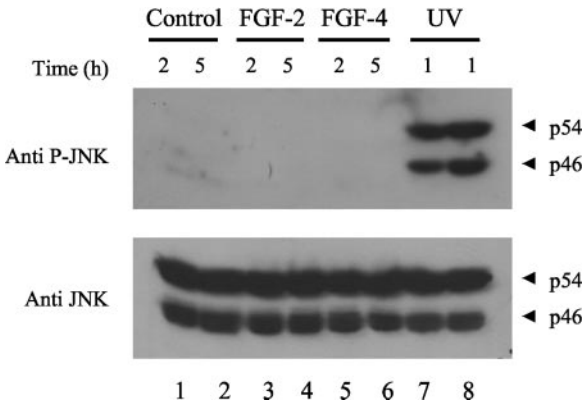

Fig. 6. FGF-2 and FGF-4 Do Not Activate JNK1 or JNK2

GH4T2 cells were serum starved overnight and stimulated with FGF-2 or FGF-4 (10 ng/ml) for the indicated times. As a control, cells were exposed to UV-C light 5000 J/m^2. Cell extracts (100 μg) were analyzed by Western blotting, as described in *Materials and Methods*. JNK activation was detected by phosphospecific (Thr183/185) JNK antibody. The blot was stripped and reprobed with total JNK antibody as a loading control. The positions of JNK1 (p46) and JNK2 (p54) are indicated by *arrows* at their corresponding molecular masses (kDa).

had no effect on the PRL promoter FGF response. The apparent modest increase in basal promoter activity in response to mAktAA was not statistically significant. Taken together, these results (Figs. 7 and 8) indicate that, in contrast to other systems, Rac-dependent FGF activation of the rPRL promoter is independent of the PI3K/Akt signaling cascade.

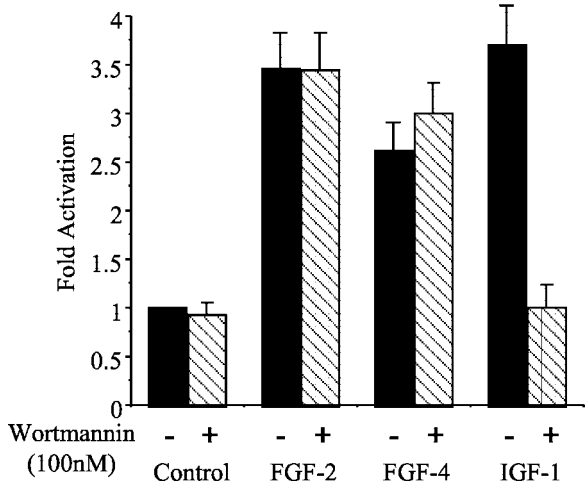

Fig. 7. PI3K Does Not Mediate FGF Stimulation of the rPRL Promoter

GH4T2 pituitary cells were cotransfected with 3 μg of pA3rPRL-425luc and 0.3 μg of pCMV βgal and serum starved for 16 h. Cells were pretreated with 100 nM Wortmannin or dimethylsulfoxide for 1 h at 37 C and then treated with 10 ng/ml FGF-2, FGF-4, 13 nM IGF-I, or diluent, as indicated, for 6 h before harvest. PRL promoter activity was determined as in Fig. 2 and is expressed as fold increase over control levels. Results are means ± SEM of four experiments, each consisting of triplicate transfections.

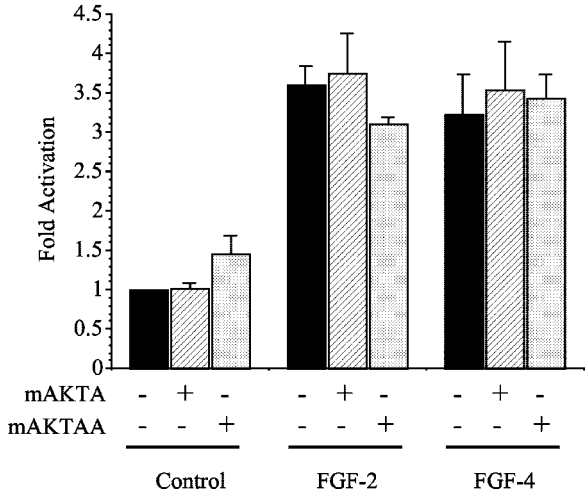

Fig. 8. Expression of Dominant Negative Akt (PKB) Does Not Block the FGF Response

GH4T2 cells were cotransfected with 3 μg pA3rPRL-425luc, 0.3 μg pCMV βgal in the presence or absence of 10 μg pmAktA or pmAktAA, or empty vector as indicated. Cells were treated with FGFs and assayed for PRL promoter activation as in Fig. 2. Results are mean ± SD of six transfections.

