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# Stimulation of Mitogen-Activated Protein Kinase Pathway in Rat Somatotrophs by Growth Hormone–Releasing Hormone

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**Growth hormone–releasing hormone (GHRH) is an important regulator of somatotroph development and function. However, GHRH signaling is still not completely understood. Signaling through the mitogen-activated protein kinase (MAPK) pathway has been observed in a wide variety of cell types but has not been explored as a mediator of GHRH action. In this study, we examined the phosphorylation of MAPK pathway intermediates in response to GHRH. After treatment of the GH4 rat somatotroph cell line with rGHRH ($10^7$ *M*) for 2.5 min, there was robust phosphorylation of MAPK not seen in vehicle-treated cells. Treatment of HeLa cells with GHRH resulted in no activation of MAPK, but activation was conferred by transfection with the GHRH receptor cDNA. MAPK activation by GHRH was dose dependent from 1 to 100 n*M*, was evident at 2.5 min, peaked at 5 min, and returned to baseline by 20 min. Pretreatment of GH4 cells with somatostatin analog BIM23014 or the MEK1 inhibitor PD98095 prevented the activation of MAPK. Finally, treatment with GHRH increased GH4 proliferation in culture, and this response was prevented by pretreatment with BIM23014 and PD98095. These results indicate that GHRH activates the MAPK pathway. Furthermore, activation of MAPK may mediate, at least in part, the effects of GHRH on somatotroph cell line proliferation. The findings support the concept that multiple pathways mediate the effects of GHRH.**

**Key Words:** Growth hormone–releasing hormone; somatotrophs; mitogen-activated protein kinase; hormone signaling.

## Introduction

Growth hormone–releasing hormone (GHRH) promotes growth hormone (GH) synthesis and secretion, as well as the differentiation and proliferation of pituitary somatotrophs *(1)*. GHRH is produced in the arcuate nucleus of the hypothalamus, is released into the pituitary portal system from the axon terminals in the median eminence, and exerts its influence on pituitary function through a G-protein-coupled receptor (GHRH receptor [GHRHr]) that is a member of the secretin/VIP/glucagon receptor subfamily *(2–4)*. However, despite the importance of GHRH in regulating somatotroph function, intracellular signaling by GHRH has not been as extensively studied as the pathways mediating the actions of other classic hypothalamic-releasing hormones (adrenocorticotropic hormone, thyrotropin-releasing hormone [TRH], gonadotropin-releasing hormone [GnRH]) or somatostatin. Consequently, our understanding of GHRH signaling remains incomplete.

Elevation of intracellular cyclic adenosine monophosphate (cAMP), either exogenously *(5,6)* or endogenously *(7)*, can mimic many, but not all, of the pituitary actions of GHRH. Although early studies established that GHRH-induced GH secretion can be mimicked by exogenous cAMP alone *(8,9)*, the elevation of cAMP cannot fully account for the secretory actions of GHRH on pituitary cells. In the dwarf rat, the intracellular cAMP response to GHRH was completely abolished, whereas 10–15% of the GH secretion persisted *(1)*. Similarly, in superfused pituitary cells *(10)*, GHRH elicited a rapid rise in intracellular cAMP, but cAMP could not itself sustain GH release. Conversely, desensitization of the GH secretory response to repeated GHRH pulses or inhibition of GH secretion with somatostatin was not temporally accompanied by a reduction in cAMP. Similarly, reduction in GH release following exposure to a GHRHr antagonist preceded the reduction in cAMP generation, and the return of GH release following removal of the antagonist occurred sooner than the recovery of cAMP generation *(11)*. Changes in GH were not preceded by changes in cAMP concentration. In ovine pituitary cells, cAMP activation of protein kinese A alone was unable to reproduce the effect of GHRH stimulation on GH release *(12)*. An examination of the effects of changing intracellular pH on GHRH-stimulated cAMP generation and GH release also demonstrated a dissociation of these two GHRH responses *(13)*. Finally, nitric oxide

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(NO) is able to completely abolish GH release in response to cAMP, while being only partially effective in inhibiting the response to GHRH, suggesting the involvement of NO-insensitive pathways *(14)*. Taken together, these results suggest that signaling by GHRH is complex, with the participation of more than one signal transduction mechanism necessary to explain fully the secretory responses of the somatotroph to GHRH.

Several alternate signaling pathways capable of mediating GHRH action have been proposed. These include cell depolarization secondary to sodium channel activation *(15)*, increased intracellular free calcium *(15–17)*, and protein kinase C (PKC) activation *(18)*. However, the relationship among GHRHr binding, generation of cAMP, and activation of these alternate pathways remains unclear.

