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Differentiation of Lactotrope Precursor GHFT Cells in Response to Fibroblast Growth Factor-2*

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Judith López-Fernández‡§¶‖, Daniela Palacios§, Ana I. Castillo§, Rosa M. Tolón§¶, Ana Aranda§, and Michael Karin‡**

From the ‡Department of Pharmacology and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0636, §Instituto de Investigaciones Biomédicas "Alberto Sols," Consejo Superior de Investigaciones Científicas and Universidad Autónoma, Madrid 28029, Spain, and ¶Unidad de Endocrinología e Instituto de Investigación, Fundación Hospital Alcorcón, 29022 Alcorcón, Spain

The mechanisms that control the emergence of different anterior pituitary cells from a common stem cell population are largely unknown. The immortalized GHFT cells derived from targeted expression of SV40 T antigen to mouse pituitary display characteristics of somatolactotropic progenitors in that they express the transcription factor GHF-1 (Pit-1) but not growth hormone (GH) or prolactin (PRL). We searched for factors capable of inducing lactotropic differentiation of GHFT cells. *PRL* gene expression was not observed in cells subjected to a variety of stimuli, which induce *PRL* gene expression in mature lactotropes. Only fibroblast growth factor-2 (FGF-2) was able to initiate the transcription, synthesis, and release of PRL in GHFT cells. However, induction of *PRL* expression was incomplete in FGF-2-treated cells, suggesting that additional factors are necessary to attain high levels of *PRL* transcription in fully differentiated lactotropes. We also show that the FGF-2 response element is located in the proximal *PRL* promoter. Stimulation of *PRL* expression by FGF-2 requires endogenous Ets factors and these factors as well as GHF-1 are expressed at low levels in the committed precursor, suggesting that these low levels are limiting for full *PRL* expression. Moreover, FGF-2 effect on lactotrope differentiation is mediated, at least partially, by stimulation of the Ras-signaling pathway. Our results suggest that, indeed, GHFT cells represent a valid model for studying lactotropic differentiation and that FGF-2 could play a key role both in initiating lactotrope differentiation and maintaining *PRL* expression.

The anterior pituitary gland represents an excellent model system for studying selective gene activation. During embryonic development, different types of hormone producing cells that are highly specialized and synthesize distinct peptide hormones are sequentially derived from a common progenitor cell population within the anterior pituitary anlagen, Rathke's pouch (1). Somatotropes, which express growth hormone (GH),[1] and lactotropes, which express prolactin (PRL), are thought to be derived from a common precursor, the somatolactotrope (2, 3).

The homeodomain transcription factor GHF-1/Pit-1 (4–6) is required both for GH and prolactin *PRL* gene activation and for emergence and expansion of both somatotropes and lactotropes (7, 8). GHF-1 transcripts are detected several days before the emergence of GH- or PRL-producing cells (9), suggesting the existence of a precursor cell for the somatolactotropic lineage. Using the 5′ GHF-1 regulatory region to target the immortalizing oncoprotein SV40 T-antigen in transgenic mice has immortalized this cell type. Mice expressing this transgene exhibit dramatic dwarfism and develop pituitary tumors, which express high levels of *GHF-1* transcripts, low levels of GHF-1 protein, and no GH or PRL (10). This expression pattern is consistent with that of GHF-1-expressing progenitors detected between embryonic days 13 and 15 in the mouse (9). A cell strain, designated GHFT, was established from these tumors. GHFT cells continue to exhibit the same phenotype as the original tumor and were therefore proposed to represent immortalized somatotrope/lactotrope progenitor (10). Thus, GHFT cells may constitute a convenient *ex vivo* system to study the mechanism of cell differentiation in an endocrine gland that itself is rather inaccessible to experimental manipulation during embryogenesis.

The aim of this work was to identify factors that can induce the lactotropic differentiation of this committed precursor and explore their mechanism of action. Our efforts were focused on those agents that are known to stimulate *PRL* gene expression in differentiated lactotropes. Multiple hormones, growth factors, and oncogenes act in conjunction with GHF-1 to regulate pituitary-specific expression of the *PRL* gene. Those factors include ligands for nuclear hormone receptors (11, 12), hypophysiotropic peptides that activate the protein kinase A or protein kinase C pathways (13–16), or ligands of tyrosine kinase growth factor receptors (17–20). Among the latter, the family of fibroblast growth factors (FGFs) appears to play an important role in pituitary organogenesis (21), in differentiation of lactotropes (22), and recently in the dedifferentiation

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‖ Supported by Fondo de Investigaciones Sanitarias Grant FIS/BAE 93/5523 and Ministerio de Educación y Ciencia Grant 42060493EX95. Present address: Servicio de Endocrinología y Nutrición, Hospital Universitario de Canarias, 38320 Santa Cruz de Tenerife, Spain.

** To whom correspondence should be addressed: Inst. de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas, Arturo Duperier 4, 28029 Madrid, Spain. Tel.: 34-91-585-4642; Fax: 34-91-585-4587; E-mail: aaranda@iib.uam.es.

[1] The abbreviations used are: GH, growth hormone; PRL, prolactin; RIA, radioimmunoassay; FGF, fibroblast growth factor; NGF, nerve growth factor; bp, base pair(s); RT, reverse transcriptase; PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid; RPA, RNase protection assay; IR-mPRL, immunoreactive prolactin.

mechanism for lactotrope tumor pathogenesis (23). Particularly, FGF-2 (or bFGF), which was originally isolated from the pituitary gland (24–26), stimulates PRL secretion from normal pituitary cells (27) and from pituitary adenomas (28). FGF-2 was recently found to stimulate the *PRL* promoter in the lactotropic GH4 cell line, and the functional components of the signal transduction pathway activated by this growth factor have been determined (29).

We report here that, among a variety of different agents tested, only FGF-2 was able to initiate the *PRL* gene transcription in GHFT cells. FGF-2 specifically stimulates *PRL* promoter activity in transient transfection assays in GHFT cells. The FGF-2 response element is located in the proximal promoter sequences, and Ets transcription factors are required for stimulation of the *PRL* promoter by FGF-2. GHFT cells express low levels of Ets factors, which could contribute to the reduced promoter responsiveness in these cells. In summary, our results indicate that FGF-2 is a strong up-regulator of *PRL* gene expression in somatolactotropic progenitors and that this factor is a strong candidate for a physiological inducer of lactotropic differentiation *in vivo* and probably also in maintaining the lactotropic phenotype of differentiated cells.

