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Prolactin Regulation by Heparin-Binding Growth Factors Expressed in Mouse Pituitary Cell Lines

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Prolactin (PRL) secretion is regulated by both inhibitory and stimulatory factors. Dopamine is the primary inhibitor, but multiple factors stimulate PRL gene expression and release. These can be divided into two categories: those that rapidly stimulate PRL release and those that induce the PRL gene followed by increased release. The pituitary intermediate lobe (IL) contains a PRL-releasing factor (PRF) that rapidly stimulates PRL release. From a mouse IL tumor, we established a nonmelanotroph cell line, mIL5, which secretes a factor that stimulated PRL gene expression and release in vitro. This PRF activity did not rapidly stimulate PRL release and bound to heparin. Our objective was to examine the regulation of PRL by heparin-binding proteins and characterize the PRF activity produced by mIL5 cells. PRL gene expression and release was determined using GH_3 cells, stably transfected with a PRL promoter/luciferase reporter (GH_3/*luc*). After screening mIL5 cells by reverse transcriptase polymerase chain reaction, we found that they expressed two heparin-binding growth factors—basic fibroblast growth factor (FGF-2) and heparin-binding epidermal growth factor (HB-EGF)—which were considered strong candidates for PRL transcriptional regulatory activity. To determine whether the activity produced by mIL5 cells is attributed to FGF-2 or HB-EGF, three approaches were used: heparin-affinity chromatography, Western blotting, and immunoneutralization. The PRF activity in conditioned media eluted from heparin with 1 *M* NaCl whereas both FGF-2 and HB-EGF eluted with >1 *M* NaCl. Neither growth factor was detectable in mIL5 cells by Western blotting. Antibodies directed against FGF-2 and HB-EGF, alone or together, did not abolish this activity from mIL5 cells. In conclusion, FGF-2 and HB-EGF are potent stimulators of PRL gene expression and release but do not account for most of the endogenous PRL gene activity in mIL5 cells. The distinct heparin-binding factor that stimulates PRL gene transcription remains to be identified.

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Introduction

Prolactin (PRL) is a protein hormone produced by anterior pituitary lactotrophs. The regulation of PRL secretion is complex, involving both stimulatory and inhibitory factors that can act either directly on the lactotrophs or indirectly at the hypothalamus *(1,2)*. Dopamine (DA), acting via DA type-2 receptors (D_2R) expressed by the lactotroph, is the primary inhibitor of PRL release *(3)*. However, numerous peptides and growth factors are capable of stimulating PRL gene expression and release in vitro. These can be broadly divided into two categories: those that rapidly stimulate PRL release from storage granules and those that induce the PRL gene expression with a subsequent increase in PRL secretion.

Peptide-releasing factors such as thyrotropin-releasing hormone (TRH) *(4)*, oxytocin *(5)*, angiotensin II *(6)*, and vasoactive intestinal peptide *(7)* rapidly stimulate PRL release. Their action is mediated by activation of specific membrane receptors, ultimately resulting in a calcium-mediated release of PRL from secretory granules. A delayed induction of the PRL gene by these peptides often follows hormone release. Growth factors such as fibroblast growth factor-1 (FGF-1) and FGF-2 also stimulate PRL release, but their action is less rapid, paralleling the increase in PRL gene expression *(8)*. The physiologic role played by these growth factors in the overall regulation of PRL remains undefined.

Several lines of evidence suggest that the posterior pituitary (neurointermediate lobe) participates in the control of PRL secretion under several physiologic conditions *(9–12)*. In addition to DA, the intermediate lobe (IL) contains a fast-acting PRL-releasing factor (PRF) that differs from known peptide releasing factors *(13,14)*, but its structure remains unresolved. Using IL tumors from proopiomelanocortin (POMC)-Tag transgenic mice *(15)*, we previously confirmed the presence of PRF in these tumors *(16)*. Recently, we developed two distinct cell lines from primary IL tumors, named mIL5 and mIL39, which differ in cellular characteristics as well as endogenous PRF activity *(17)*.