While our understanding of the secretory actions of GHRH remains incomplete, even less is known about the intracellular pathways mediating the proliferative effects of GHRH on somatotrophs. Analogs of cAMP or somatotroph-targeted expression of cholera toxin in transgenic animals induce GH synthesis and cellular proliferation in primary somatotroph cells in culture *(7,19)*. Conversely, GH promoter-driven overexpression of a dominant negative variant of cAMP-responsive element binding protein (CREB) in transgenic mice leads to decreased GH synthesis and somatotroph hypoplasia *(20)*, a finding interpreted to indicate that inhibition of the transcriptional effects of cAMP prevents the genomic and proliferative effects of GHRH. However, phosphorylation/activation of CREB can occur by alternate intracellular pathways *(21–23)*, rendering the interpretation of these earlier experiments more difficult. The concept that GHRH-induced somatotroph proliferation is a result of cAMP generation without activation of other intracellular signaling pathways is not well supported in the literature and would represent a nearly unique example of cAMP-promoted cell proliferation *(24)*.

The mitogen-activated protein kinase (MAPK) pathway has been implicated in cellular proliferation in a wide variety of cell types *(25)*, including proliferative responses to other G-protein receptor ligands *(26–29)*. Although GHRH clearly plays an important role in regulating somatotroph proliferation, the participation of the MAPK pathway in GHRH signaling has not previously been examined. We report here that GHRH induces robust, dose-dependent activation of MAPK signaling components in the GH4 rat somatotroph cell line, that this activation of MAPK is associated with somatotroph proliferation, and that this activation is antagonized by somatostatin.

## Results

To determine whether GHRH activates MAPK, rat GH4 cells were serum starved as indicated, followed by treatment with rat GHRH (rGHRH) ($10^7$ *M*) or vehicle. Epidermal growth factor (EGF), a known activator of MAPK in somatotrophs *(30)*, was used as a positive control. Activation of MAPK was examined by Western blot analysis of equally loaded protein samples with a phospho-MAPK specific antibody. As shown in Fig. 1, after exposure to vehicle, no phosphorylation of MAPK was evident. However, after exposure to rGHRH for 2.5 min, there was robust stimulation of MAPK in GH4 cells. Activation of MAPK by EGF was evident after 10 min.

Activation of MAPK by GHRH is specific for cells expressing the GHRHr. As shown in Fig. 2, exposure of HeLa cells to GHRH at doses as high as 100 n*M* resulted in no activation of MAPK, whereas exposure EGF (25 n*M*) resulted in activation similar to that seen in GH4 cells. However, transfection of HeLa cells with a mammalian expression vector encoding the rGHRHr cDNA conferred the ability to activate MAPK in response to 1 n*M* GHRH. The reduced activation of MAPK by GHRH in transfected HeLa cells seen in Fig. 2. compared with that seen in wild-type GH4 cells (Fig. 1) reflects the fact that only a minority of HeLa cells in the culture are successfully transfected by the protocol used (data not shown). Similarly, rGHRH had no effect on MAPK activation in α-T3 thyrotrophs *(31)* that lack GHRH receptors.

The MAPK response to GHRH was dose dependent. GH4 cells were exposed for 3 min to GHRH (1–1000 n*M*) or vehicle. For comparison, the ED50 for cAMP generation in response to GHRH is approx 10n*M (2)*. Following chemiluminescent visualization (ECL, Amersham), the image density of the phospho-MAPK band was quantitated by video densitometry as described in Materials and Methods. As seen in Fig. 3, GHRH promoted distinct MAPK activation at doses as low as 1 n*M*. This response peaks at 100 n*M*, similar to that for cAMP, and then decreases at higher doses.

To determine the time course of MAPK activation in response to GHRH, GH4 cells were exposed to 1 or 10 n*M* GHRH for 1–20 min. As shown in Fig. 4, GHRH activation of MAPK is rapid and followed by rapid extinction, characteristics shared by MAPK activation by other hormones and growth factors. Furthermore, the details of the time course depend on the dose of GHRH. In response to 1 n*M* GHRH, activation was evident at 2.5 min, peaked at 5 min, and returned to baseline or slightly below by 20 min. When the higher dose of 10 n*M* was used, MAPK phosphorylation peaked at 2.5 min followed by steady decay.