EXPERIMENTAL PROCEDURES

Cell Culture—GHFT cells were grown as described previously (10). Experiments were performed in a defined serum-free (Dulbecco's modified Eagle's medium-high glucose) medium without phenol red, containing insulin (10 μg/ml), sodium selenite (50 nM), human transferrin (10 μg/ml), ascorbic acid (10 μg/ml), 0.1% bovine serum albumin (fraction V), sodium pyruvate, glutamine, penicillin, and streptomycin. Cells were maintained at least overnight in this defined medium before the beginning of the experiments. GH4C1 and HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. For the experiments, the cultures were shifted to medium containing 10% AG1X8 resin-charcoal-stripped newborn calf serum and 24 h later shifted to serum-free medium. Treatments were administered in serum-free medium.

Polymerase Chain Reaction after Reverse Transcription (RT-PCR)—Total RNA was isolated from cells as described previously (30). One μg of total RNA was used in RT-PCR reaction. The reverse transcription of RNA to cDNA (using cloned murine leukemia virus reverse transcriptase) and subsequent amplification (using GeneAmp® PCR process and AmpliTaq® DNA polymerase) were performed all in a single reaction tube to avoid cross-contamination after first strand synthesis. RNA was copied to cDNA using random hexamers. To increase the specificity and sensitivity of PCR amplification, the "hot start" technique was used to suppress primer annealing to non-target sequences. AmpliWax™ PCR Gem 100 (Perkin-Elmer) was added to each single reaction tube containing a subset of amplification reagent for this proposal. For amplification of PRL cDNA, the specific primers 5′-CCCGAATACATCCTATCAAGAGCC-3′ and 5′-TTGATGGGCAATTTGGCACCTCAG-3′ were used. These primers amplified a fragment of 263 bp. As an internal control, the amplified cDNA fragment spanned two spliced exons, such that when genomic DNA was amplified the corresponding bands were larger due to the presence of an intron.

RNase Protection Assay (RPA)—PRL mRNA was detected by RPA. Total RNA from mouse pituitary was used as positive control. mRNA from GHFT and HeLa cells was isolated by Oligotex™ direct mRNA kit (Qiagen). The mouse PRL cDNA was inserted into pGEM2, and after *Xho*I linearization an antisense riboprobe was generated using SP6 RNA polymerase and [α-^{32}P]UTP. The run-off transcription was allowed to proceed for 60 min at 37 °C. The reaction was stopped by digesting the DNA template with 10 units/μl DNase I for 15 min at 37 °C. The probe was purified from a polyacrylamide gel, eluted with the RNAid™ kit (Bio 101), and hybridization was performed overnight at 50 °C. The hybridization solution contained 80% formamide, 40 mM PIPES, pH 6.4, 1 mM EDTA, and 0.4 M NaCl. After hybridization, samples were digested using RNase-ONE™ (Promega; 50 units/sample, 50 min at 30 °C), precipitated with ethanol, and separated on a 6% polyacrylamide, 8 M urea gel. Autoradiography of the RPA showed a double protected fragment of 280 bp. Identical amounts of poly(A)$^+$ RNA (16 μg) of each experimental group were used, except for total mouse pituitary RNA, that served as a positive control, where 0.5 and 2 μg of total RNA were used.

mPRL Radioimmunoassay (RIA)—RIA for mouse PRL was performed in duplicate as described previously (31). RIA components were purchased to Dr. Parlow (Pituitary Hormones and Cancer Center, Harbor-UCLA Medical Center). Iodination of mPRL with ^{125}I was conducted using the chloramine-T method. Rabbit anti-mouse PRL serum (anti-mPRL AFP-131078) was used at a final dilution of 1/200,000 and samples were incubated for 18–24 h at room temperature prior to addition of secondary antibody. Medium samples were compared with a standard curve prepared with reference preparation (AFP-6476C), as described previously (31). The assay sensitivity was 0.48 ng/ml. After 48 of incubation with or without FGF-2, culture media (8 ml) from GHFT and HeLa cells were collected, frozen at −80 °C, lyophilized, and resuspended in 100 μl of phosphate buffer to load directly into the RIA.

Plasmids and Transient Transfections—Constructs containing different fragments of the rat *PRL* promoter fused to luciferase or chloramphenicol acetyltransferase were described previously (12, 20, 33). Expression vectors for GHF-1, c-Ets-1, dominant negative Ets-1 (encoding the DNA binding domain of c-Ets-2), oncogenic Ha-*ras*$^{Val\text{-}12}$, and the dominant inhibitory Ha-*ras*$^{Asn\text{-}17}$ mutant (20) were also used in the transfection assays. Cells were transfected with calcium phosphate and chloramphenicol acetyltransferase and luciferase activity determined as described previously (12, 20). Reporter plasmids (1 μg/plate) were transfected alone or in combination with the amounts of expression vectors indicated in the corresponding figures. In all experiments the amount of DNA was kept constant by addition of the same amount of an "empty" expression vector.

Gel Retardation Assays—Assays were performed with nuclear extracts (32) from GHFT, GH4C1, and HeLa cells. The labeled *PRL* promoter fragment −176 to −101 was obtained by PCR using the oligonucleotides 5′-cccaagcttTGGCCACTATGTCTTCCT-3′ and 5′-CAATCATCTATTTCCGTCAT-3′ as primers. The first oligonucleotide was previously end-labeled with [^{32}P]ATP using T4-polynucleotide kinase. For the binding reaction, the extracts were incubated on ice for 15 min in a buffer (20 mM Tris HCL (pH 7.5), 75 mM KCl, 1 mM dithiothreitol, 5 μg/μl bovine serum albumin, 13% glycerol) containing 3 μg of poly(dI-dC) and then for 15–20 min at room temperature with approximately 50,000 cpm of labeled DNA fragment. DNA-protein complexes were resolved on 6% polyacrylamide gels in 0.5× TBE buffer. The gels were then dried and autoradiographed at −70 °C.