FGF Activation of the PRL Promoter Is Mediated by PLCγ

PLCγ binds to activated FGF receptors (44) and is a critical component of FGF-mediated signal transduc-

tion (45), undergoing tyrosine phosphorylation and activation in response to FGFs (1, 2). We have previously shown that FGF activation of rPRL transcription is mediated by PKCδ (8), which is typically activated by PLCγ-catalyzed production of diacylglycerol (46). As shown in Fig. 9, using tyrosine 783 phosphospecific antibodies (Cell Signaling, Beverley, MA), FGF stimulation led to an increase in PLCγ phosphorylation. Bands were quantitated using a Kodak EDAS digital imaging system and Kodak 1D image analysis software (Eastman Kodak Co., Rochester, NY), and phospho-PLCγ was normalized to total PLCγ. Treatment with FGF-2 resulted in a 7-fold increase in PLCγ phosphorylation (Fig. 9, lane 2), whereas FGF-4 led to a 3.4-fold increase in tyrosine phosphorylation of PLCγ (Fig. 9, lane 3). To determine the functional role of PLCγ in FGF stimulation of the rPRL promoter, we used 1-O-octadecyl-2-*O*-methyl-*rac*-glycero-3-phophorylcholine (ET-18-OCH_3), an inhibitor of phosphatidylinositol-specific PLCγ (47) (Calbiochem, La Jolla, CA). Pretreatment (for 30 min to 1 h) of GH4 cells with ET-18-OCH_3 resulted in inhibition of FGF-2 and FGF-4 activation of the rPRL promoter (Fig. 10A). The reduction in rPRL promoter FGF response was dose dependent and consistent with the reported IC_{50} (15 μM) for this compound (not shown). As a control for nonspecific toxicity, ET-18-OCH_3 had no effect on V12 Ras induction of rPRL promoter activity, indicating a specific effect on the FGF signaling pathway (Fig. 10A). Additionally, treatment of GH4 cells with an inhibitor of phosphatidylcholine-specific phospholipases, tricyclodecan-9-yl-xanthate, potassium salt (D609, Calbiochem), which does not inhibit PLCγ (48), had no effect on FGF (or V12Ras) activation of PRL promoter activity (Fig. 10B). Taken together, these results show that activation of the rPRL promoter is mediated via PLCγ and that FGF induces phosphorylation of PLCγ in GH4 cells. However, overexpression of T17Nrac had no effect on FGF-induced PLCγ phosphorylation (not shown), suggesting that Rac1 is not upstream of PLCγ in the FGF signal transduction pathway.

DISCUSSION

FGFs play a critical role in pituitary development and function and regulate both synthesis and secretion of the lactotroph-specific hormone PRL (1, 2). FGFs and their receptors have also been implicated in the formation and progression of tumors of the pituitary (5, 9–12) and other endocrine hormone target tissues (3–5). We have previously shown that FGF-2 and FGF-4 regulate the tissue-specific transcription of the PRL gene via an atypical signaling pathway, which is independent of Ras and mediated by PKCδ activation of the MAPK (ERK) pathway. Here we show that FGF activation of the rPRL promoter is dependent on Rac1, a member of the Rho monomeric G-protein family, and PLCγ. Expression of dominant negative Rac1 inhibited FGF-stimulated rPRL promoter activity, whereas analogous RhoA and Cdc42 constructs had no effect on PRL transcription (Fig. 2). Moreover, in contrast to canonical Rac-dependent signal transduction pathways (16, 30, 34, 37), the tissue-specific PRL FGF response is mediated via activation of MAPK (ERK) and is independent of the JNK and PI3K/Akt signaling cascades.

We have shown that FGFs stimulate phosphorylation of PLCγ and that inhibitors of PLCγ selectively block FGF induction of rPRL promoter activity (Figs. 9