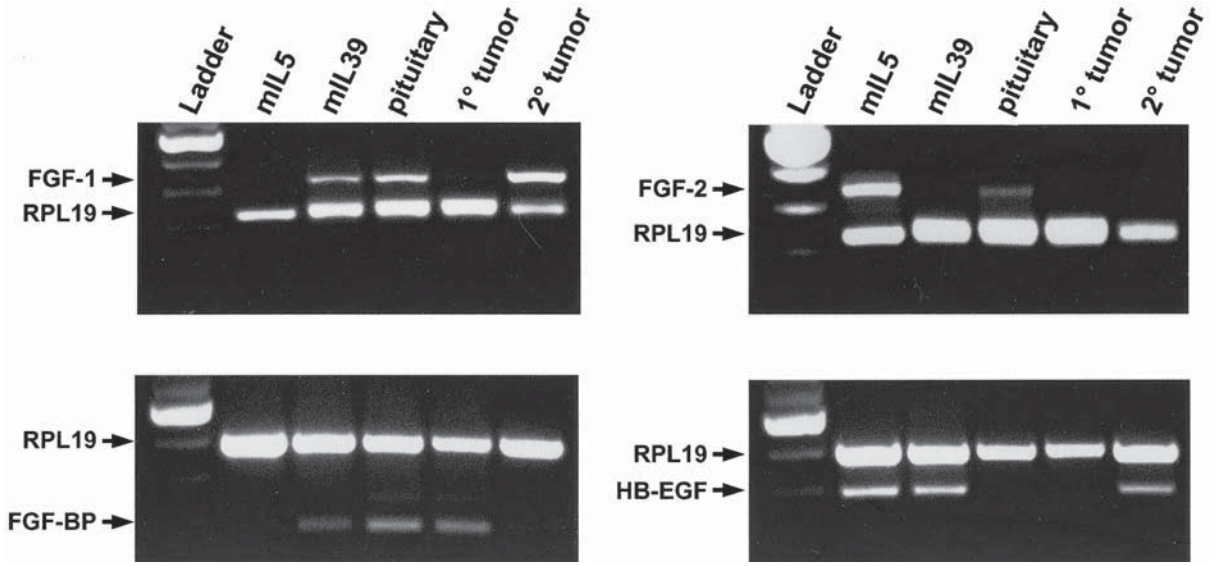

Fig. 1. Expression of FGF-1, FGF-2, FGF-BP, and HB-EGF in mIL5, mIL39, mouse pituitary, and IL tumors (1º and 2º), as determined by RT-PCR. (**Top left**) FGF-1, expected product size of 440 bp; (**top right**) FGF-2, expected product size of 450 bp; (**bottom left**) FGF-BP, expected product size of 291 bp; (**bottom right**) HB-EGF, expected product size of 403 bp. All samples included primers for RPL19, an internal control, expected product sizes of 500 or 333 bp. Ladder, 100 bp.

The mIL39 cells express the POMC and D_2R genes, which define them as melanotrophs. Coculture experiments with GH_3 cells, a somatolactotroph cell line, showed that mIL39 cells contain little, if any, PRL-releasing activity. However, the mIL5 cells, whose cellular phenotype is unknown, secrete a potent stimulator of PRL gene expression and release *(17)*. Preliminary characterization indicates that it is a heparin-binding protein (>5000 mol wt) that does not rapidly stimulate PRL release in vitro. The present article compares genes expressed by the two mIL cell lines, investigates the regulation of PRL by heparin-binding growth factors, and further characterizes this activity produced by the mIL5 cells. The specific objectives of our study were to examine the expression of select heparin-binding growth factors by the mIL cell lines and evaluate their ability to induce the PRL gene, compare their heparin-binding property to that of the activity in mIL5 cell extract and conditioned media (CM), and to determine whether these growth factors account for the PRL-regulating activity in mIL5 cells.

Results

Selected Genes Expressed by mIL Cell Lines, IL Tumors, and Mouse Pituitary

During the initial characterization of PRL gene's regulatory activity from mIL5 cells, we found that it binds heparin. Consequently, we examined whether mIL cells express FGF-1, FGF-2, FGF-binding protein (FGF-BP), or heparin-binding epidermal growth factor (HB-EGF), all of which are heparin-binding proteins that may function as PRL gene transcription inducers. Utilizing reverse transcriptase polymerase chain reaction (RT-PCR), we compared the expression of these heparin-binding proteins in mIL cells, mouse pituitary, as well as primary and secondary POMC-Tag tumors. As evident in Fig. 1, the mIL cell lines differ in their pattern of gene expression. The mIL5 cells express FGF-2, but not FGF-1, whereas mIL39 cells express FGF-1, but not FGF-2. Of the two cell lines, only mIL39 expresses FGF-BP, but both express HB-EGF. The normal mouse pituitary gland expresses FGF-1, FGF-2, and FGF-BP, with a weak and inconsistent expression of HB-EGF. Primary IL tumors express only FGF-BP, whereas secondary tumors express both FGF-1 and HB-EGF, but not FGF-BP.

In an attempt to identify the cell lineage of mIL5 cells, we used RT-PCR to screen for select gene products known to be expressed in the pituitary (Tables 1 and 2). Among the hormones examined, only follistatin (a heparin-binding protein) was expressed by both cell lines, while POMC was expressed exclusively by mIL39 cells. The only receptor expressed by both cell lines was estrogen receptor α (ERα), while mIL39 expressed receptors for DA, corticotropin-releasing factor (CRF), and leptin. Notably, ERβ and the PRL receptor were undetectable in either cell line. Table 2 also summarizes the expression of heparin-binding growth factors by the two cell lines. This gene screening failed to resolve the lineage of mIL5 cells.

Comparison of PRL Induction by Heparin-Binding Growth Factors

The stimulation of PRL gene expression in GH_3/*luc* by FGF-1 and FGF-2 is shown in Fig. 2. FGF-1 stimulated the PRL gene in a linear manner from 10 to 200 ng/mL, reaching a fivefold induction at the highest dose tested, with an ED_{50} of 30 ng/mL; FGF-1 <5 ng/mL was ineffective. FGF-2

Table 1
PCR Primers for Select Mouse Genes and Expected Product Sizes[a]