Western Blot Analysis—The levels of GHF-1 and Ets were determined by immunoblot analysis in GHFT, GH4C1, and HeLa cells. Cell extracts were prepared in a lysis buffer supplemented with a mixture of protease and phosphatase inhibitors (32). Equal amounts of proteins (100 μg) were suspended in SDS sample buffer and resolved by 12% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose membrane and, after blocking in 5% dried milk, were probed with a 1/1000 dilution of rabbit polyclonal antibody generated against GHF-1 (5), and with 1/500 dilution of an antibody (sc112, Santa Cruz Biotechnology) that recognizes Ets-1 and Ets-2. Antigen-antibody complexes were detected by chemiluminescence.

RESULTS

Screening for Factors That Induce PRL Expression in GHFT Cells—To identify extracellular factors capable of inducing *PRL* expression in GHFT precursor cells, we tested several hormones, peptides, and growth factors known to have a stimulatory effect on synthesis and/or release of PRL in differentiated lactotropes. We arbitrarily divided the factors into 3 groups. In group I, we analyzed the effects of ligands of nuclear hormone receptors that were demonstrated to transactivate the *GHF-1* and/or the *PRL* genes, including vitamin D_3 (12, 33), retinoic acid (34, 35), and both. 17β-Estradiol, a strong stimulator of *PRL* gene expression, was tested alone and in combination with retinoic acid and/or vitamin D3. In group II, we analyzed the peptides thyrotropin releasing hormone, epidermal growth factor, insulin, insulin-like growth factor-I, vasoactive intestinal peptide, and pituitary adenylate cyclase activating polypeptide 1–38. We also checked combinations of two, three, and four of these factors, along with combinations of group I substances. Group III included human nerve growth factor-β (NGF-β; Ref. 36) and FGF-2 (22), growth factors implicated in differentiation of cultured neonatal pituitary cells. We also checked these factors in combination with group I and group II agents. All treatments were performed under the same

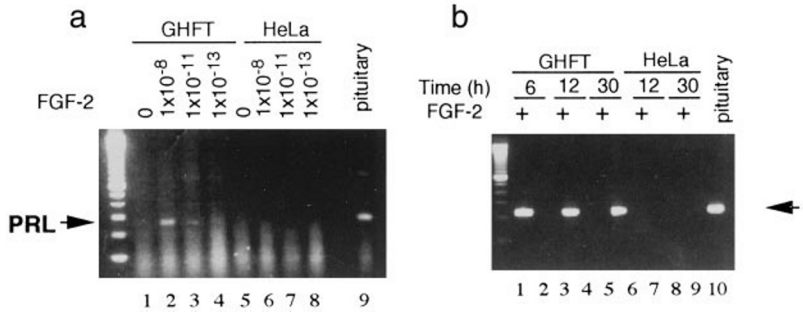

FIG. 1. **FGF-2 induces *PRL* gene expression in GHFT cells.** *a*, representative experiment of FGF-2 dose response on *PRL* gene expression in GHFT cells. GHFT and HeLa cells were treated for 24 h with the indicated concentrations of FGF-2, RNA was extracted, and RT-PCR performed. The band corresponding to the correct length *PRL* mRNA-derived product is indicated. Appearance of a larger band indicates DNA contamination. *Lanes 1–4*, GHFT cells treated with 0, 1×10^{-8}, 1×10^{-11}, and 1×10^{-13} M FGF-2, respectively. *Lanes 5–8*, HeLa cells with the same treatments. *Lane 9*, RNA from mouse pituitary (used as a positive control). *b*, representative experiment of FGF-2 time-course on *PRL* gene expression in GHFT cells. Cells were treated with 10 nM FGF-2 for the time periods indicated or with an equal volume of vehicle (*lanes 1–6*). As a negative control, HeLa cells were also incubated in the presence of absence of 10 nM FGF-2 for 30 h (*lanes 7* and *8*). *Lane 9*, RNA from mouse pituitary.

conditions for at least 48 h. After treatment, total RNA was isolated from cells, and expression of genes for PRL, GH, and GHF-1 was examined by RT-PCR.

This screen revealed that only when FGF-2 was included in the experimental treatment, expression of *PRL* mRNA was detectable. None of the other agents either alone or in combination were able to induce *PRL* transcripts in GHFT cells (data not shown). Fig. 1*a* shows a representative experiment of dose response of FGF-2 effect on GHFT cells. The expected amplified PRL band was obtained in cells treated with 10 nM FGF-2 for 24 h. This band was amplified when mouse pituitary RNA was used as a positive control but not when HeLa cell RNA was used as a negative control. Although 10 nM FGF-2 was the most effective dose in inducing *PRL* gene expression, incubation with 0.01 nM FGF-2 was sufficient to produce a weak detectable signal. Expression of *PRL* mRNA was detectable within 6 h of treatment with FGF-2 and remained elevated for at least 30 h (Fig. 1*b*). The same treatment did not cause the appearance of *PRL* mRNA in HeLa cells. Under all conditions at which FGF-2 induced *PRL* gene expression, no induction of *GH* gene expression was detectable. However, GHFT cells continued to express GHF-1, a transcription factor necessary for *GH* and *PRL* gene expression (data not shown).

Detection of PRL Transcripts by RNase Protection Assay—To confirm that *PRL* gene expression was properly initiated after FGF-2 treatment, RPA was used (Fig. 2*a*). The expected protected double fragment corresponding to properly initiated PRL RNA was found in samples of FGF-treated GHFT cells (*lane 6*) but not in the untreated cells (*lane 5*). Longer exposures of the autoradiogram (up to 2 weeks) confirmed the results, demonstrating the presence of the double protected fragment of *PRL* mRNA only in the positive control (total RNA from mouse pituitary) and in the newly differentiated precursor (GHFT cells after FGF-2 exposure) (Fig. 2*b*). Therefore, RPA confirmed that FGF-2 was able to promote *PRL* expression. However, the levels of *PRL* transcripts produced by GHFT-treated cells were much lower than those expressed in mouse pituitary (*lanes 3* and *4*). These results confirm that, although FGF-2 appears to be an important factor for the initiation of *PRL* gene expression in GHFT cells and is able to initiate lactotrope differentiation, other factors are required to attain the high levels of *PRL* gene transcription found in the pituitary gland.