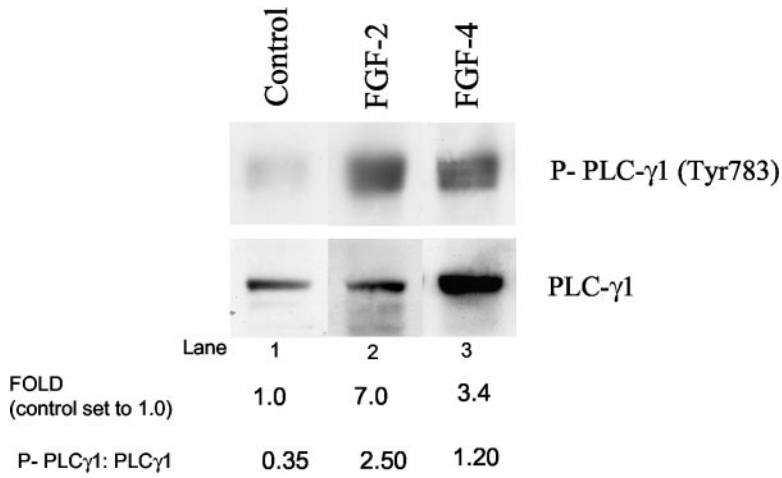

Fig. 9. FGF Induces Phosphorylation/Activation of PLCγ1

GH4 cells were serum deprived for 24 h and subsequently stimulated with 10 ng/ml FGF-2 or 10 ng/ml FGF-4. Cells were harvested at time 0 or 1 h after stimulation. Extracts were resolved by SDS-PAGE on 8% gels and immunoblots were performed with antibodies specific for p-PLCγ1(Tyr783) (Cell Signaling) or pan PLCγ1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Bands were quantitated using a Kodak EDAS digital imaging system and Kodak 1D image analysis software. Phospho-PLCγ1 was normalized to total PLCγ1, and fold changes relative to control (set at 1.0) were calculated.

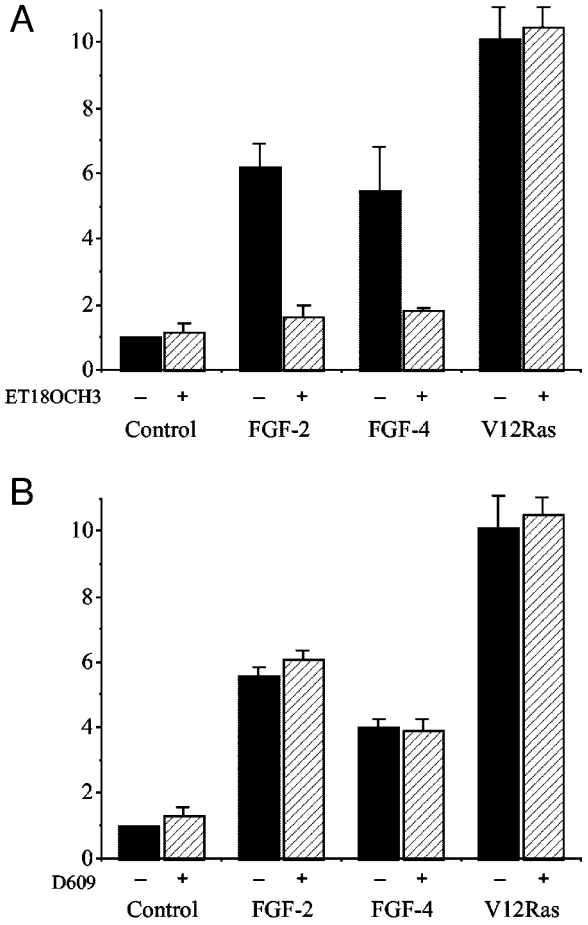

Fig. 10. The Phosphatidylinositol-Specific PLC Inhibitor, ET-18-OCH_3, Inhibits FGF-Induced rPRL Promoter Stimulation

GH4T2 pituitary cells were cotransfected with 3 μg of pA3rPRL-425luc and 0.3 μg of pCMV βgal $\pm$ 2 μg pSVRas as indicated. Cells were pretreated 16 h post transfection with A) 30 μM ET-18-OCH_3 or B) 50 μM D609 inhibitors at the indicated concentrations for 30 min and then stimulated with 10 ng/ml of FGF-2 or FGF-4. Six hours post treatment, cells were harvested and assayed as described in Fig. 2. Results are means $\pm$ SEM of three experiments, each consisting of triplicate transfections.

and 10). A number of reports have implicated PLCγ in Rac-dependent signaling (49–53). In these reports, PLCγ has been described to act both upstream and downstream of Rho family GTPases. In our system, expression of dominant negative Rac1 had no effect on FGF-induced PLCγ phosphorylation, indicating that PLCγ is either upstream or parallel to Rac1 in this signal transduction pathway. This is consistent with the majority of published reports, including EGF, macrophage-colony stimulating factor, and platelet-derived growth factor-mediated Rho GTPase-dependent signaling (50, 52, 53).