Mouse Genes	Accession #	Sense primer	Antisense primer	Product size (bp)
CRF-R	X72305	TGCCAGGAGATTC TCAACGAAG	CCGAACATCCAGA AGAAGTTGG	320
D_2R	X55674	CG**C**AGCAG**T**CGA GCTTTCAGA	GCTCATCGTCTTA AGGGAGGT	402, 315
ERα	M38651	GGTC**C**AATTCTGA CAATCGACG	TT**T**CGTAT**C**CCGC CTTTCATC	319
ERβ	U81451	AACCTCAAAAGAG TCCTTGGTGTG	AACACTTGCGAAG TCGGCAG	327
FGF-1	M30641	AAGGGGAGATCA CAACCTTCGC	GGGCAGAAACAA GATGGCTTTC	440
FGF-2	M30644	ATCACCTCGCTTC CCGCAC	TCAGCTCTTAGCA GACATTG	450
FGF-4	M30642	AAAAGGCTTCGG CGGCTCTACTG	TTCATGGTAGGCG ACACTCGGTTCC	353
FGF-BP	U49641	ATGCAGATGGGCT GTGACTGAG	TCCTTCCTGGGCT TCGTGTTTC	291
FOLLISTATIN	Z29532	TGTAATCGGATTT GCCCAGAGC	AAGAAGCACGCCA GAAGAGCAG	348
GALANIN	Z23069	TCACAGGCAAGAG GGAGTTACAAC	TCAAAGCAGAGAA CAGAGGATTGG	305
GH	X02891	CTACAAAGAGTTC GAGCGTGCC	TAGGTTTGCTTGA GGATCTGC	342
HB-EGF	L07264	CGTCGGTGATGCT GAAGCTC	TGGTAACCAGGGA GGCATTTG	403
LEPTIN-R	U58861	GAATGACGCAGG GCTGTATGTC	GAACTGCTTTCAG GGTCTGGTG	348
POMC	J00611 J00612	TCCTGCTTCAGAC CTCCATAGA	GGAAGTGACCCAT GACGTACTT	201
PRL	X02892	TCAGCCTCTGCCA ATCTGTTCC	TCGAGGACTGCAC CAAACTGAG	275
PRL-R	L14811	CAAGGAAACATTC ACCTGCTGG	TGCAGCGAGTCTG GACAAGATAC	506
RPL19	M62952	AGTATGCT**T**AGGC TACAGAAG	TTCCTTGGTCTTA GACCTGCG	500
RPL19	M62952	**C**GAAATCGCCAAT GCCAACTC	TGCTCCATGAG**A**A T**C**CGCTTG	333
VEGF	M95200	GCTCTCTTGGGTG CACTGGA	CACCGCCTTGGCT TGTCACA	431, 563, 635

[a] CRF-R, corticotropin-releasing factor receptor; D_2R, dopamine type 2 receptor; ERα, estrogen receptor α; ERβ, estrogen receptor-β; FGF-1, acidic fibroblast growth factor; FGF-2, basic fibroblast growth factor; FGF-4, hst/K fibroblast growth factor; FGF-BP, FGF-binding protein; GH, growth hormone; HB-EGF, heparin-binding epidermal-like growth factor; POMC, proopiomelanocortin; PRL, prolactin; PRL-R, prolactin receptor; RPL19, ribosomal protein L19; VEGF, vascular endothelial growth factor. Underlined and bold bases designate mismatches against mouse gene sequences (derived from rat gene sequences).

was 100-fold more potent than FGF-1, with an ED_{50} of 0.2 ng/mL or 10 p*M*. The induction of the PRL gene by FGF-2 results in a Gaussian-shaped curve that plateaus at 1 ng/mL and declines at concentrations >20 ng/mL. No change in cell number was observed with either FGF-1 or FGF-2 at any dose tested during the 18-h incubation period (data not shown). The next experiment demonstrated that HB-EGF can stimulate both PRL gene expression and release from GH_3/*luc* cells (see Fig. 3). At 0.5 ng/mL, HB-EGF and FGF-2 increased PRL release five- and ninefold, respectively ($p < 0.05$). Neither of the growth factors examined stimulated PRL release in <4 h (data not shown).

Heparin-Binding Properties of FGF-2, HB-EGF, mIL5 Cell Extract, and CM

Since FGF-2 and HB-EGF are expressed by mIL5 cells (Fig. 1) and stimulate PRL (Fig. 3), it was important to examine whether these growth factors account for the PRF activity

Table 2
Genes Expressed in Mouse Pituitary Cell Lines, as Determined by RT-PCR[a]

	mIL5	mIL39
Hormones		
POMC	–	+
PRL	–	–
GH	–	–
Follistatin	+	+
Galanin	–	–
Receptors		
PRL-R	–	–
D_2R	–	+
ERα	+	+
ERβ	–	–
CRF-R	–	+
Leptin-R	–	+
Growth factors		
FGF-1	–	+
FGF-2	+	–
FGF-4	–	–
FGF-BP	–	+
HB-EGF	+	+
VEGF	+	+

[a]See Table 1 for abbreviations.

in mIL5 cells. In the first experiment, we compared the heparin-binding profiles of FGF-2, HB-EGF, and PRF activity in mIL5 cell extract and CM. Figure 4 shows that both FGF-2 and HB-EGF eluted from heparin with >1 *M* NaCl. All the PRL-regulating activity in either CM or cell extract was heparin bound since no activity was detected in the column flow-through. This activity in CM eluted from heparin between 0.05 and 1 *M* NaCl, while that in the cell extract eluted between 0.05 and 1.5 *M* NaCl, showing partial overlap with either FGF-2 or HB-EGF (Fig. 4).