Detection of PRL Secretion—After mRNA production, the next steps in expression of a polypeptidic hormone include translation, post-translational processing and secretion to the extracellular environment. Fig. 3 shows the effect of FGF-2 on PRL secretion by GHFT cells. Immunoreactive PRL (IR-mPRL) was essentially undetectable in medium from either untreated GHFT cells or from HeLa cells treated for different time periods with FGF-2 (Fig. 3*a*). However, following FGF-2 treatment, mPRL gradually accumulated in the culture medium and reached a level of 2 ng/ml after 24 h of treatment. This stimulatory effect was not lost after longer incubation intervals (72 and 120 h). As shown in Fig. 3*b*, treatment with a low dose of FGF-2 (0.1 nM) for 48 h was enough to produce detectable PRL secretion. Detection of PRL in the cell culture supernatants confirms that FGF-2 initiates differentiation of GHFT cells into PRL-expressing and secreting lactotropic cells.

Induction of PRL Promoter Activity by FGF-2—To analyze the elements that mediate increased *PRL* gene transcription in response to FGF-2 treatment, transient transfection experiments with reporter plasmids containing different fragments (Fig. 4*a*) of the rat *PRL* promoter were performed. As shown in Fig. 4*b*, incubation of GHFT cells with FGF-2 increased the activity of a promoter construct which contains the PRL distal enhancer (between −1.8 and 1.5 kilobase pairs) ligated to the −422/+34 *PRL* promoter fragment. In five independent experiments, incubation for 8–9 h with 1 nM FGF-2 increased luciferase activity by 2.7 ± 0.3-fold ($p < 0.001$). A 24-h incubation stimulated activity by 1.9 ± 0.2 ($p < 0.01$). A construct extending only to −422 bp, which does not contain the distal enhancer, was also stimulated by FGF-2 (Fig. 4*b*). In contrast, a plasmid in which the −78/+34 promoter fragment was ligated to the distal enhancer was not significantly activated by FGF-2. The activity of the −38/+34 fragment was very low and was not affected by FGF-2. These data demonstrate that the elements responsible for FGF-2 responsiveness are contained between nucleotides −422 and −78. These results are in agreement with the previous observation that FGF-2 induction of the *PRL* promoter in GH4 cells maps to this region. A more detailed mapping was performed with plasmids extending to −176, −101, and −70 bp, and to better resolve the effect of FGF-2 the transfections were performed in GH4C1 cells, in which incubation with FGF-2 produced a stronger stimulation of *PRL* promoter activity (Fig. 4*c*). A similar increase (9-fold) was found with constructs containing either the entire 5′-flanking region (3 kilobase pairs) or extending only to −176 bp. However, stimulation decreased to a mere 2-fold when sequences between −176 and −101 were deleted, and disappeared upon a deletion to −70. Thus, the region between −176 and −101 bp of the *PRL* promoter, which contains a GHF-1 binding site overlapping with an Ets binding site (37, 38), significantly contributes to the induction of promoter activity by FGF-2. The role of the proximal Ets binding sites in the residual stimulation by FGF-2 of the reporter that extents −101 bp is demonstrated by

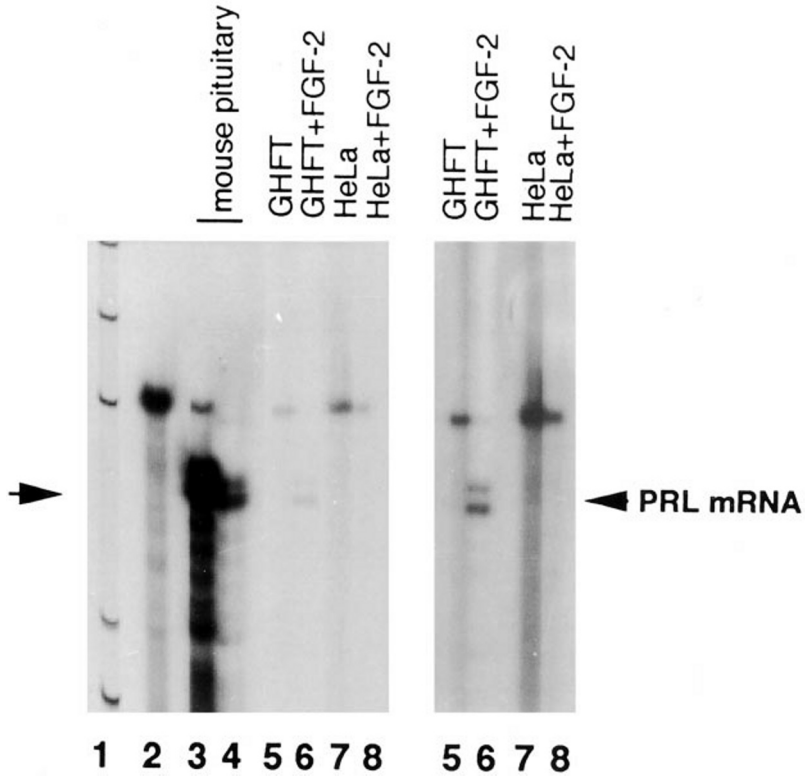

FIG. 2. **RNase protection assay of PRL mRNA.** Autoradiograms of a RNase protection assay with RNA from mouse pituitary, GHFT cells, and HeLa cells. The *left panel* shows the autoradiogram after overnight exposure, and the *right panel* shows *lanes 5–8* of the same autoradiogram after a 10-day exposure. *Lane 1*, size markers; *lane 2*, undigested *PRL* probe; *lanes 3* and *4*, RNA from mouse pituitary (2 and 0.5 μg of total RNA, respectively); *lane 5*, RNA from untreated GHFT cells; *lane 6*, RNA from GHFT cells after exposure to 10 nM FGF-2 for 48 h; *lane 7*, RNA from untreated HeLa cells; *lane 8*, RNA from HeLa cells treated with 10 nM FGF-2, used again as a negative control. Poly(A)$^+$ RNA (16 μg) from GHFT and HeLa cells was used in the assays. A double protected fragment of *PRL* mRNA indicated by an *arrow* is detected in *lane 6* (GHFT cells treated with FGF-2) and in *lanes 3* and *4* (mouse pituitary RNA loaded as a positive control). All other lanes are negative even after long exposure times.

