Fibroblast growth factor receptor 4 (FGFR4) mediates signaling to the prolactin but not the FGFR4 promoter

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Yu, Shunjiang, Lei Zheng, Sylvia L. Asa, and Shereen Ezzat. Fibroblast growth factor receptor 4 (FGFR4) mediates signaling to the prolactin but not the FGFR4 promoter. Am J Physiol Endocrinol Metab 283: E490–E495, 2002.—Fibroblast growth factor receptors (FGFRs) have been implicated in a multitude of activities. Signaling of the 23 members of the FGF family is mediated through FGFR1–4. We show that FGF-19, which selectively binds FGFR4, can induce prolactin (PRL) but not growth hormone expression. FGFR-19 also stimulated MAPK activation, an effect that was abrogated by a soluble dominant negative (dn) form of FGFR4. The response of the pituitary PRL promoter to FGF maps to an Ets-Pit1 binding site. We have previously shown that the hematopoietic zinc finger-containing transcription factor Ikaros (Ik) regulates FGFR4 as part of an overlapping site with that for an Ets-type factor in the FGFR4 promoter. Thus, we examined whether FGF-19 might regulate its own receptor through the Ets-Ik element in the FGFR4 promoter. Ets stimulated and dn-Ets inhibited basal FGFR4 and PRL promoter activity. In contrast, Ets enhanced FGF-19-induced PRL activation but failed to confer an effect on FGF-19 on the FGFR4 promoter. We conclude that FGFR4 mediates FGF-19 signaling to the PRL promoter. Our data also suggest a possible functional role for Ik in sorting Ets signals to the FGFR4 promoter, as distinct from the PRL promoter, where Ets partners with Pit1.

fibroblast growth factor receptor 4; fibroblast growth factor-19; pituitary; Ikaros; Ets

THE PITUITARY IS THE SITE OF SYNTHESIS AND THE TARGET OF fibroblast growth factors (FGFs) that modulate hormone production and cell proliferation (6). FGF-2 (bFGF) was originally identified in bovine pituitary and is overexpressed in pituitary tumors, with the highest levels noted in aggressive tumors (7, 8). FGF-4 has also been found in transforming DNA sequences from human prolactin (PRL)-secreting pituitary tumors (12). FGF ligands have also been shown to stimulate PRL secretion (6). Little is known, however, about the mechanism of this effect and the receptors involved in mediating it.

FGF signaling is potentially mediated through one of four FGFRs, a complex family of transmembrane receptor tyrosine kinases (11). Each prototypic receptor is composed of three immunoglobulin (Ig)-like extracellular domains, two of which are involved in ligand binding, a single transmembrane domain, a split tyrosine kinase, and a COOH-terminal tail with multiple autophosphorylation sites (11). We have recently shown that targeted expression of a truncated pituitary tumor-derived FGFR4 (ptd-FGFR4) to the pituitary results in tumor formation (10). In marked contrast to the oncogenic effects of ptd-FGFR4, expression of full-length wild-type FGF4 failed to stimulate adenoma formation in the pituitaries of transgenic mice (10). In the current study, we sought to examine other functions of full-length FGFR4 in the pituitary.

Although multiple FGFs interact with each of the four FGFRs, we took advantage of FGF-19 as a novel high-affinity heparin-dependent ligand that shows exclusive binding to FGFR4 (23) to analyze FGFR4 function in a complex cell system. In addition to demonstrating an effect on MAPK activation and PRL gene expression, we investigated the molecular mechanisms of FGF4 signaling to the PRL promoter and compared them with those to the FGFR4 promoter itself. We present evidence that a member of the Ets family of transcription factors is a functional nuclear target that sorts and integrates distinct FGFR4-generated MAPK signals to the PRL and FGFR4 promoters.

MATERIALS AND METHODS

Cell culture. The rat pituitary GH4C1 cell and the human embryonic kidney HEK 293 cell line were propagated in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) with high glucose supplemented with 10% fetal bovine serum (FBS; Sigma, Oakville, ON), 2 mM glutamine, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin. Twenty-four hours before transfection, cells were plated with DMEM containing 10% serum. Primary human pituitary samples were obtained at the time of transsphenoidal pituitary surgery, as previously described (3).