FGF-2 and HB-EGF Are Undetectable by Western Blotting in mIL5 Cells

The next experiment examined the presence of FGF-2 and HB-EGF proteins in mIL5 cells by Western blotting. Figure 5 shows that FGF-2 was undetectable in any heparin fraction of mIL5 cell extract, at a detection limit of <5 ng. Rat C_6 glioma cells, which express FGF-2 *(18)*, were used as a positive control. FGF-2 was detected only in the 1.5 *M* NaCl heparin fraction of C_6 cell extract (Fig. 5, bottom). FGF-2 was undetectable in heparin fractions of CM from either mIL5 or C_6 cells (data not shown). In addition, HB-EGF was undetectable by Western blotting in heparin fractions from either mIL5 cell extract or CM (data not shown).

Immunoneutralization with Anti-FGF-2 and Anti-HB-EGF

Because FGF-2 and HB-EGF stimulate PRL production at concentrations undetectable by Western blotting, we used

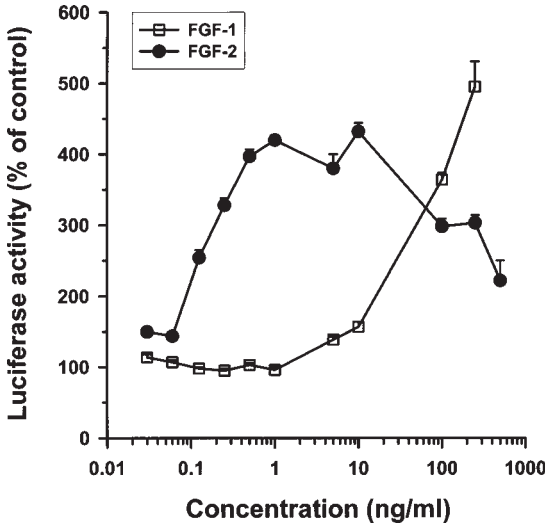

Fig. 2. Induction of PRL gene expression in GH_3/*luc* cells by FGF-1 and FGF-2. Cells were incubated with the growth factors for 18 h, followed by quantitation of luciferase activity in cell lysates by luminometry. Each value is the mean ± SEM of four determinations from a representative experiment.

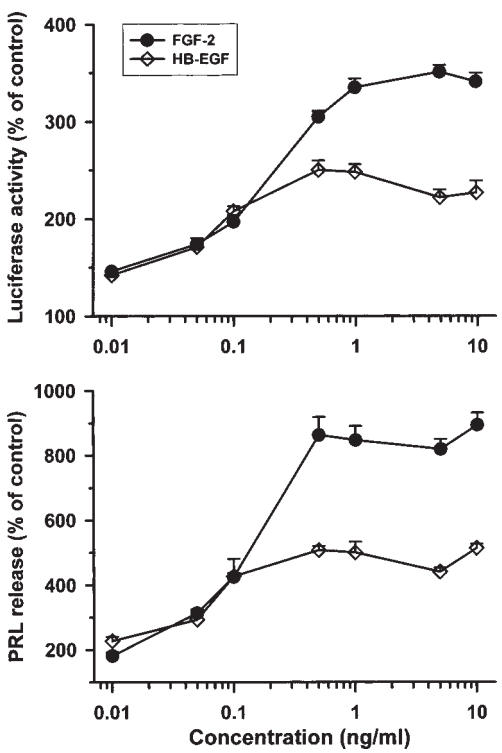

Fig. 3. Stimulation of PRL gene expression (**top**) and release (**bottom**) from GH_3/*luc* cells by FGF-2 and HB-EGF. PRL concentrations in media were determined by RIA after 18 h of incubation with the growth factors. See Fig. 2 for other details.

neutralizing antibodies to determine whether either growth factor accounted for the activity in mIL5 cells. As shown in Fig. 6, the antibodies decreased the PRL gene induction by recombinant FGF-2 (200 pg/mL) by 90% ($p < 0.05$) but decreased gene induction in nonfractionated mIL5 extract

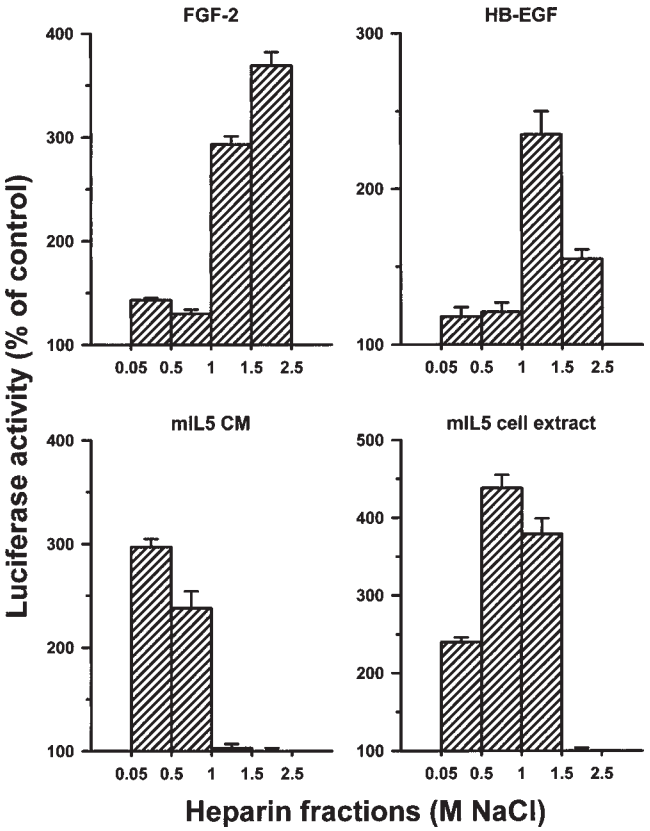

Fig. 4. Comparison of elution profiles of FGF-2, HB-EGF, and PRL gene regulatory activity from mIL5 cell extract or CM from heparin-affinity columns. Fractionation was performed with a discontinuous NaCl gradient at pH 7.4. Aliquots were analyzed for PRL gene induction using GH_3/*luc* cells. See Fig. 2 for other details.