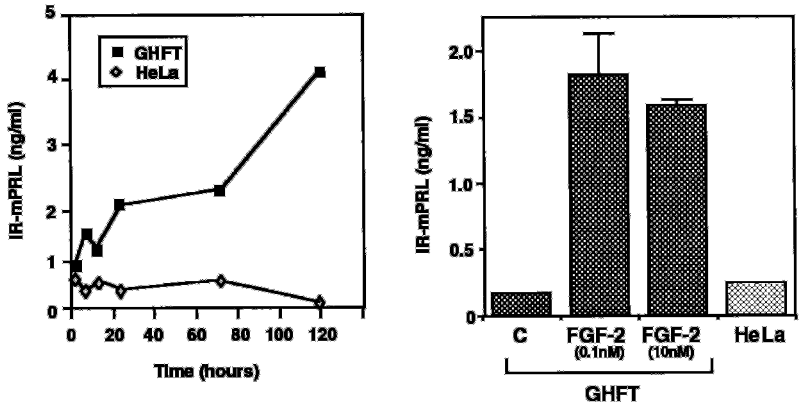

FIG. 3. **Effect of FGF-2 treatment on PRL release in GHFT cells.** *a*, IR-mPRL was determined in the culture media of GHFT and HeLa cells after exposure to FGF-2 (10 nM) for 2, 6, 12, 24, 72, and 120 h. A representative experiment out of three is shown. *b*, accumulation of IR-mPRL was analyzed in media from HeLa cells and from GHFT cells treated for 48 h with 0, 0.1, and 10 nM FGF-2. The data shown are the mean ± S.D. obtained from five independent cultures.

the finding that the −101mut reporter in which the Ets binding sites were rendered non-functional (20) did not show a significant response to FGF-2 (Fig. 4*c*).

Role of GHF-1, Ets, and Ras in PRL Promoter Stimulation by FGF-2—In different experiments, basal *PRL* promoter activity was found to be consistently lower in precursor GHFT cells than in the PRL-producing GH4C1 cells. As both GHF-1 and Ets factors appear to play an important role in *PRL* gene transcription, we tested the possibility that a lower expression of these transcription factors in the precursor cells could contribute to low promoter activity. Indeed, as analyzed by gel retardation assay with the prolactin promoter fragment −176 to −101, GHFT cells expressed lower levels of GHF-1 than GH4C1 cells (Fig. 5*a*). GHF-1 and Ets protein levels were then compared by Western blotting of GHFT, GH4C1, and HeLa cell extracts. This analysis confirmed the reduced content of GHF-1 in GHFT cells. The anti-GHF-1 antibody recognized the characteristic 31- and 33-kDa doublet in pituitary cells, which was less abundant in GHFT cells (Fig. 5*b*). In the blot shown in the figure, obtained after a long exposure, two other weaker bands of 36 and 28 kDa were observed in GH4C1 cells, and no bands were detected in HeLa cells. In addition, the levels of endogenous Ets factors were markedly lower in GHFT cells than in GH4C1 or HeLa cells (Fig. 5*b*). To functionally determine the role of these factors in basal *PRL* promoter activity as well as in its induction by FGF-2, we examined the influence of ectopically expressed c-Ets-1 alone or in combination with GHF-1 on *PRL* promoter activity. Overexpression of GHF-1 and/or c-Ets-1 did not further activate the *PRL* promoter in GH4C1 cells that already contain high endogenous levels of these factors (20). However, cotransfection with the c-Ets-1 vector increased the activity of the *PRL* promoter in GHFT cells and overexpression of GHF-1 further enhanced this activation (Fig. 5*c*). After overexpression of c-Ets-1 and GHF-1, *PRL* promoter activity in GHFT cells was quite similar to that found in GH4C1 cells. These results suggest that the endogenous levels of GHF-1 and Ets factors are limiting in GHFT cells and confirm the important role of Ets proteins in activation of this promoter.

Ras acts synergistically with Ets and GHF-1 to stimulate *PRL* promoter activity in lactotropic cell lines (20, 37, 38). An impaired Ras activation, which is a strong stimulator of *PRL* promoter activity (37), may be responsible for the decreased FGF-2 responsiveness of GHFT cells. However, coexpression of