We next examined the effect of somatostatin, a physiological antagonist of GHRH, and of PD98095, an agent that prevents MAPK phosphorylation by blocking the activity of the MAPK kinase MEK1 *(32)*, on GHRH activation of MAPK. GH4 cells were exposed to the somatostatin analog BIM23014 or PD98095 for 10 mins, followed by 10 n*M* GHRH for 1–5 min. The resulting activation of MAPK was examined as described and compared to the response to GHRH without pretreatment with the inhibitor. As seen in Fig. 5A, exposure to vehicle had no effect on MAPK phosphorylation. GHRH (10 n*M*)

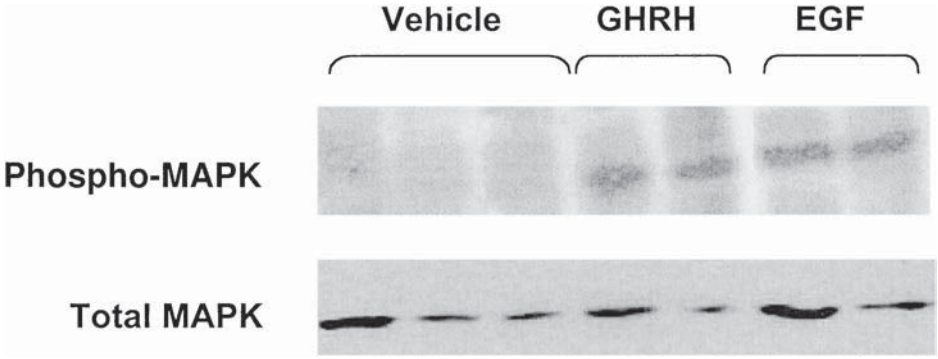



Fig. 1. GHRH induces phosphorylation of MAPK in GH4 cells. Serum-starved GH4 cells ($2.5 \times 10^6$ cells/dish) were exposed for 2.5 min to GHRH (10 n*M*) or for 10 min to EGF (25 n*M*). Equal protein samples of cell extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to nitrocellulose, Western blot analysis with antiphosphospecific MAPK antibody, and visualization by chemiluminescence as described in Materials and Methods. After visualization, membranes were stripped as described in Materials and Methods and reprobed with antibody to p42/p44 MAPK (Total MAPK) to verify equal protein loading. Each lane represents the extract of an independent dish.

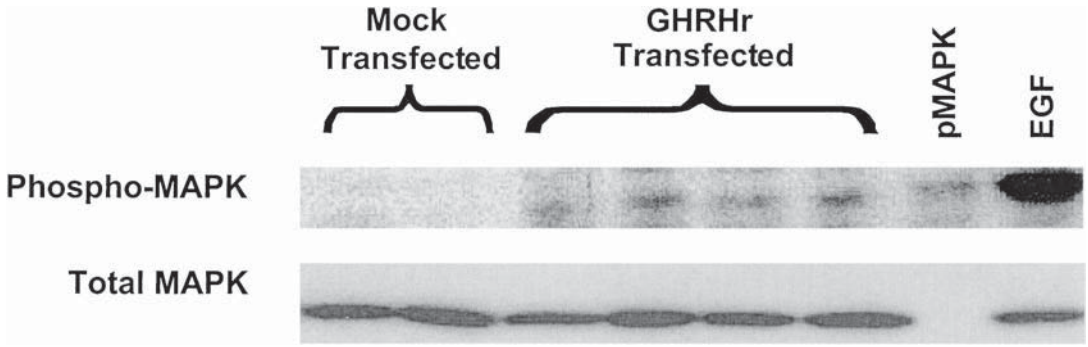



Fig. 2. GHRH induces MAPK phosphorylation in HeLa cells transiently transfected with GHRHr. HeLa cells ($2.5 \times 10^6$ cells/200 μL of Dulbecco's modified Eagle's medium [DMEM]/10% fetal calf serum [FCS]) were transfected with 5 μg of pcDNA3.1 or 5 μg of vector containing full-length GHRHr cDNA at 220 V and 500 μF. Following incubation overnight in 3 μL of DMEM/0.6% FCS, cells were serum starved for 4 h. Mock transfected HeLa cells were treated with 100 n*M* GHRH for 2.5 min or 25 n*M* EGF for 10 min. GHRHr transfected cells were treated with 1 n*M* GHRH for 2.5 min. Equal protein samples of cell extracts were separated by SDS-PAGE followed by transfer to nitrocellulose, Western blot analysis with antiphosphospecific MAPK antibody, and visualization by chemiluminescence as described in Materials and Methods. Phospho-MAPK standard was run in parallel as a control (pMAPK). After visualization, membranes were stripped as described in Materials and Methods and reprobed with antibody to p42/p44 MAPK (Total MAPK) to verify equal protein loading. Each lane represents the extract of an independent dish.