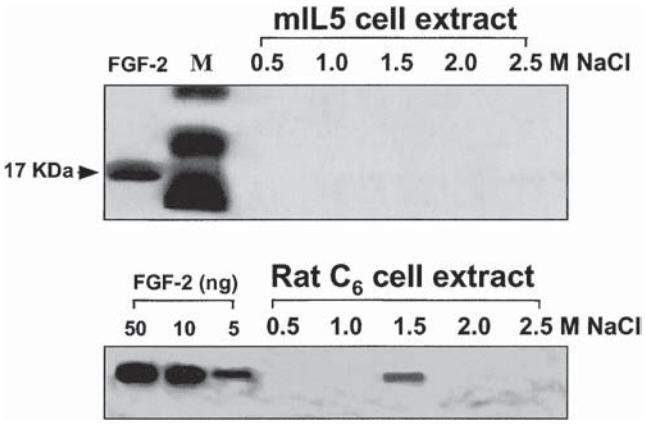

Fig. 5. Western blot analysis for FGF-2 in mIL5 and C_6 cell extracts fractionated on heparin. (**Top**) FGF-2 (17 kDa) was undetectable in any heparin fractions of mIL5 cell extract. (**Bottom**) FGF-2 was detected in the 1.5 *M* NaCl heparin fraction of C_6 cell extract. Recombinant human FGF-2 was used as a standard, with a detection limit of <5 ng. This is a representative experiment repeated two to three times. M, molecular weight markers.

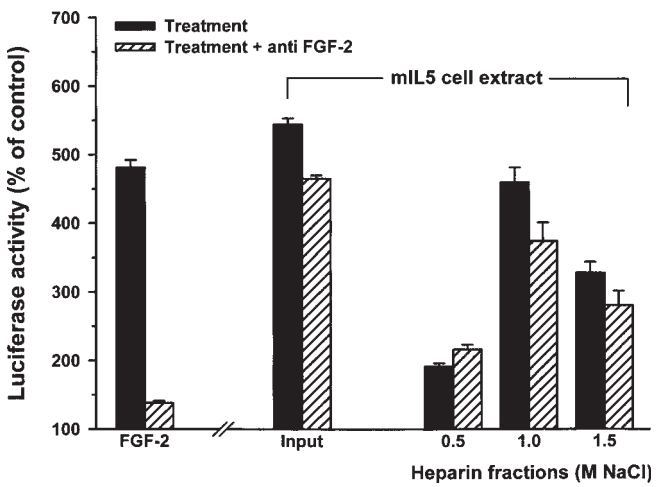

Fig. 6. Comparison of ability of FGF-2 antibodies to neutralize PRL gene induction by recombinant FGF-2 and mIL5 extract. The antibodies reduced the PRL gene induction of FGF-2 (200 pg/mL) to 10% of control (FGF-2 alone) but to 82% of the PRF activity from nonfractionated cell extract (input). The FGF-2 antibodies were ineffective in reducing PRF activity in any heparin fraction of the cell extract. See Materials and Methods and Fig. 2 for other details.

(input) by only 18% ($p < 0.05$). The FGF-2 antibodies did not significantly reduce the PRL gene regulatory activity in any heparin fraction of mIL5 extract (Fig. 6). Comparable results were obtained with mIL5 CM (data not shown). Figure 7 demonstrates that HB-EGF antibodies abolished the PRL gene induction by recombinant HB-EGF (1 ng/mL) but had no effect on this activity in mIL5 CM. A combination of both antibodies was also ineffective in neutralizing the majority of this activity in mIL5 CM (data not shown).

Discussion

We have shown that two heparin-binding proteins, FGF-2 and HB-EGF, are potent stimulators of PRL gene expression and release from GH_3 cells in vitro. The mouse pituitary mIL5 cells expressed the mRNA for both growth factors and secreted a protein that binds heparin and stimulates PRL gene transcription. However, the heparin-binding properties of FGF-2 and HB-EGF differed from the endogenous activity in mIL5 cells, and Western analyses did not detect either FGF-2 or HB-EGF proteins in these cells. Furthermore, antibodies against both FGF-2 and HB-EGF effectively blocked the induction of the PRL gene by these growth factors but were ineffective in reducing the majority of the activity in mIL5 cell extract or CM. These data suggest that FGF-2 and HB-EGF are not responsible for most of the PRL gene transcription induction by mIL5 cells and raise the possibility of a distinct heparin-binding protein with such properties.