Plasmids. Promoter analysis of the human FGFR4 gene was performed as previously described (24). Full-length human FGFR4 (10) and soluble dominant negative FGFR4 (sFGFR4) (9) were used as previously described. The orien-
**Results**

Expression of FGF-19. Although FGF4 is expressed by pituitary GH4C1 cells (24), we were not able to detect significant amounts of expression of its selective ligand (23) FGF-19 in the pituitary. Figure 1 depicts RT-PCR analysis of human pituitary specimens of different adenoma types. Despite a positive signal for the housekeeping PGK-1 gene, these samples failed to yield a product with FGF-19 primers. As previously crosslinked, prehybridized, and hybridized at 55°C with the rPRL or GAPDH cDNA. Blots were washed and autoradiographed for 24–48 h.

**Western blot analysis.** Protein concentrations were determined by the Bio-Rad protein assay. Equal amounts of protein (50 μg) from cell lysates or media were solubilized in 25× SDS sample buffer and separated on SDS 8% polyacrylamide gel and transferred to nitrocellulose. Apparent molecular weights were determined by comparison with concurrently electrophoresed standards. PRL and GH protein levels were determined using the following antibodies: polyclonal antisera to rPRL or GH [donated by the National Hormone and Pituitary Program (NHPP), National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Child Health and Human Development, Bethesda, MD] applied at dilutions of 1:8,000 and 1:50,000, respectively, or a polyclonal affinity-purified rabbit antisera directed against the carboxy terminus of hFGFR4 (Santa Cruz). Immunoblotting with a monoclonal anti-His antibody (Invitrogen) was used to confirm His-tagged sFGFR4 expression. Total and phosphorylated MAPK was detected by specific antisera that recognize the p42/44 corresponding to Erk1/2 members of the MAPK family (New England Biolabs). An actin control was performed using a monoclonal antibody (Sigma) at 1:500. Blots were scanned for densitometric analysis.

Statistical analyses. Data are expressed as means ± SE. Differences were examined by one-way ANOVA or Student’s t-test, both with a significance level of <0.05.
described (23), the human colon adenocarcinoma cell line SW480 displayed markedly elevated levels of FGF-19 message (Fig. 1).

**FGF-19-mediated FGFR4 activation induces PRL expression.** We took advantage of the FGFR4-selective ligand FGF-19 to determine whether it can activate FGFR4 and stimulate pituitary PRL expression. Because the rodent FGF-19 has not yet been cloned, we used recombinant human FGF-19 and transfected rat pituitary GH4 cells with the human FGFR4 to establish the response to this FGF. Figure 2A depicts Western blotting analysis of lysates from GH4 cells treated as in A with FGF-1 or FGF-19 with rPRL (top) or GAPDH (bottom) cDNA reveal induction of PRL gene expression that is more pronounced by FGF-1 (5-fold) than FGF-19 (2-fold) as assessed by densitometry.

![Fig. 2](http://ajpendo.physiology.org/)

**Fig. 2.** Effect of FGF-19 on prolactin (PRL) expression. A: GH4 cells were transiently transfected with empty vector (pcDNA; left) or full-length human FGF receptor 4 (FGFR4, right) and treated for 24 h with FGF-1 or recombinant human FGF-19 (50 ng/ml) in the presence of added heparin (10 U/ml). Total cell lysates were subjected to Western blotting with an antibody that recognizes rat (r)PRL (top) or actin (bottom). Note stimulation by FGF-19 on PRL expression, an effect that was further enhanced by FGFR4 transfection as evidenced by increased basal as well as FGF-19-induced stimulation. B: densitometric analysis of mean values from 3 independent experiments. C: Northern blotting hybridization of RNA from GH4 cells treated as in A with FGF-1 or FGF-19 with rPRL (top) or GAPDH (bottom) cDNA reveal induction of PRL gene expression that is more pronounced by FGF-1 (5-fold) than FGF-19 (2-fold) as assessed by densitometry.