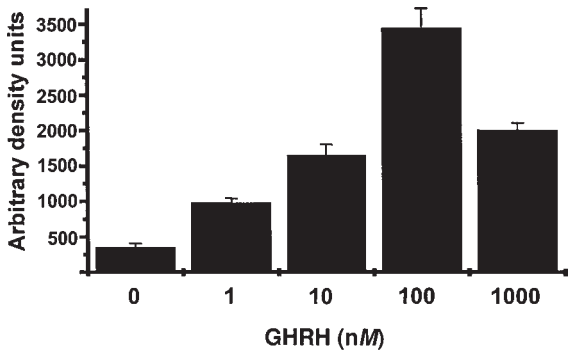



Fig. 3. GHRH induction of MAPK phosphorylation is dose dependent. Serum-starved GH4 cells ($2.5 \times 10^6$ cells/dish) were exposed for 3 min to rat GHRH in doses ranging from 1 to 1000 n*M* or vehicle alone. Equal protein samples of cell extracts were separated by SDS-PAGE followed by transfer to nitrocellulose, Western blot analysis with antiphosphospecific MAPK antibody, and visualization by chemiluminescence. Optical density (OD) of resulting autoradiographs following chemiluminescence was determined using video densitometry. Bars represent mean OD ± SEM ($n = 3$).

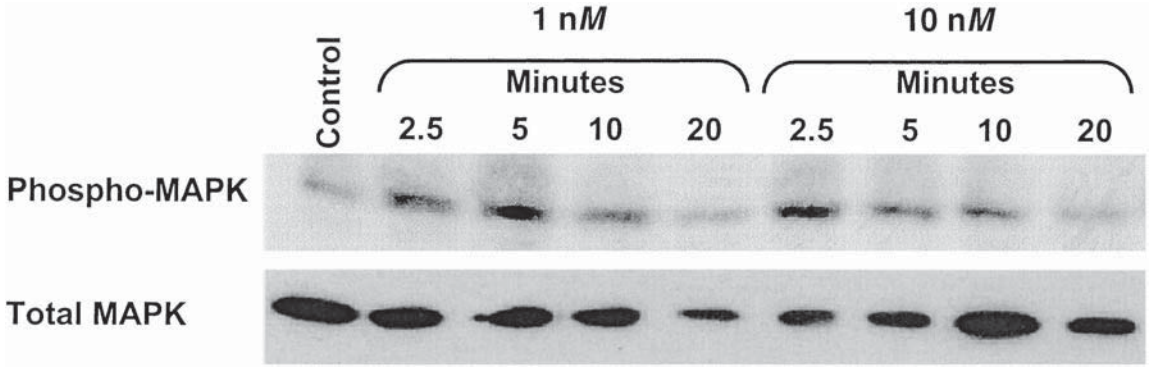



Fig. 4. Time course of MAPK activation by GHRH is dose dependent. Serum-starved GH4 cells ($2.5 \times 10^6$ cells/dish) were exposed (2.5–20 min) to rGHRH (1 or 10 n*M*) or vehicle alone. Equal protein samples of cell extracts were separated by SDS-PAGE followed by transfer to nitrocellulose, Western blot analysis with antiphosphospecific MAPK antibody, and visualization by chemiluminescence. After visualization, membranes were stripped as described in Materials and Methods and reprobed with antibody to p42/p44 MAPK (Total MAPK) to verify equal protein loading. Each lane represents the extract of an independent dish.

resulted in the expected activation of MAPK, with a peak at 2.5 min followed by a rapid decline to below baseline. BIM23014 by itself had no effect on MAPK activation. However, pretreatment with BIM23014 resulted in complete inhibition of MAPK activation in response to GHRH. Similarly, as shown in Fig. 5B, pretreatment with PD98095 completely prevented the activation of MAPK by GHRH.

Activation of MAPK has been implicated in the regulation of receptor-induced cellular proliferation in a wide variety of systems. To investigate the potential role of MAPK in GHRH-induced somatotroph proliferation, we examined the proliferation of GH4 cell proliferation in response to GHRH in the absence and presence of inhibitors of MAPK. As shown in Fig. 6, treatment with GHRH increased somatotroph cell number between 24 and 48 h ($p < 0.01$) compared with cells maintained in serum-ree medium. Stimulation of somatotroph proliferation by GHRH has been demonstrated previously using primary rat pituitary somatotrophs *(5)*. However, this is the first demonstration of the effect of GHRH on a somatotroph cell line. Pretreatment of the cells with BIM23014, which inhibits activation of MAPK by GHRH, completely prevented the GHRH-induced increase in cell number, but had no effect on basal GH4 proliferation (Fig. 6A). Similarly, pretreatment of the cells with PD98095 prevented GHRH-induced cell proliferation (Fig. 6B). Furthermore, unlike BIM23014, PD98095 inhibited the proliferation of GH4 cells in the absence of GHRH stimulation, confirming that the MAPK pathway likely plays a role in basal somatotroph proliferation.

## Discussion

The results presented herein demonstrate that GHRH activates the MAPK pathway in the GH4 rat somatotroph cell line. This activation is dose dependent in a physiological dose range, rapid, and reversible. Furthermore, the activation is prevented by somatostatin, a physiological antagonist of many of the effects of GHRH, as well as by the MEK inhibitor PD98095. Finally, the activation of MAPK by GHRH appears to play an important role in mediating the effects of this hypothalamic neuropeptide on somatotroph cell line proliferation. These results broaden our current understanding of intracellular signaling by GHRH and raise the possibility that distinct intracellular pathways mediate the various actions of GHRH on somatotroph cells.