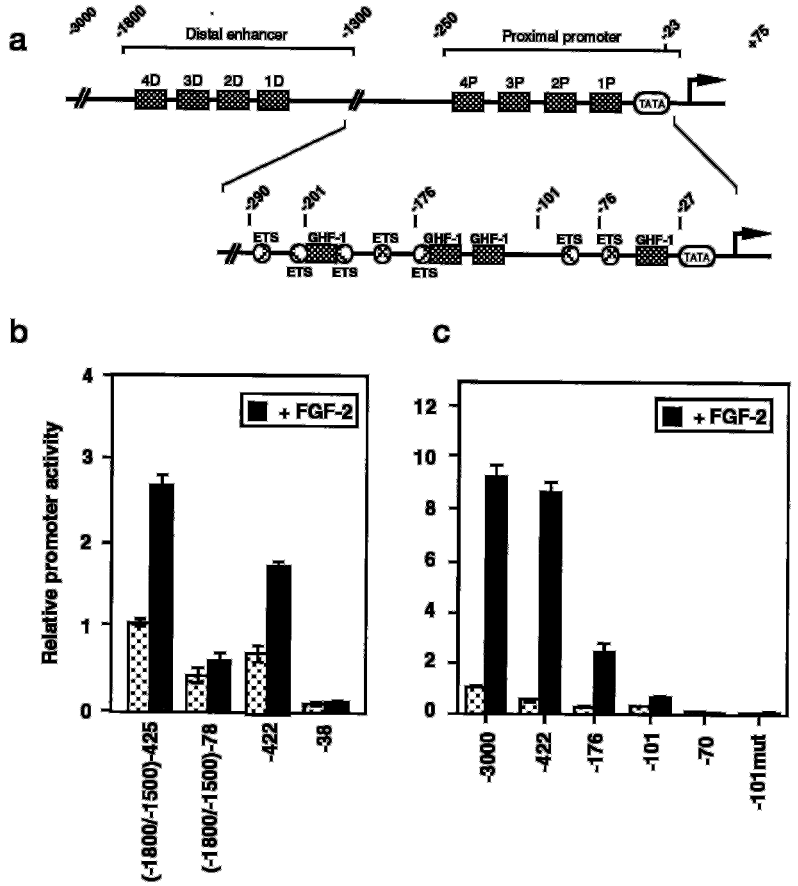

FIG. 4. **FGF-2 activates the *PRL* promoter.** *a*, schematic structure of the 5′-flanking region of the *PRL* promoter depicting the positions of the GHF-1 binding sites in the proximal promoter (*1P–4P*) and the distal enhancer (*1D–4D*). The positions of the Ets binding sites in the proximal promoter are also shown. In the *lower panels* transient transfection experiments were performed in GHFT cells (*b*) and GH4C1 cells (*c*) treated with 1 nM FGF-2 for 9 h. The plasmids used, containing different *PRL* promoter fragments, are indicated in the *ordinates*. Results are expressed as -fold activation relative to the full-length promoter activity, and each data point represents the mean ± S.D. obtained from four independent cultures. Similar results were obtained in two additional experiments.

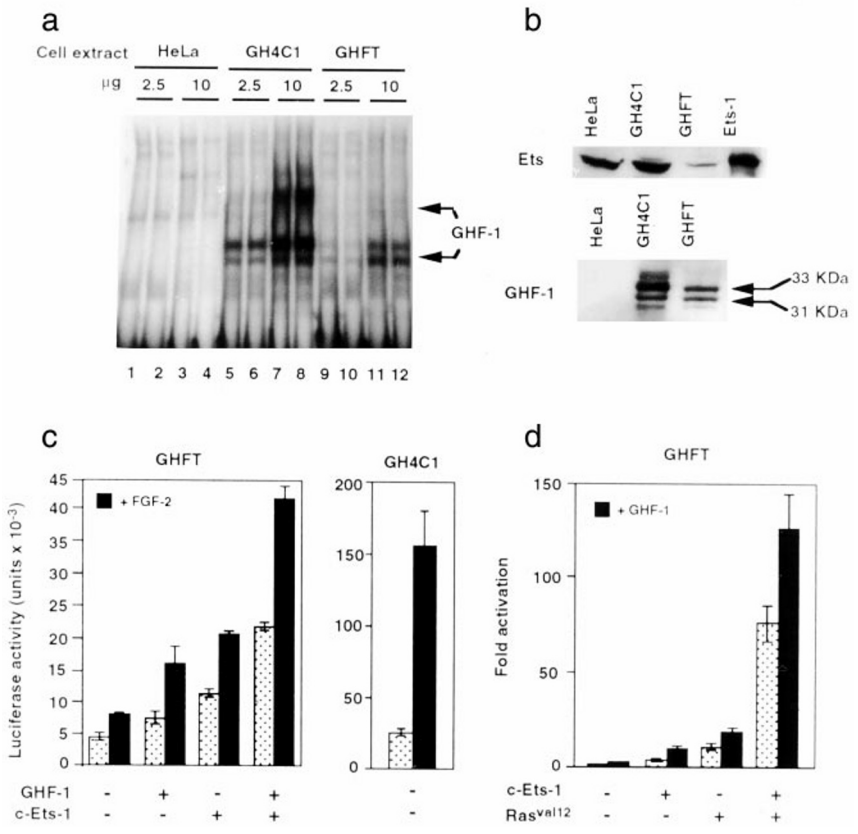

FIG. 5. **GHF-1 and Ets factors in GHFT cells.** *a*, gel retardation assays with extracts from HeLa, GH4C1, and GHFT cells and a labeled *PRL* promoter fragment containing sequences from −176 to −101. *b*, protein extracts were subjected to Western blot analysis with antibodies against Ets factors (*upper panel*) and GHF-1 (*lower panel*). *In vitro* translated c-Ets-1 (5 μl) was used as a control with the anti-Ets antibody. *c*, GHFT cells were cotransfected with the *PRL* promoter construct containing the distal enhancer ligated to sequences −422/+34 and expression vector encoding GHF-1 (0.4 μg) and or c-Ets-1 (0.5 μg). Cells were incubated for 24 h in the presence and absence of 10 nM FGF-2 and luciferase activity determined. Reporter activity was determined in parallel in GH4C1 cells after the same treatment. *d*, luciferase activity was measured in GHFT cells cotransfected 24 h before with the reporter plasmid and expression vectors for c-Ets-1, GHF-1, and/or the oncogenic Ras^{Val-12} mutant (5 μg). All transfection data shown are the mean ± S.D. obtained from triplicate cultures. A representative experiment out of three is shown.

constitutively active Ras^{Val-12} in GHFT cells potentiated *PRL* promoter activity by more than 100-fold in cells cotransfected with c-Ets-1 and GHF-1 (Fig. 5*d*).

Ets factors are targets for FGF-activated Ras-dependent signal transduction pathway responsible for *PRL* promoter activation in GH4 cells (29). To analyze the role of endogenous Ets