Because MAPK is considered to be an important mediator of FGF signaling (13), we examined whether FGF-1 and FGF-19 can similarly activate MAPK in wild-type and FGFR4-transfected HEK 293 cells. We chose HEK 293 cells for this experiment, as we had previously shown that these cells do not express significant amounts of FGFR1–3 (10). Both ligands induced phosphorylation of MAPK (Fig. 3, B and C). Moreover, co-transfection of a soluble dn FGFR4 isoform lacking the transmembrane and cytoplasmic do-
mains (9) attenuated FGF-19 as well as FGF-1-induced MAPK activation (Fig. 3, B and C). In contrast to the MAPK response to FGF-19 in FGFR4-transfected HEK 293 cells, untransfected GH4 cells demonstrated a more robust response to FGF-1 than to FGF-19 (Fig. 3A). These findings are consistent with the high selectivity of FGF-19 for FGFR4 compared with FGF-1, which is also capable of activating FGFR1–3 that are co-expressed in the pituitary (1).

FGFR4 transduces signaling to the PRL but not to the FGFR4 promoter. Rat pituitary GH4 cells express PRL, whose basal and FGF-mediated stimulation occurs through a well described bipartite complex formation between Ets and Pit-1 (20). Figure 4A demonstrates that FGF-19 can activate the PRL promoter but not influence its own receptor FGFR4. Treatment of GH4 cells with use of the non-FGFR4-selective ligand FGF-1 resulted in induction of PRL promoter activity by nearly eightfold. As noted at the level of mRNA, the highly FGFR4-selective ligand FGF-19 resulted in a more modest twofold induction of PRL activity compared with FGF-1. We suspect that this difference is determined, at least partially, by the expression of multiple FGFRs in the pituitary (1). Neither FGF-19 nor FGF-1 had influence on FGFR4 promoter activity, however (Fig. 4A; right).

Ets binding sites contribute to PRL and FGFR4 basal promoter activity. We have previously shown that over-expression of Ets1 results in activation of the FGFR4 promoter, an effect that was attenuated with disruption of the overlapping Ik binding site (24). Co-transfection of Ets1 and Ik1 resulted in a greater degree of wild-type promoter stimulation compared with Ets1 alone, suggesting a functional synergism between these two factors in pituitary FGFR4 regulation (24). To further determine the significance of the Ets binding site within the FGFR4 minimal promoter, we compared the effect of co-transfection of wild-type Ets1 or a dn form of Ets on the FGFR4 as well as the PRL promoter. Figure 4B reveals that Ets1 transfection can activate either promoter by ~300%, whereas dn-Ets inhibits both promoters compared with vector control-transfected GH4 cells.

FGF-19 and FGF-1 enhance Ets1 signaling to the PRL but not to the FGFR4 promoter. Given the importance of MAPK in FGFR4 signaling, together with our findings on the role of Ets in regulating FGFR4 expression, we asked whether the Ik/Ets element may mediate FGF-19 responsiveness to the FGFR4 promoter. To answer this question, we examined the response of the FGFR4 and PRL promoters co-transfected with Ets1 and treated with FGF-1 or FGF-19 (Fig. 4C). Treatment with FGF-19 as well as FGF-1 resulted in further enhancement (2- and 6-fold, respectively) of Ets1-mediated activation of the PRL promoter (Fig. 4C, left). Moreover, dn-Ets resulted in attenuation of the FGF-19 and FGF-1 responses on the PRL promoter (Fig. 4C). Despite the effects of FGF-19 and FGF-1 on Ets1 activation of the PRL promoter, both FGF ligands failed to stimulate Ets1-induced FGFR4 activity (Fig. 4C, right). Similarly, co-transfection of Ik1 did not confer an effect for FGF-1 or FGF-19 on the FGFR4 promoter (data not shown). Moreover, no significant effects on GH gene activity were noted in the presence of FGF-1 or FGF-19 treatment, co-transfection of Ets1, or both FGF treatment and Ets1 co-transfection (data not shown).