Several heparin-binding growth factors, known to regulate cell growth, differentiation, and survival, are expressed in the pituitary *(19)*. Heparin sulfate proteoglycans are abun-

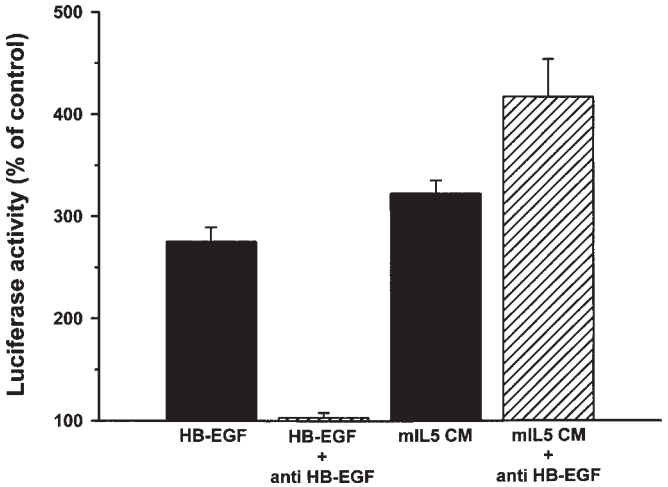

Fig. 7. Comparison of ability of HB-EGF antibodies to neutralize PRL gene induction by recombinant HB-EGF and mIL5 CM. The antibodies abolished the PRL gene induction of HB-EGF (1 ng/mL) but had no effect on PRF activity of the CM (100 µL/well). See Materials and Methods and Fig. 2 for other details.

dant in the pituitary extracellular matrix and the residual cleft that separates the anterior from the intermediate lobe *(20)*. They play a role in sequestering certain proteins via sequence-specific interactions. Changes in the extracellular environment, such as ion concentration or activation of proteolytic enzymes, can liberate proteins bound to heparin and make them readily available to target cells *(21)*. Both FGF-1 and FGF-2 were isolated from the pituitary, but their precise cellular origin remains unclear *(22)*. They differ in their heparin-binding properties, with FGF-1 eluting from heparin with 0.5 *M* NaCl and FGF-2 eluting with 1.5 *M* NaCl *(22)*. Both growth factors can stimulate proliferation of fibroblasts, smooth muscle, and endothelial cells, but neither affected GH_3 cell growth under our culture conditions. Since both lack putative signal sequences, the mechanism by which they are released from the cells is unclear *(23)*. Trafficking of these proteins to the extracellular environment and their release from proteoglycans might involve apoptosis, proteolysis, and/or the FGF-BP.

FGF-1 and FGF-2 have been reported as PRL stimulators *(24–26)*, and the FGF-BP has been implicated as a general mediator of their action *(27)*. Our results demonstrate that FGF-2 is one of the most potent inducers of the PRL gene, with a half-maximal stimulation at a concentration of 10 p*M* (Fig. 2). By contrast, TRH, a well-characterized regulator of PRL release, requires a concentration of 10 n*M* to equally stimulate PRL gene transcription. FGF-2 is extremely potent at low doses, but its dose response curve is Gaussian, with higher doses producing less induction of the PRL gene. FGF-1 requires much higher doses than FGF-2 to stimulate the PRL gene and produces a linear dose response. One might speculate that a combination of these growth factors could have a significant effect on PRL production by lactotrophs.

The mIL5 cells, established from a primary POMC-Tag IL tumor but of an unknown cell lineage, produce and secrete a potent activator of the PRL gene. This activity required a minimum of 4 h of incubation with GH_3/*luc* cells to induce either PRL gene expression or release. Preliminary data obtained with primary anterior pituitary cells also show a slow effect on PRL release. Therefore, this PRL stimulatory activity does not conform with the properties of classic PRFs, which affect hormone release within minutes. Instead, it belongs to a class of heparin-binding proteins (e.g., FGF-2) that exert an indirect effect on PRL release via induction of PRL gene transcription. Our data suggest that mIL5 produces a PRL regulatory activity that is distinct from FGF-2. First, it has a different heparin-binding property than FGF-2. Second, although FGF-2 expression was detected by RT-PCR, the protein was undetectable by Western blots of cell extract or CM, while it was detected in C_6 glioma cells. Third, to rule out the possibility that the FGF-2 protein was translated at very low levels, we used neutralizing antibodies that suppressed the PRL gene transcriptional activity by recombinant FGF-2, but caused minor reduction of the activity of mIL5 cells. Collectively, these data suggest that the bulk of this activity in mIL5 cells is not owing to FGF-2.

Other heparin-binding proteins expressed by mIL5 cells, including HB-EGF, vascular endothelial growth factor (VEGF), and follistatin, were examined as possible candidates for PRL gene regulatory activity. HB-EGF, a member of the EGF family of growth factors, is a membrane-bound protein that is processed to yield a 74 amino acid–secreted product *(28)*. HB-EGF stimulates growth of fibroblasts and smooth muscle cells but not endothelial cells *(29)*, while its action on PRL has not been reported. Our study is the first demonstration of PRL gene induction and release by HB-EGF (Fig. 3). The action of HB-EGF could be mediated by either the EGF receptors *(30)* or the ErbB4 *(31)* receptors that are expressed in the pituitary. The RT-PCR data (Fig. 1) show that HB-EGF is not consistently expressed in the normal mouse pituitary or in primary POMC-Tag tumors but is detectable in both mIL cell lines and secondary tumors. Although HB-EGF binds heparin, it differs in affinity from PRF activity in mIL5 cells. Furthermore, the lack of detection of HB-EGF by Western blot in mIL5 cells and ineffective immunoneutralization of the endogenous activity by HB-EGF antibodies (Fig. 7) suggest that HB-EGF is not produced in large enough quantities to account for PRL gene activation by mIL5 cells.