We have shown that FGF stimulation of PRL transcription requires Rac 1 but not Cdc42 or RhoA. Isoform-specific responses to Rho family members have been implicated in other pituitary/hypothalamic signaling pathways. TRH inhibition of the Ether-a-go-go-related potassium channel in GH4 cells is mediated by Rho, whereas the mutually antagonistic stimulation of Ether-a-go-go-related activity by thyroid hormone (T_3) is dependent on Rac (30). With respect to gene expression, GnRH stimulation of the glycoprotein hormone LHβ promoter is inhibited by dominant negative forms of Rac and Cdc42 (24, 54). Both FGFs and GnRH induce ERK activation in pituitary cells, via PKC, in a Ras-independent pathway (8, 55). However, in contrast to FGF-mediated activation of the PRL promoter (Figs. 5 and 6), GnRH activation of LHβ transcription is dependent on JNK activity (24). Like the rPRL promoter FGF response (7, 8) (Figs. 1 and 4), adhesion-related kinase mediates repression of GnRH transcription via Rac-dependent activation of an ERK signaling pathway that is also independent of Ras (27). Moreover, both FGF stimulation of PRL transcription and adhesion-related kinase-mediated repression of the GnRH promoter are mimicked by transfection of constitutively active Q61Lrac (Fig. 2A and Ref. 27). Thus, the critical role of Rac in FGF regulation of PRL promoter activity provides further evidence for the importance and versatility of Rho family proteins in pituitary-specific signal transduction pathways.

The role of Rho family GTPases in FGF signaling has not been extensively characterized. Rac1 and JNK activation are required for FGF-2-induced DNA synthesis in MCF-7 breast cancer cells (20), and Rac is necessary for FGF-1- and FGF-2-mediated membrane ruffling in breast cancer cells, a response not observed in normal breast epithelium (56). Conversely, FGF-induced growth of astrocyte processes is antagonized by Rac1 and RhoA (21). With respect to transcriptional regulation by FGFs, FGF-2 activates serum response factor (SRF) gene expression, in murine fibroblasts, utilizing both RhoA- and Ras-dependent pathways that target distinct response elements in the SRF promoter (23). In addition, activated Rac1, Cdc42, and Ras (but not RhoA) stimulate the FGF1C and FGF1D promoters while Ras and Rac1 activate expression of the FGF2 gene (57). Interestingly, the FGF- and/or Rac-responsive DNA *cis* elements in the SRF, FGF and, as we have shown, rPRL promoters each map to composite elements containing binding sites for Ets transcription factors (7, 23, 57). Hence, Ets family members may be important nuclear targets of Rac-dependent transcriptional regulation. However, FGF activation of the interstitial collagenase promoter via a bipartite Ets-AP1 element, is independent of Rho and Rac (58).

Rho G-proteins are acknowledged activators of JNK and p38 MAPK kinase cascades (16). However, they have also been shown to activate the ERK 1 and 2 MAPK pathway (27, 59). In addition, Rho proteins can cooperate with Raf to indirectly activate (60) or facilitate the formation of MEK1-ERK signaling complexes (61). Here we show that dominant negative Rac1 inhibits FGF activation of ERK1 and 2 and that expres-

sion of activated Rac1 induces ERK phosphorylation (Fig. 4). Moreover, we have previously demonstrated that FGF-2 and FGF-4 do not activate Raf kinase in GH4 cells (7). Thus, our data are consistent with a direct, rather than permissive, role for Rac1 in FGF-induced ERK activation. In this respect, FGF regulation of rPRL transcription is similar to the Rac1-dependent activation of the plasminogen activator inhibitor (PAI-1) promoter in response to TGFβ (30). TGFβ selectively activated ERK, but not JNK, and stimulated PAI-1 promoter activity via a PKC-dependent, Ras-independent pathway. Furthermore, dominant negative Rac1, but not analogous RhoA or Cdc42 constructs, blocked both FGF activation of the rPRL promoter (Fig. 2) and TGFβ induction of PAI-1 transcription (62). Similarly, IL-6-induced phosphorylation and transactivation of signal transducer and activator of transcription 3 is dependent on sequential activation of Rac1, MKK4, and PKCδ, but not JNK (63). PKC is also required for Rac-dependent activation of cyclin D1 expression (64) and GnRH stimulation of the LHβ-subunit promoter, which is mediated by Rac and Cdc42 (24, 54). Thus, consistent with the results reported herein, Rac appears to be a characteristic element of PKC-mediated, hormone- and growth factor-dependent transcriptional regulation.