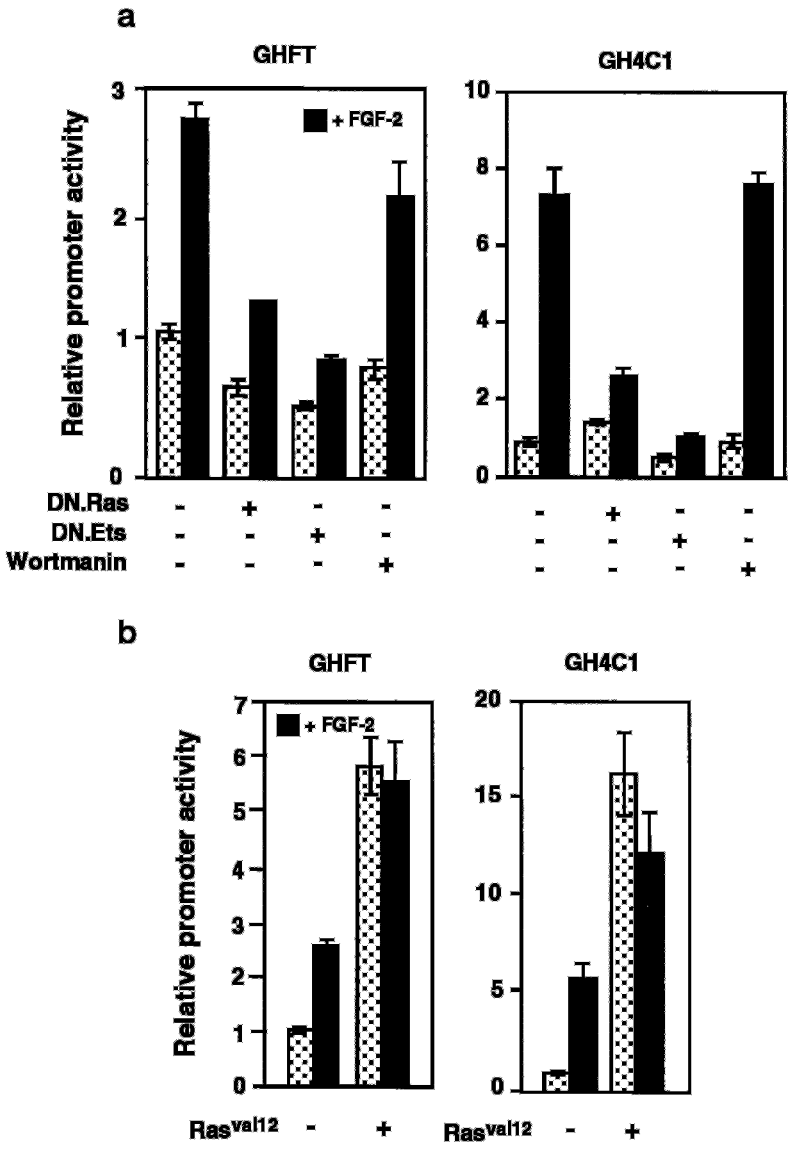

FIG. 6. **Role of endogenous Ets factors, Ras, and phosphoinositol 3-kinase on the FGF-2 response.** *a*, GHFT (*left panel*) or GH4C1 cells (*right panel*) were transfected with the same reporter construct as in Fig. 5*c* and 4 μg of expression vectors encoding dominant negative (*DN*) mutants of Ras or Ets. The cells were treated for 8 h with 1 nM FGF-2. When indicated, the cells were preincubated for 30 min with 100 nM wortmannin before the addition of the growth factor. *b*, GHFT and GH4C1 cells were transfected with the *PRL* promoter construct and 1 μg of $Ras^{Val\text{-}12}$. Luciferase activity was determined in cells incubated in the presence and absence of FGF-2. In both panels, data are expressed relative to the values obtained in the corresponding untreated cells transfected with the reporter plasmid alone. Each data point represents the mean ± S.D. obtained from triplicate cultures, and similar results were obtained in an additional experiment.

transcription factors in FGF-2-induced *PRL* gene expression in GHFT cells, the influence of expression of the DNA-binding domain of c-Ets-2, which results in a dominant negative effect, was examined. Overexpression of the ETS domain significantly reduced the induction of *PRL* promoter activity by FGF-2 in both GHFT and GH4C1 cells (Fig. 6*a*). These results confirm the involvement of Ets-proteins in FGF-2-induced *PRL* gene transcription.

Ets proteins, which mediate transcriptional responses to mitogen-activated protein kinases (MAPK), are an important element in *PRL* promoter stimulation by growth factors, which cause Ras activation (39). However, it was described that FGF-2 induction of *PRL* promoter activity was independent of Ras in GH4 cells (29). We therefore examined the effect of the dominant negative $Ras^{Asn\text{-}17}$ mutant on *PRL* promoter activation by FGF-2 in GHFT and GH4C1 cells. Expression of the dominant negative Ras reduced the response to FGF-2 not only in GHFT cells, but also in GH4C1 cells (Fig. 6*a*). However, a partial response to FGF-2 was still found. This suggests that stimulation of the *PRL* promoter by FGF-2 is at least partially Ras-dependent. Therefore, although Ras and Ets appear to be required for a full FGF-2 responsiveness, activation of additional pathways is also important. This prompted us to investigate whether the phosphoinositol 3-kinase signaling pathway, which contributes to *PRL* promoter induction by insulin-like growth factor-I in GH4C1 cells (20), could also be involved in the stimulatory effect of FGF-2. Incubation of GHFT or GH4C1 cells with wortmannin, a specific inhibitor of phosphoinositol 3-kinase, had little if any effect on induction of the *PRL* promoter by FGF-2 (Fig. 6*a*). Therefore, this signaling pathway does not appear to be required for induction of *PRL* gene expression by FGF-2. On the other hand, as illustrated in Fig. 6*b*, expression of oncogenic $Ras^{Val\text{-}12}$ mimicked the effect of FGF-2 and caused marked *PRL* promoter activation in both cell types. Under these conditions in which the Ras signaling pathway is maximally activated, treatment with FGF-2 did not produce further stimulation.

DISCUSSION

Lactotropes are post-mitotic cells whose principal function is the synthesis and secretion of PRL. During development, the hierarchy of regulatory events that leads to generation of lactotropes remains to be elucidated. Immortalization of neuroendocrine cells at specific stages of differentiation by targeted oncogenesis has been successfully used to establish clonal cell lines representing different steps in a developmental cell lineage (10, 39, 40). In this work we show that FGF-2 initiates *PRL* gene transcription and *PRL* secretion in GHFT cells, suggest-

ing that this growth and differentiation factor could play a role in the generation of the lactotropic phenotype. These findings also suggest that GHFT cells can be used as a model system to analyze the progression from the committed somatolactotrope cell precursor to the terminally differentiated *PRL*-expressing cell.

