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Peptides Derived from Pro-Growth Hormone–Releasing Hormone Activate p38 Mitogen-Activated Protein Kinase in GH3 Pituitary Cells

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Posttranslational processing of the pro-growth hormone–releasing hormone (proGHRH) peptide can result in the formation of at least two peptide products: GHRH and the C-terminal peptide, GHRH-related peptide (GHRH-RP). While cyclic adenosine monophosphate transduces many of the actions of GHRH, other pathways also have been implicated in its actions. The aims of this study were to examine and characterize the activation of mitogen-activated protein kinase (MAPK) pathways by GHRH, and GHRH-RP in pituitary-derived GH3 cells, as well as the activation of the transcription factors that serve as substrates for these kinases. GHRH rapidly increased p44/p42 MAPK activity in GH3 cells in a protein kinase A–dependent and a protein kinase C–independent manner and stimulated the activation of the transcription factor Elk-1. By contrast, GHRH-RP and p75-92NH2 had no effect on p44/p42 MAPK phosphorylation in these cells. Additionally, we determined that all three peptides, GHRH, GHRH-RP, and p75-92NH2, rapidly and specifically increase phosphorylation of p38 MAPK and stimulate the activation of the nuclear factor CHOP. These are the first studies to demonstrate the activation of Elk-1 by GHRH and the activation of p38 MAPK and CHOP by GHRH, GHRH-RP, and p75-92NH2. We conclude that members of the GHRH family of peptides differentially activate multiple intracellular signaling pathways and suggest that the biologic actions of GHRH may be far more diverse than previously thought.

Key Words: GHRH; MAPK; GH_3 cells; growth hormone-releasing hormone related peptide; signal transduction.

Introduction

Growth hormone–releasing hormone (GHRH) is produced by nerve fibers located in the medial basal region of the arcuate nucleus and is transported, via the portal circulation, to the pituitary, where it functions as the main evocative stimulus for growth hormone (GH) synthesis and release *(1)*. Circulating GHRH is derived from a 104 amino acid precursor peptide that is posttranslationally processed, resulting in the production of several bioactive peptides. These include GHRH (amino acids 30–74 of preproGHRH) and a second peptide, GHRH-related peptide (GHRH-RP). The sequence of this latter peptide is composed of the C-terminal, thirty amino acids (amino acids 75–104) of preproGHRH *(2)*. Additional data suggest that GHRH-RP is further processed to a truncated peptide, which has been designated p75-92NH2.

In cultured rat Sertoli cells, both GHRH-RP and p75-92NH2 stimulate the expression of stem cell factor (SCF), a growth, differentiation, and migration-promoting cytokine that is essential for both hematopoietic and gonadal germ cell homeostasis *(3–6)*. The regulation of SCF expression by GHRH-RP was confirmed in vivo, using transgenic mice, which constitutively overexpress only the GHRH-RP portion of the precursor peptide without interference from excess GHRH. GHRH-RP excess, in these animals, induced overexpression of SCF in multiple tissues including the bone marrow and spleen *(7)*, where these increases were associated with significant increases in myeloid and erythroid progenitor cell cycling rates.

GHRH regulates GH synthesis and release through stimulation of adenylate cyclase and the accumulation of cyclic adenosine monophosphate (cAMP) *(8)*. There are also additional reports concerning alternative pathways that GHRH may activate, including phospholipase C and the inositol phosphate pathway *(9–11)*. Zeitler and Siriwardana *(12)* and Pombo et al. *(13)* reported that GHRH also stimulates p44/p42 (ERK1/2) mitogen-activated protein kinase (MAPK) in the rat GH4 cell line and in primary somatotrophs, respectively. Recently, we have determined that GHRH-RP and p75-92NH2 stimulate the phosphorylation and activation