Activation of the MAPK pathway by G-protein-coupled receptors has been reported in a number of systems *(26–28,31)*, including adrenergic receptors, muscarinic receptors, as well as receptors for the hypothalamic neuropeptides TRH *(28)* and GnRH *(31)*. Therefore, it would appear that activation of MAPK by GHRH, although not previously reported, is not unexpected and may be a relatively common feature of signaling by neuroendocrine G-protein-coupled receptors.

GHRH appears to be a critical trophic factor promoting development and proliferation of the pituitary somatotroph population *(5,6)*. In animal models, GHRH deficiency or resistance leads to failure in somatotroph development *(33–35)*. In the *Gsh-1* knockout mouse *(33)*, absence of GHRH expression in the arcuate nucleus is accompanied by profound GH deficiency and dramatically reduced somatotroph population in the adult pituitary. Similarly, GHRH resistance in the Little (*lit*) mouse and the Dwarf (*dw*) rat *(34–39)* is accompanied by GH deficiency and a 95% deficit in the number of pituitary somatotrophs; the other pituitary cell types are intact.

Although these experiments strongly imply an important role for GHRH in somatotroph proliferation in vivo, the effect of GHRH on proliferation of somatotrophs in vitro has been more difficult to document. GHRH has been shown to stimulate proliferation of rat somatotrophs in primary culture, but this response was slow and resulted in an increase of only a few percent of cells after many weeks of treatment *(5)*. Similarly, GHRH stimulates expression of c-fos in primary rat somatotrophs, a response thought to represent early stages in cellular proliferation *(6)*.

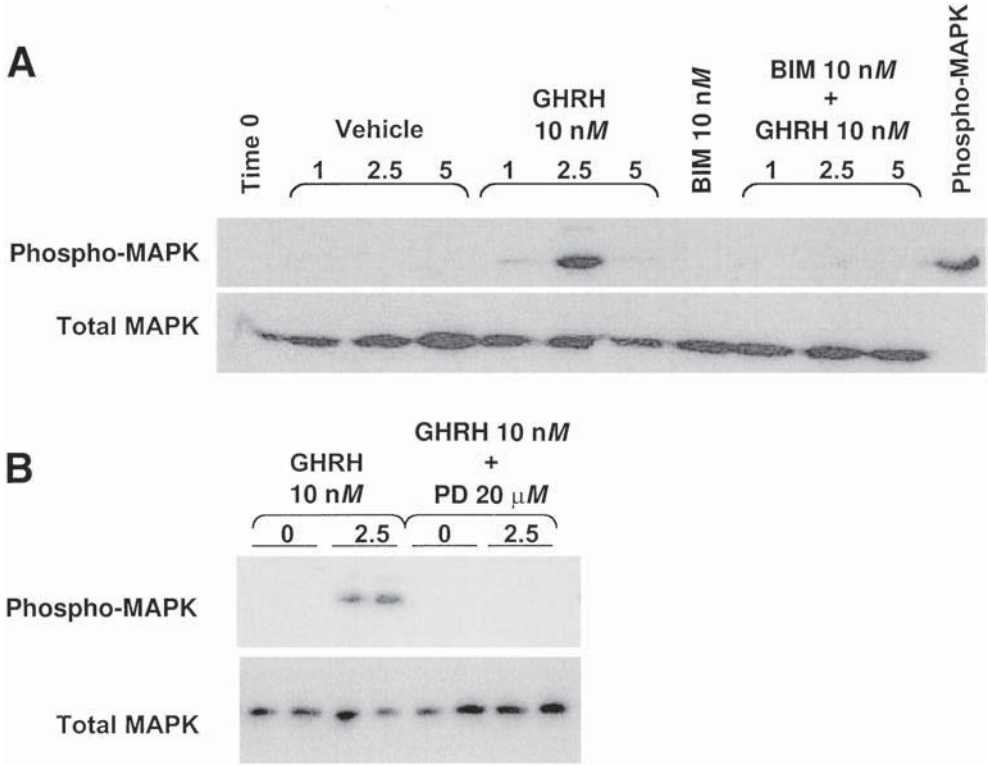
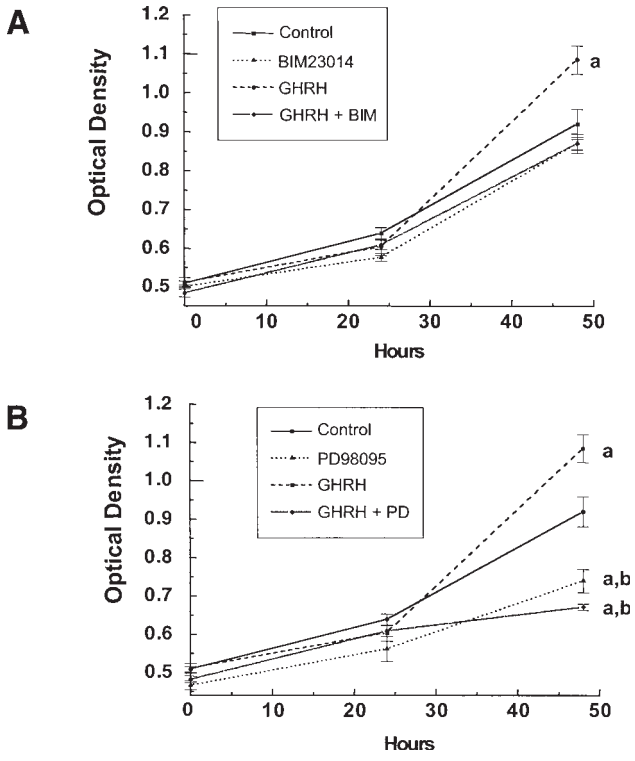