Fig. 4. Comparison of PRL and FGFR4 promoter regulation by FGF-19 and FGF-1. A: GH4 cells were stimulated with FGF-1 or FGF-19 after transfection with PRL (−422)-Luc promoter (left) or FGFR4 P(−115/+99)-Luc minimal promoter (right). Cells were incubated for 24 h before harvesting for luciferase (Luc) and β-galactosidase (β-gal) assays. Note that FGF-19 and FGF-1 induce PRL but not FGFR4 promoter activity. B: GH4 cells were transiently co-transfected either with the minimal FGFR4 promoter construct P(−115/+99)-Luc or PRL (−422)-Luc, as indicated, along with equal amounts of the expression vector Ets1, or its corresponding empty vector control, or pApr EtsZ-neo [encoding dominant negative (dn) Ets] or its corresponding empty control vector and 20 ng of pCMV-β-gal to control for transfection efficiency. C: GH4 cells co-transfected with the PRL or FGFR4 promoter and Ets1 or dn-Ets were treated with FGF-19 or FGF-1 as described in A. Note that FGF-19 and FGF-1 treatments further enhance Ets1-mediated activation of the PRL, an effect which was attenuated by dn-Ets. Unlike the effects on PRL, neither FGF ligand could appreciably influence FGFR4 promoter activity. All results are presented as means ± SD of 3 separate experiments, each performed in triplicate.
DISCUSSION

Our data identify a role for FGF-19 and its receptor FGFR4 in the pituitary. FGF-19 stimulates MAPK, which in turn phosphorylates and stimulates Ets. Ets binds the promoter in the PRL and the FGFR4 genes, but Ets has a pivotal role in sorting MAPK-generated signals through interaction with Pit-1 to the PRL promoter and through Ik to the FGFR4 promoter. These events confer selectivity of the unique responses generated by individual FGF-FGFR interactions.

The biological importance of the FGF family is underscored by the expanding number of ligands that now includes at least 23 members with varying mitogenic, angiogenic, and hormone-regulatory functions (4, 16). The FGFR domains involved in ligand binding are complex. FGFR1, -2, and -3 bind to or mediate responsiveness to both FGF-1 and FGF-2, whereas FGFR4 binds FGF-1 with 10-fold greater affinity than FGF-2 (19, 22). Although the prototypic receptor has three Ig-like domains, FGFR isoforms that lack the first Ig-like domain are still efficiently activated by FGFs (5); it has been suggested that the first domain may even decrease the binding affinity of FGFs and heparin. The second and third Ig-like domains are implicated in FGF binding in a highly specific fashion for each ligand: FGF-1 binds to loop 2 of FGFR2 but not to loop 3; FGF-7 binds to loop 3 but not to loop 2. FGF-7 binds to the loop 3 IIIb variant, whereas FGF-2 binds the IIIc variant. FGF-1 binds both variants equally (18). FGF-19, however, uniquely binds intact FGFR4 (23). We took advantage of this property to specifically define the role of FGFR4 in the pituitary that commonly expresses multiple types of FGFRs.

As with other tyrosine kinase receptors, FGFRs are activated by dimerization, resulting in autophosphorylation and subsequent recruitment of intracellular signaling proteins, notably phospholipase C-γ (PLC-γ) (11, 17). A 28-amino acid peptide containing Tyr766 of FGFR1 has been identified as the major binding site for PLC-γ; the analogous residue is conserved in all FGFRs, with Tyr764 being the putative site in FGFR4. Activated FGFR1 and FGFR4 homodimers interact with PLC-γ (21). DNA synthesis and cell proliferation are equally induced by FGFR1 and FGFR4 (21). Mutations in the activation loop of kinase domains of FGFR1, FGFR3, and FGFR4 have revealed that all three receptor domains can equally transform NIH 3T3 cells, induce neurite outgrowth in PC12 cells, and phosphorylate Shp2, PLC-γ, and MAPK (13). Using FGF-19 as a selective ligand for FGFR4, we now show that FGF-19 through intact FGFR4 can activate MAPK and that this response is associated with PRL induction.