Follistatin is a 288 amino acid heparin-binding protein that interacts with the β-subunit of both activin and inhibin and modulates follicle-stimulating hormone secretion *(32)*. Follistatin did not stimulate PRL gene expression or release from GH_3/*luc* cells at any dose tested. Another heparin-binding protein, VEGF, is a potent mitogen and permeability factor for the endothelia that exists in several isoforms *(33)*. Like follistatin, VEGF did not affect PRL gene transcription. Notably, FGF-4/hst protein also binds heparin

and can stimulate PRL *(34)*. However, since the mRNA for this protein was not detected by RT-PCR in any mIL cells, FGF-4 was not further explored.

Another objective was to characterize further the two mIL cell lines, which differ in morphology, growth rates, and gene expression *(17)*. Unlike mIL5 cells, which do not express POMC or D_2R, mIL39 cells are classified as melanotrophs. Using RT-PCR we have demonstrated the expression of FGF-1 and HB-EGF, possibly accounting for the weak stimulation of PRL by CM from mIL39 *(17)*. These endogenous growth factors may play a role in the robust proliferation of these cells in vitro or in IL tumor formation in vivo. The proliferative capacity of melanotrophs is suppressed by DA, acting through D_2R *(35)*. The maintenance of cell number in the adult IL is owing to a balance between proliferation and apoptosis, and a disruption of this balance might account for the sensitivity of the IL to tumorigenesis *(36,37)*. The expression of both CRF receptor and leptin-R by mIL39 cells provides a valuable cellular model for studying the involvement of these receptors and their ligands in POMC regulation in the melanotrophs.

In summary, this investigation addressed several important issues. First, compared to classic releasing/inhibiting factors, the role of heparin-binding growth factors in pituitary physiology in general and PRL regulation in particular has been underappreciated. Second, the availability of two distinct cell lines from the IL provides an opportunity for elucidating the function of this inaccessible tissue. Finally, identification of a novel PRF from the IL has been hampered by the sensitivity of PRL gene transcription and release to multiple factors and will require a creative approach.

Materials and Methods

Chemicals

Beetle luciferin and reporter lysis buffer were from (Promega, Madison, WI). Tri-Reagent was from Molecular Research Center (Cincinnati, OH). The protease inhibitors leupeptin, aprotinin, pepstatin A, phenylmethylsulfonyl fluoride and NaF were from Sigma (St. Louis, MO). Superscript II, RNAsin, DNase I, and *Taq* DNA polymerase were from Gibco-BRL (Gaithersburg, MD).

Culture Media

RPMI-1640, Ham's F10, Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, fetal bovine serum (FBS), horse serum (HS), and penicillin-streptomycin (Pen-Strep) were all from Gibco-BRL. ITS and ITS+ premix supplement were from Collaborative Biomedical Products (Bedford, MA). Poly-D-lysine and geneticin (G418 were from Sigma, and gelding serum was from Colorado Serum).

Growth Factors/Hormones, Antibodies, and Chromatography

Recombinant human FGF-1, FGF-2, HB-EGF, and mouse VEGF164 were from (R&D Systems, Minneapolis, MN). Follistatin was from National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Neutralizing rabbit polyclonal antibodies, anti-FGF-2 and anti-HB-EGF, were from American Diagnostica (Greenwich, CT). HiTrap Heparin was from Amersham-Pharmacia (Uppsala, Sweden), and protein A Affi-gel was from Bio-Rad (Hercules, CA).

Animals

Mice

POMC-Tag transgenic mice were generated by Dr. Malcolm Low (Vollum Institute, Portland, OR) as previously described *(15)* and used to establish a breeding colony at Cincinnati. Genotyping on DNA extracted from tail clips was done by PCR with primers for SV40 large T-antigen *(17)*. Primary IL tumors were removed from adult heterozygote females. Tumors were homogenized in 50 m*M* phosphate buffer (PB) with 50 m*M* NaCl, pH 7.4, in the presence of protease inhibitors to generate the cellular extract used for heparin affinity chromatography and Western blots. Total RNA was obtained with Tri-Reagent for RT-PCR analysis. To generate secondary tumors, primary tumors were dissociated with 0.1% trypsin, and 5×10^6 cells/0.1 mL of saline were injected subcutaneously into female athymic *nu/nu* mice (Harlan, Indianapolis, IN). Mice were sacrificed after large secondary tumors had developed (2–4 wk), and RNA was extracted as just described.

Cell Culture

mIL Cell Lines

The mIL5 and mIL39 cell lines were established from primary IL tumors of adult female POMC-Tag transgenic mice as previously described *(17)*. Cultures were maintained in RPMI-1640 medium supplemented with 10% FBS and 1% Pen-Strep at 37ºC under 5% CO_2. CM from mIL5 cells grown to 75% confluence in Nunclon 185-cm^2 flasks (Nunc, Copenhagen, Denmark) were collected after a 3-d incubation with 30 mL of serum-free medium (SFM) composed of DMEM:F10 (1:1) supplemented with 1% ITS. Protein and RNA extracts were obtained as described earlier.