Fig. 5. GHRH induction of MAPK phosphorylation is inhibited by somatostatin and MEKK inhibitor. **(A)** Serum-starved GH4 cells (2.5 × $10^6$ cells/dish) were exposed to vehicle or rGHRH (10 n*M*) for 1–5 min with or without preincubation for 10 min with the somatostatin analog BIM23014, **(B)** Serum-starved GH4 cells (2.5 × $10^6$ cells/dish) were exposed to rGHRH (10 n*M*) for 2.5 min with or without preincubation for 10 min with the MEKK inhibitor PD98095 (20 μ*M*). Equal protein samples of cell extracts were separated by SDS-PAGE followed by transfer to nitrocellulose, Western blot analysis with antiphosphospecific MAPK antibody, and visualization by chemiluminescence as described in Materials and Methods. Phospho-MAPK standard was run in parallel as a control. After visualization, membranes were stripped as described in Materials and Methods and reprobed with antibody to p42/p44 MAPK (Total MAPK) to verify equal protein loading. Each lane represents the extract of an independent dish.




We demonstrate herein that GHRH stimulates proliferation of GH4 cells in culture, increasing proliferation by approx 25% after 48 h. Although a small increase, this result is reproducible, confirms in vitro the implication drawn from the animal models, and provides an experimental system in which to investigate the mechanism of the proliferative effects of GHRH .

Agents that prevent activation of MAPK obliterate proliferation of somatotroph cell lines in response to GHRH. Thus, the MEK inhibitor PD98095 completely blocks the effects of GHRH on both activation of MAPK

Fig. 6. GHRH promotes increased proliferation of GH4 somatotrophs, and this effect is blocked by somatostatin and inhibition of MEKK. GH4 cells (3000 cells/well in 96-well plates in 200 μL of DMEM) were serum starved for 48 h, followed by preincubation with BIM23014 (10 n*M*) **(A)**, PD98095 (20 μ*M*) **(B)**, or vehicle for 10 min. Cells were then treated with GHRH (10 n*M*) or vehicle, and treatments were renewed every 24 h. At appropriate time intervals, plates were assayed using the Cell Titer AQ assay, and OD was determined at 490 n*M*. Points represent mean ± SEM ($n = 6$ for each treatment at each time point). a = $p < 0.01$ vs control; b = $p < 0.01$ vs GHRH-treated cells.

and cell proliferation. Furthermore, PD98095, at a dose that is highly selective for inhibition of MAPK activity *(32)*, reduced the basal rate of somatotroph proliferation, suggesting a role for MAPK in basal proliferation, as has been observed in a wide variety of cell types. These results strongly imply that GHRH promotes somatotroph cell line proliferation, at least in part, through activation of the MAPK pathway.

GHRH and somatostatin are physiological antagonists that reciprocally regulate somatotroph function and proliferation. The interaction of GHRH and somatostatin to regulate GH secretion and synthesis has been well studied and involves antagonistic effects on multiple intracellular mediators including cAMP, intracellular calcium, and cell depolarization. However, the interaction of these two neuropeptides to regulate somatotroph proliferation is less well understood. Somatostatin has antiproliferative effects in both pituitary and extrapituitary tissues *(40,41)*, an effect that is thought to be mediated by interaction with the MAPK cascade *(42)*. The effect of somatostatin on phosphorylation of MAPK appears to depend on the receptor subtype(s) available to mediate the signal. Studies in vitro indicate that MAPK phosphorylation/activation is increased by SSTR1 *(43)* and SSTR4 *(44)* but inhibited by SSTR2A and SSTR5 *(45,46)*. While multiple somatostatin receptor subtypes are expressed on pituitary somatotrophs *(47)* and GH4 cells *(48)*, SSTR2 and SSTR5 predominate. Therefore, we hypothesized that antagonistic effects of these two agents on MAPK activation mediate the reciprocal effects of somatostatin and GHRH on somatotroph proliferation. In the experiments reported herein, a long-acting, relatively SSTR2-specific analog of somatostatin, BIM23014, effectively blocked both activation of MAPK and GH4 proliferation by GHRH. Somatostatin, unlike PD98095, had no effect on basal proliferation. Thus, not only does activation of MAPK appear to mediate the effects of GHRH on somatotroph proliferation, reciprocal effects of these two neuropeptides on MAPK activation may account for their physiological antagonism in the regulation of somatotroph populations.