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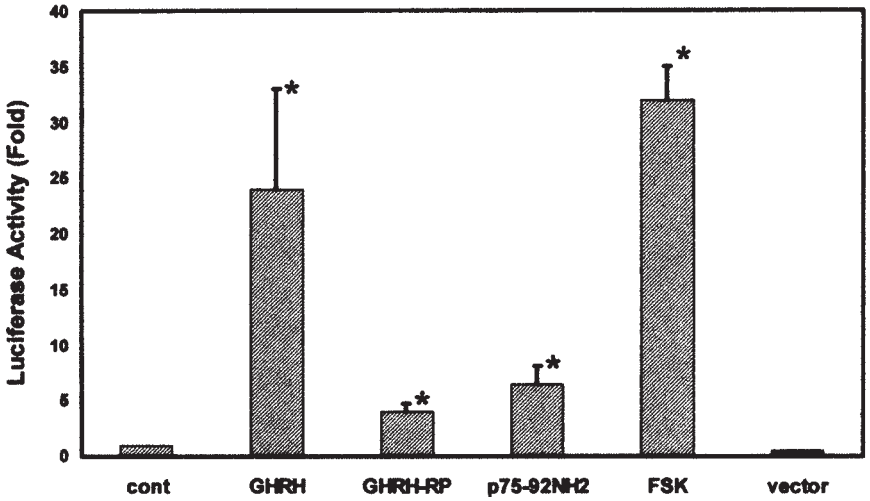

Fig. 1. Activation of a CREB-responsive promoter by GHRH, GHRH-RP, and p75-92NH2 peptides. GH3 cells (3.5×10^5 cells/well) were cotransfected with the CREB reporter system and treated with vehicle (cont), GHRH, GHRH-RP, p75-92NH2 (100 n*M* each), or forskolin (FSK; positive control, 10 μ*M*). Cells transfected with the reporter plasmid alone served as vector controls (vector). Results shown are the mean ± SEM of the fold increase in luciferase activity in peptide-treated cells as compared with control cells. Results were calculated from nine transfections performed in three separate experiments. Results were normalized to β-galactosidase activity. $*p < 0.05$.

of the transcription factor CREB in the testes *(2,4)*. However, the receptor (or receptors) that mediate the actions of GHRH-RP and p75-92NH2 have not been identified.

These above findings indicate that GHRH signaling is more complex than previously believed, involving multiple intracellular pathways. Our objectives were to investigate GHRH activation of alternative signal transduction pathways in pituitary cells and to compare this to the signaling pathways activated by the novel peptides GHRH-RP and the p75-92NH2.

Results

GHRH, GHRH-RP, and p75-92NH2 Increase Activity of CREB-Responsive Promoter in GH3 Cells

Transcription assays were used to determine whether GHRH, GHRH-RP, and p75-92NH2 activate the transcription factor CREB. Following treatment of cells with each of the peptides, phosphorylation of the CREB activation domain was measured indirectly by increases in luciferase activity of the GAL-4/CREB-driven promoter. GHRH consistently stimulated luciferase activity greater than 20-fold, whereas GHRH-RP and p75-92NH2 only moderately increased activation 3.5- and 6.3-fold, respectively, in GH3 cells (Fig. 1). In GH3 cells treated with forskolin (positive control), CREB reporter activation increased as expected.

GHRH, but Not GHRH-RP and p75-92NH2, Induces Transcription of ELK-1-Responsive Promoter

GHRH has recently been shown to stimulate MAPK activity. One of the major downstream targets of p44/p42 MAPK is the transcription factor Elk-1. To determine whether GHRH activates Elk-1, GH3 cells were cotransfected with DNA reporter gene constructs that specifically monitor Elk-1 activation and then treated with GHRH, or the proGHRH-derived peptides, GHRH-RP and p75-92NH2. GHRH significantly increased Elk-1 activity approx 25-fold greater than that seen in untreated GH3 cells. By contrast, GHRH-RP and p75-92NH2 had no effect on Elk-1 activity (Fig. 2). In cells treated with phorbol-12-myristate-13-acetate (PMA), Elk-1 activity was dramatically increased by approx 60-fold.