C_6 Glioma Cells

Rat C_6 glioma cells, obtained from the American Type Culture Collection (ATCC) (Rockville, MD), were grown in F10 medium supplemented with 12.5% HS, 2.5% FBS, and 1% Pen-Strep at 37ºC under 5% CO_2. Cells were grown to 75% confluence, harvested by scraping, and protein extracts were obtained as described earlier.

GH_3/Luc Cells

GH_3 cells, obtained from ATCC, were stably transfected with 2.5 kb of rat PRL promoter ligated upstream of a firefly luciferase reporter gene as described previously *(38,39)*. The GH_3/*luc* cells were maintained in F-10 medium supplemented with 15% heat-inactivated gelding serum and 50 μg/mL of G418. Under these conditions the cells were maintained as floating colonies with a low and stable basal luci-

ferase activity. The GH_3/*luc* cells were used to evaluate PRL regulation, as judged by both gene expression and release, described later.

Reverse Transcriptase Polymerase Chain Reaction

Total RNA (5 μg) was reverse transcribed using Superscript II and random hexamers as described previously *(17)*. PCR was performed on 0.5–1 μg of the RT products using *Taq* DNA polymerase. The composition of the primer pairs, all designed to span introns, is described in Table 1. Each PCR reaction also included a primer pair for ribosomal protein L19 (RPL19), serving as an internal control. Mouse tissues served as positive controls, and omission of the RT step was used as a negative control. Annealing temperatures varied with the different primer sets, but the melting and extension temperatures were 94 and 72ºC, respectively. Each temperature step in the PCR cycle was 45 s for a total of 30 cycles. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide and photographed.

PRL Induction

PRL Gene Expression

GH_3/*luc* cells (10,000 cells/well) were plated on poly-D-lysine-coated 96-well plates and incubated in SFM with 1% ITS+ for 24 h. Cells were washed twice with SFM and incubated (200 μL/well) with the test materials for 18 h. Media were analyzed for PRL release by radioimmunoassay (RIA) (see next section), the cells were lysed in 60 μL of lysis buffer, and duplicate 20-μL samples were transferred to black 96-well plates (Packard, Downers Grove, IL). After adding 80 μL of luciferin substrate, luciferase activity, as a measure of PRL gene expression, was quantified by luminometry using a Packard TopCount.

PRL Release

The concentration of PRL in medium collected from GH_3/*luc* cells was determined by a modified RIA *(17)* using a rat PRL RIA kit from NIDDK with RP-3 as a reference preparation. Briefly, 20-μL aliquots were diluted in 10 m*M* phosphate-buffered saline containing 0.1% bovine serum albumin and added to white 96-well plates (Packard) in a final volume of 100 μL. After adding 50 μL each of primary antibody (S9; 1:45,000) and iodinated rat PRL (18,000 cpm), the plates were incubated for 2 d at 4ºC. Protein-A (1:5; 50 μL) was then added and the plates were centrifuged at 4000*g* for 10 min. The supernatant was aspirated, and the pellets were dissolved in 20 μL of 0.1 *M* NaOH, followed by the addition of 200 μL of scintillation fluid (Microscint 20; Packard). The plates were sealed with TopSeal (Packard), and after vigorous shaking, radioactivity was counted using a Packard TopCount. The limit of sensitivity for the RIA was 125 pg/well.

Heparin Affinity Chromatography

One- or 5-mL heparin-Sepharose columns were equilibrated in 50 m*M* PB with 50 m*M* NaCl at pH 7.4. Cell extract, CM, or recombinant growth factors were fractionated stepwise with NaCl (0.05–2.5 *M*) in 50 m*M* PB at pH 7.4. Fractions were desalted and concentrated by centrifugation using spin filters (Ultrafree-15 or -4; Millipore, Bedford, MA) with a 5000 mol wt cutoff. Aliquots were taken for either the GH_3/*luc* bioassay, Western blotting, or immunoneutralization as described next.

Western Blotting

Extracts or CM from mIL5 cells or rat C_6 glioma were fractionated on a heparin affinity column as just described. Each fraction was dialyzed (3500 mol wt cutoff) against water overnight at 4ºC, concentrated, denatured under reducing conditions, and 50 μg of proteins from each fraction were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 13.5% gel. Proteins were transferred to nitrocellulose, incubated with FGF-2 antibodies (3 μg/mL) overnight at 4ºC, and detected using an ECL chemiluminescence kit and X-ray film (Amersham).

Immunoneutralization

Recombinant human FGF-2 and HB-EGF were diluted in SFM to a concentration of 2 ng/mL. Aliquots (0.5 mL) of FGF-2; HB-EGF; as well as mIL5 extract, heparin fractions, or CM were incubated with either anti-FGF-2 or anti-HB-EGF (at a final concentration of 20 μg/mL) for 18 h at 4ºC. After a 3-h incubation with 50 μL of protein A–coupled agarose beads, samples were centrifuged at 10,000*g* to pellet the antibody-antigen complexes. The supernatants were diluted with equal volumes of SFM containing 1% ITS+, and 200 μL/well was incubated for 18 h with GH_3/*luc* cells and analyzed for PRL induction as described earlier.

Data Analyses

All experiments were performed at least twice. Data from the GH_3/*luc* bioassay represent four samples, each analyzed in duplicate. PRL values are expressed as a percentage of control, i.e., GH_3/*luc* cells incubated alone. Data were analyzed by analysis of variance followed by student's *t*-test, and significance was accepted with a value of $p < 0.05$.

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