The mechanism by which G-protein-coupled receptors, including GHRH, activate the MAPK pathway is unclear. Growth factor receptors generally activate MAPK through a phosphorylation cascade involving Grb2-SOS, ras, Raf, and Mek *(25)*. On the other hand, G-protein-mediated activation of MAPK may occur through alternate pathways, including indirect activation mediated through βγ stimulation of PI3-kinase or a membrane-bound src-like kinase *(49–51)*. Alternatively, activation of ERK by PKC has recently been reported *(26,27)* for TRH stimulation of GH3 cells *(28)* and GnRH stimulation of αT3-1 cells *(31)*. Finally, cAMP may activate ERK via a Raf-1-independent route *(52)*, possibly involving B-Raf *(53)*. The pathway mediating GHRH activation of MAPK is currently under examination in our laboratory, with particular attention to the question, IS MAPK activation distinct from, or coupled to, generation of cAMP by this ligand?

In summary, we have demonstrated here the novel finding that GHRH leads to dosedependent and reversible activation of the MAPK pathway in the GH4 rat somatotroph cell line, a standard model for somatotroph function. Furthermore, we have demonstrated that activation of MAPK mediates, at least in part, the effects of GHRH on GH4 proliferation in vitro. Finally, we have shown that reciprocal actions of somatostatin and GHRH on the MAPK pathway may mediate the effects of these physiological antagonists in the regulation of somatotroph proliferation. These findings expand our current understanding of intracellular GHRH signaling and raise the exciting possibility that, as now seems to be the case for a wide variety of ligands *(54)*, several distinct and mutually interacting signaling pathways may mediate the effects of GHRH.

## Materials and Methods

### *Cell Culture*

Previous studies have suggested that GH4 cells do not respond to GHRH with increased secretion of GH *(55,56)*. Therefore, it has been assumed that these cell lines do not have GHRH receptors. However, synthesis of GH by these cell lines is minimal and using GH secretion as the end point limits the sensitivity of assessment of response to GHRH. As shown in Fig. 7, GHRHr mRNA is easily isolated from GH4 cells by polymerase chain reaction (PCR) and is present in quantities grossly equivalent to that present in rat pituitary cells (Fig. 7A). Furthermore, GH4 cells respond to GHRH with increased intracellular cAMP production, indicating the presence of an intact intracellular signaling response to GHRH (Fig. 1B). Therefore, we utilized GH4 cells for the present studies to avoid the technical problems associated with the use of primary pituitary cells in short-term signaling studies.

GH4 cells were maintained in DMEM supplemented with 15% horse serum/2.5% FCS in a humidified incubator at 37°C in 5% $CO_2$. Cells were passaged at 75% confluency using phosphate-buffered saline (PBS)/EDTA. Prior to experiments, cells ($2.5 \times 10^6$/plate) were transferred to 6-cm culture dishes in 15% horse serum/2.5% FCS. After 24 h, cells were serum starved overnight in 3 mL of DMEM with 0.6% FCS prior to use in the following experiments, unless otherwise stated.

### *Transfection*

HeLa cells ($2.5 \times 10^6$ cells/200 μL of DMEM/10% FCS) were transfected by electroporation with 5 μg of empty vector (pcDNA3.1; Invitrogen, Carlsbad, CA) or 5 μg of vector containing full-length GHRHr cDNA *(57)* at 220 V and 500 μF. Following incubation overnight in 3 mL of DMEM/0.6% FCS, cells were serum starved for 4 h and treated with GHRH or vehicle as described.