The effect of FGF-2 on the somatolactotrope precursor is not surprising in view of previous observations of the effect of FGF family members on *PRL* gene expression. For instance, FGF-2 and FGF-4 are abundant in human pituitary tumors, can stimulate PRL secretion from cultured pituitary adenomas, and may even be involved in the development and progression of these tumors (23, 28). Indeed, recent data have demonstrated the early involvement of FGF-2 in prolactinoma pathogenesis (41).

In the rat pituitary tumor cell lines GH3 and GH4, it has been shown that FGFs increase *PRL* mRNA (42, 43) and *PRL* promoter activity in transient transfection assays (29, 42). Our data confirm these observations and demonstrate that *PRL* transcription is initiated by FGF-2 in GHFT cells, which prior to that treatment do not produce any PRL. Therefore, by inducing *PRL* expression and secretion, the hallmarks of lactotropes, FGF-2 converts these murine somatolactotropic progenitors into early lactotropic precursors. The level of *PRL* expression after FGF-2 exposure in GHFT cells was low but specific and with physiological significance because the doses capable to induce lactotropic differentiation are within the calculated K_d of cellular binding sites for FGF-2 (44, 45). The stimulatory effect of FGF-2 caused not only *PRL* gene transcription but also hormonal synthesis and secretion. Conversion of these precursor cells into fully differentiated lactotropes that express high levels of PRL will probably require additional factors that remain to be identified. As the anterior pituitary is composed of a complex network of endocrine and non-endocrine cells, which undoubtedly cooperate to assist each other development, a complete lactotrope development most likely requires endocrine, paracrine, and autocrine mechanisms, which are not present in a single cell population such as GHFT cells.

In contrast to FGF-2, other agents that have a strong stimulatory action on *PRL* gene expression in differentiated lactotropes were unable to induce *PRL* gene transcription in GHFT cells. In agreement with our results, incubation of newborn rat anterior pituitary cells with FGF, but not with other hypophysiotropic peptides, increases significantly the percentage of PRL-producing cells (22). Therefore, in different mammalian species, FGF factors appear to play a role in differentiation and function of lactotropes. It is particularly interesting that NGF did not elicit *PRL* gene expression in GHFT cells, because this factor is able to support the proliferation and differentiation of lactotropes in cultures of pituitary cells prepared from early postnatal rats (36). Our findings suggest that NGF could modulate *PRL* expression only in differentiated lactotropes, after FGF-2 has triggered the differentiation process. This also occurs during differentiation of cells of the sympathoadrenal lineage, in which FGF-2 initiates differentiation and NGF promotes further maturation and survival (46).

Although FGF-2 is able to cause *PRL* gene transcription in GHFT cells, the induced level of *PRL* transcripts is low. An appropriate threshold or constellation of transcription factors required for full activation of the *PRL* promoter may be lacking in GHFT cells, thereby explaining the low level of *PRL* gene transcription. The proximal region of the *PRL* gene is sufficient to mediate transcriptional responses to several hormones in mature lactotropes, and this region contains several binding sites for the pituitary specific transcription factor GHF-1, as well as for Ets factors. Gel retardation experiments with the −176/−101 *PRL* promoter fragment demonstrated that indeed the concentration of nuclear factors that bind to these sequences is significantly lower in GHFT cells than in the differentiated cell line GH4C1. The most abundant factor that binds to these sequences is GHF-1, and our results show that GHFT cells express less GHF-1 protein than GH4C1 cells do. Furthermore, we have also observed that the levels of Ets factors, which also play a major role on *PRL* gene transcription (47), are markedly lower in GHFT cells. That both types of transcription factors are present in limiting concentrations in GHFT cells is functionally proved by the finding that elevated expression of GHF-1 or c-Ets-1 causes a marked increase in *PRL* promoter activity. By contrast, overexpression of GHF-1 or Ets-1 in differentiated GH4C1 cells, where the amounts of endogenous factors are sufficient for maximal stimulation of *PRL* promoter activity, does not result in further *PRL* transcription.

In keeping with previous observations (29), we have mapped the FGF responsiveness of the *PRL* promoter in GHFT cells to sequences containing Ets binding sites. Furthermore, our results demonstrate that endogenous Ets factors are the nuclear targets for the FGF-2 signal transduction pathway. Since Ets factors have also been established as nuclear acceptors for the Ras-MAPK pathway (42) and FGFs activate this signaling pathway, induction of lactotrope differentiation of GHFT cells by FGF-2 is likely to involve the Ras-MAPK signaling pathway as well. However, it had been reported that FGF-mediated induction of the *PRL* promoter in GH4 cells was not mediated via Ras but was dependent on MAPK (29). Our data do not confirm these observations in GHFT cells, as expression of a dominant negative Ras mutant was able to reduce *PRL* promoter activation. It is possible that FGF-2 could activate the promoter via a Ras-dependent pathway in the precursor cells, but that after terminal lactotrope differentiation it relies on a Ras-independent pathway. Nevertheless, this is unlikely since the dominant-negative Ras mutant had a similar inhibitory effect on the FGF-2 response also in GH4C1 cells. On the other hand, our results also show that the dominant inhibitory Ras blocks only partially the FGF-2 response. Taken together our results suggest that activation of the Ras pathway leading to phosphorylation of an Ets factor mediates, at least part of the *PRL* induction by FGF-2, but other, still unidentified, Ras-independent pathway(s) also contribute to this response.

In summary, differentiation of GHFT cells can be induced by FGF-2 through an, at least in part, Ras-dependent pathway where GHF-1 and Ets are limiting factors. Therefore, FGF-2 is a strong candidate for initiating the processes of lactotrope cell differentiation *in vivo* and, probably, also for maintaining the normal lactotrope phenotype of differentiated cells. Undoubtedly, other still unknown factors should act in concert with FGF-2, either simultaneously or sequentially, to induce a fully differentiated lactotrope phenotype. The availability of immortalized lactotrope progenitors has provided an invaluable tool for analysis of the mechanisms of developmental regulation of *PRL* gene expression. Further investigation with this model might be also useful for defining the signaling system that controls growth and dedifferentiation of an inaccessible central endocrine gland in which the more frequent tumors are the lactotrope adenomas. However, caution is necessary to extrapolate the results obtained in a SV40-transformed cell line derived from a pituitary tumor to the *in vivo* mechanisms of lactotrope development.

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