In summary, we have shown that FGF stimulation of the rPRL promoter is dependent on PLCγ and Rac1, acting via an atypical MAPK-dependent Rac signal transduction pathway, independent of JNK and the PI3K cascade. Consistent with our results, FGF regulation of DNA synthesis (20), cell morphology (21, 56), and transcription (23) have also been shown to be Rac dependent. Thus, Rac proteins regulate multiple FGF-dependent cellular functions. Together with previous reports discussed above, our results suggest that, in addition to their classical roles in regulation of cell morphology and motility, Rho GTPases may represent archetypal components of Ras-independent, PKC-mediated growth factor signal transduction pathways modulating tissue-specific gene expression.

MATERIALS AND METHODS

Cell Culture and Transfections

GH4T2 rat pituitary tumor cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 12.5% horse serum and 2.5% fetal calf serum (referred to as full serum) (Invitrogen) and 10 U/ml penicillin and 10 μg/ml streptomycin (Invitrogen). Cells were maintained at 37 C in 5% CO_2.

Transient transfections were carried out by electroporation, as described previously (33, 65). Briefly, media were changed 4–12 h before each transfection, and cells were harvested at 50–70% confluency and electroporated in full serum as described (33). After electroporation, 200 μl cells ($3–5 \times 10^6$) were plated in 3 ml DMEM without serum for a final concentration of 0.94% serum to achieve low levels of endogenous growth factors. Cells were incubated for 16–24 h and treated with FGF-2 or FGF-4 (R & D Systems, Minneapolis, MN), or diluent (0.1% BSA in PBS) at a final concentration of 10 ng/ml to the existing media. FGF responses were assayed 6 h post treatment. Electroporations were performed in triplicate for each condition within a single experiment, and experiments were repeated using different plasmid preparations. Total DNA was kept constant using appropriate empty vectors. Luciferase and β-galactosidase assays were performed as previously described (33, 65).

Plasmid Constructs

The promoter constructs pA3-425rPRLluc and pSV40β-gal have been described previously (66). The plasmids pRK5-T17NRac1 and pRK5-Q61LRac1, encoding dominant negative and constitutively active forms of Rac1, respectively (27), were provided by Dr. Gary Bokoch (Scripps Research Institute, La Jolla, CA). Flag-tagged dominant negative Cdc42 and RhoA constructs were a generous gift of Dr. Scott Weed (University of Colorado Health Sciences Center, Denver, CO). These vectors were generated by subcloning *Bam*HI/*Eco*RI fragments containing the GTPase mutants from pRK5-N17Cdc42 and pRK5-N19RhoA (31) into *Bam*HI/*Eco*RI cut pcDNA3-Flag2AB (67). Dominant negative PKB/Akt constructs, pCIS-2mAktA (T308 to A), and pCIS-2mAktAA (T308 to A and S473 to A) (42, 43) were obtained from Dr. Matthew Ringel (Washington Hospital Center, Washington, DC) and dominant negative JNK expression plasmids, pSRαJNK1APF and pSRαJNK2APF (36), were obtained from Dr. Lynn Heasley (University of Colorado Health Sciences Center).

Pharmacological Reagents

The PI3K inhibitors, Wortmannin and Ly 294002, and the PLCγ inhibitors, D609 and ET-18-OCH_3, were obtained from Calbiochem (San Diego, CA). Pharmacological reagents were prepared and stored as per the manufacturer's specifications and used at the concentrations indicated in the specific experiments. Cells were treated with the indicated pharmacological reagents for 30 min to 1 h at 37 C before FGF stimulation.

Western Blot Analysis

GH4T2 cells were serum starved overnight and treated with 10 ng/ml FGF-2 or FGF-4 or the equivalent volume of diluent for the indicated times. Cells (10^7) were washed in ice-cold PBS and harvested in 500 μl RIPA buffer [PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, Complete Protease Inhibitor Cocktail (Roche Clinical Laboratories, Indianapolis, IN)]. Equal amounts of protein (50–100 μg), as determined by the Pierce Mini BCA protein assay (Pierce Chemical Co., Rockford, IL), were resolved by electrophoresis on 10% polyacrylamide-sodium dodecyl sulfate gels and transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA) and probed with the indicated antibodies as described (8). MAPK, JNK, and PLCγ antibodies were obtained from Cell Signaling, and anti-FLAG M2 antibody was purchase from Amersham Pharmacia Biotech (Piscataway, NJ). Membranes were stripped for reprobing using Restore Western Blot Stripping Buffer (Pierce Chemical Co.) according to the manufacturer's directions.

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