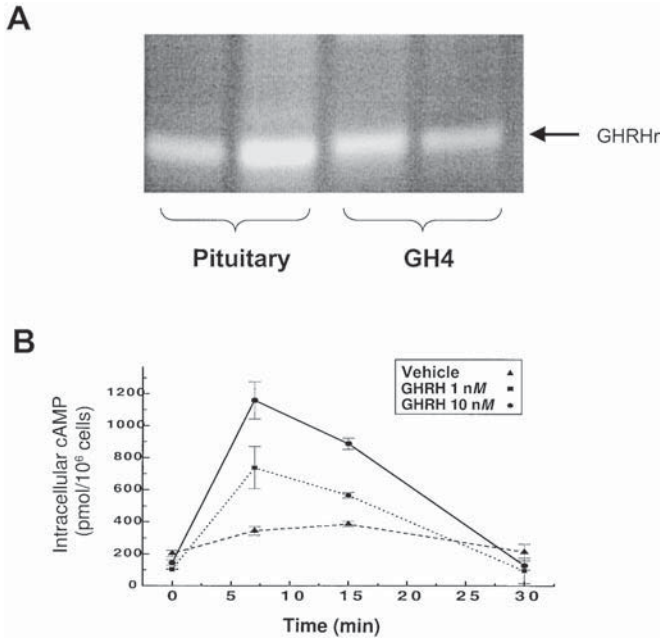



Fig. 7. GH4 cells contain GHRHr mRNA and respond to GHRH with cAMP generation. **(A)** GH4 cells were grown in culture as described in Materials and Methods, and total RNA was extracted with RNAstat 60 (Tel-Test) according to the manufacturer's directions. For comparison, adult male rats were decapitated following $CO_2$ narcoses. Rat pituitaries were removed rapidly and frozen on dry ice, and the total RNA was extracted with RNAstat 60. Reverse transcriptase (30 cycles) was performed on 2 μg of total GH4 and pituitary RNA as previously described *(32)* using primers that amplify a 240-bp product (5'TTGCTGAACCTGTGGGGAGTTG3'and 5'GGGTCT GAGCCAAAATGAGAGAAG3'). PCR products were electrophoresed through 1% agarose and visualized with ethidium bromide. **(B)** Serum-starved GH4 cells ($2.5 \times 10^6$ cells/dish) were exposed for 0–30 min to GHRH (10 or 100 n*M*). At indicated times, cells were lysed in assay buffer and the generation of intracellular cAMP was determined on cell lysates using the Biotrak cAMP EIA kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Values represent mean ± SEM ($n = 6$).

### *Western Blot Analysis*

Serum-starved cells were treated with varying doses of rGHRH (Sigma, St. Louis, MO) or vehicle (0.1% bovine serum albumin (BSA), 0.01*M* acetic acid, 0.1 m*M* ascorbic acid) alone. Treatment with EGF (25 n*M* for 10 min) served as a positive control. In some experiments, cells were preincubated for 10 min with the somatostatin analog BIM23014 (10 n*M*) (Bachem, Philadelphia, PA) or the MEK1/MAPK inhibitor PD98095 (20 μ*M*) (New England Biolabs, Beverly, MA). At the indicated times after treatment, the medium was aspirated, and the cells were quickly rinsed with cold PBS and extracted directly into 200 μL of sample loading buffer (0.25 *M* Tris, pH 6.8; 2% SDS; 10% β-mercaptoethanol; 30% glycerol; 0.01% bromophenol blue). Extract from equal numbers of cells in an equal volume of sample was separated by electrophoresis through 10% SDS-PAGE and electrotransferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were immunoblotted with primary antibodies to the tyrosine-phosphorylated form of MAPK (New England Biolabs)(1:1000), followed by horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad, Hercules, CA) (1:10,000 in blocking buffer). Protein-antibody complexes were detected by chemiluminescence (ECL; Amersham) according to the manufacturer's protocol, followed by autoradiography (Kodak Biomax MR, Eastman Kodak, Rochester, NY). Following autoradiography, membranes were stripped for 30 min at 50°C according to the ECL protocol and reprobed with a p42/p44 MAPK antibody ("total MAPK") as described for the phospho MAPK antibody. OD of autoradiographs following chemiluminescence was determined using video densitometry (alphaimager 2000; Alpha Innotech, San Leandro, CA).

### *Cell Proliferation*

GH4 cells were plated (3000 cells/well) in 96-well plates in 200 μL DMEM and serum-starved for 48 hours to quiesce the cells. A separate 96-well plate was used for each time point. Cells were then treated with 20 μ*M* PD98095, 10 n*M* BIM 23014, or vehicle for 10 min, followed by GHRH (10 n*M*), FCS (final concentration of 10%), or vehicle ($n = 6$ wells for each treatment at each time point). Media were renewed every 24 h. At appropriate time intervals, plates were assayed for cell count using the Cell Titer AQ assay (Promega, Madison, WI) according to the manufacturer's directions. OD of the bioreduced MTS (Owen's reagent: 3[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) was determined at 490 n*M* on an MRX OD reader (Dynatech, Chantilly, VA).

### *Statistical Analysis*

Where indicated, data were analyzed by one-way ANOVA followed by post-hoc analysis with the Neuman-Keuls test.

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