FGFs have been shown to induce PRL gene transcription (20); however, the role of FGFR4 in mediating this response has not been identified, and, indeed, little is known about which FGF/FGFR interactions are involved in mediating FGF signaling in the pituitary. Previous studies using FGF-2 and FGF-4 in GH4 cells revealed that activation of the PRL promoter is indepen-dent of Ras and Raf-1 but requires MAPK (20). We thus sought to determine whether FGFR4 is involved in transducing the FGF signal to the PRL gene. We show, for the first time, that the FGFR4-selective ligand FGF-19 (23) can activate MAPK and the PRL promoter, thus defining a contribution for FGFR4 in mediating pituitary FGF signaling to the PRL promoter. As anticipated, PRL stimulation elicited by FGF-19 was not as robust as that induced by the non-FGFR-selective FGF-1 ligand, consistent with FGF-1-mediated effects through multiple FGFRs that are expressed in the pituitary (1). The only other previously known function of FGF-19 has been a synergistic interaction with Wnt-8c in initiating inner ear development (14). In contrast to the effects of FGF-19 on the PRL promoter, FGF-19 did not influence the regulation of its own receptor. These findings are in contrast to those in the FGFR2 promoter, where FGFR2 is down-regulated in response to FGF ligand stimulation (2).

In our analysis of the FGFR4 promoter, we characterized a 214-bp fragment of the 5’ region that is important for activity. Further mapping identified an ~40-bp fragment (~64/25) that demonstrated strong binding with nuclear extracts from pituitary GH4 cells (24). This fragment contained functional binding sites for the zinc finger-containing transcription factor Ikflanked by two sites for Sp1 and overlapping with an Ets-type factor. The identification of Ets as a transcription factor in pituitary FGFR4 regulation was interest-

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**Fig. 5.** Schematic illustration of proposed sorting of MAPK signals by Ets partnerships. PRL and FGFR4 promoters contain 2 key functional Ets binding sites. In the case of the PRL promoter, a composite Ets/Pit-1 element confers FGF responses, and another downstream site co-localizes with basal transcription. Similarly, the FGFR4 promoter also contains 2 distinct Ets binding sites with a functional composite element that binds Ets and the zinc finger transcription factor Ikaros (Ik) as shown. Even though FGF-1 and FGF-19 signaling converge at MAPK (ERK1/2), these pathways are likely to target distinct Ets factors and their transcriptional partners. In the case of FGFR4, the Ets/Ik-1 functional partnership appears to sort distinct basal and FGF-stimulated signaling pathways to this receptor’s promoter. This model provides a mechanism by which the combination of distinct Ets partnerships and their binding sites transduces specific growth factor responses.
ing, because the pituitary is well known to express Ets as an important regulator of hormone gene expression (20).

The PRL promoter FGF response element (FRE) has been mapped to two Ets binding sites, and co-transfection of dn-Ets inhibits FGF activation (20). That MAPK is upstream of Ets in this signaling cascade is corroborated by our demonstration that dnEts abrogates the PRL response to FGF stimulation (Fig. 4). One of the FRE sites co-localizes with an Ets/Pit-1 composite. We have previously shown through co-transfection and mutational analysis that Ik1 and Ets1 functionally interact in regulating basal FGFR4 promoter activity (24). It was, therefore, particularly relevant to determine whether the Ets/Ik composite is important in determining pituitary FGFR4 regulation in response to FGF stimulation. Despite the effect of Ets on basal and FGF-stimulated PRL promoter activity, the introduction of Ets induced basal FGFR4 promoter activity but was not sufficient to confer an effect for FGF-1 or FGF-19 on the FGFR4 promoter, as shown schematically in Fig. 5. These data are in agreement with a pivotal role for Ets in sorting MAPK-generated signals. In this instance, Ets appears to sort and integrate distinct FGF-induced MAPK signals through interaction with Pit-1 to the PRL promoter. In contrast, the functional interaction between Ets and Ik does not appear to be sufficient to confer a similar effect of FGF-19 onto the FGFR4 promoter.

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REFERENCES