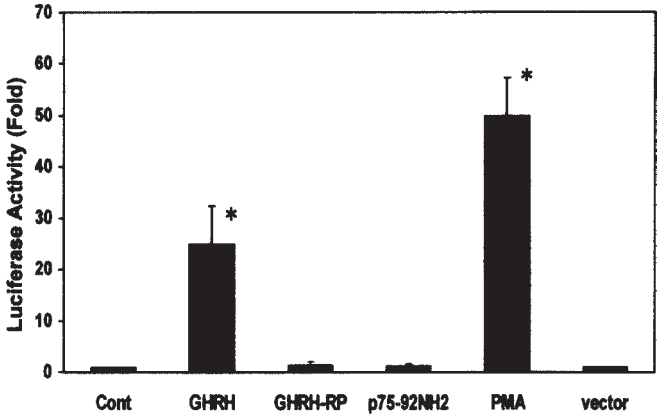

Fig. 2. Transcriptional activation of an Elk-1-responsive promoter by GHRH. GH3 cells (3.5×10^5 cells/well) were cotransfected with the pFA/Elk-1 expression plasmid and the pFR/luc reporter gene and treated with GHRH, GHRH-RP, p75-92NH2 (100 n*M* each), or PMA (1 μ*M*). Results shown are the mean ± SEM of the fold increase in luciferase activity in peptide-treated cells as compared with control cells and represent the results of 18 transfections from 6 separate experiments. Luciferase activity was normalized to β-galactosidase activity. $*p < 0.05$.

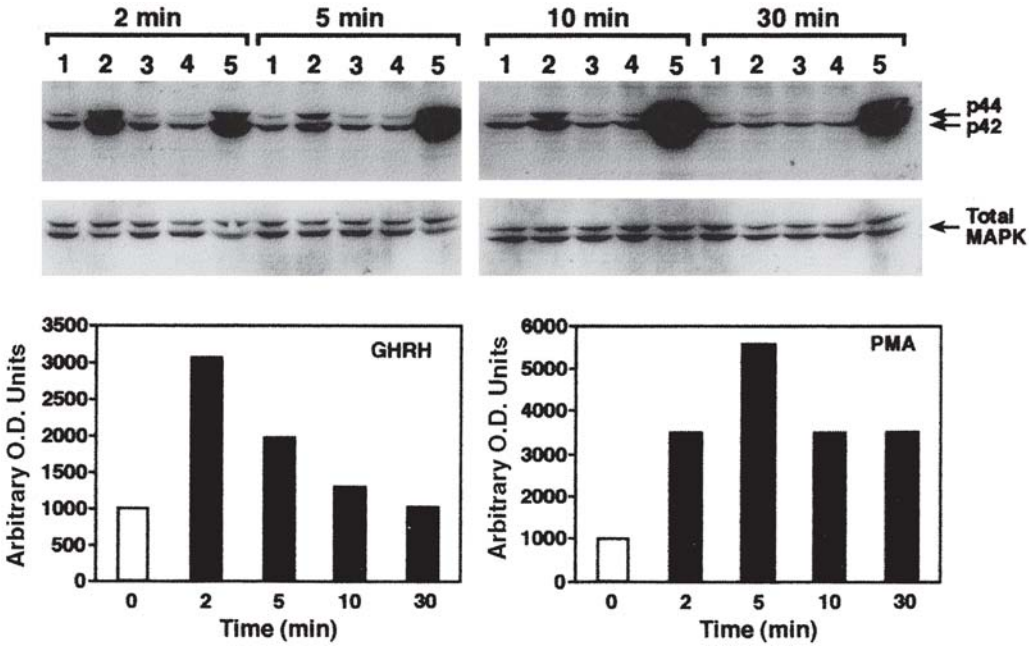

Fig. 3. Time course for p44/p42 MAPK activation by GHRH in GH3 cells. (**Top**) GH3 cells were incubated for 2, 5, 10, and 30 min with vehicle control (lane 1 at each time point), GHRH (100 n*M*, lane 2 at each time point), GHRH-RP and p75-92NH2 (100 n*M*, lanes 3 and 4, respectively, at each time point; negative controls), or PMA (1 μ*M*, lane 5 at each time point; positive control). Western blot analyses for phosphorylated (top) and total p44/p42 MAPK (bottom) were performed. (**Bottom**) Densitometric analysis of Western blots for phosphorylated p44/42 following treatment with GHRH or PMA. The results shown are representative of three individual Western blot experiments.

MAPK Activation is Necessary for GHRH Stimulation of Elk-1

To understand the mechanisms responsible for GHRH-stimulated Elk-1 in GH3 cells, phosphorylated p44/p42 levels were monitored following GHRH treatment. Cells were treated with GHRH (10 or 100 n*M*) and cell extracts analyzed by Western blotting using antibodies that specifically recognize phosphorylated p44/p42 MAPK. GHRH rapidly increased phosphorylation with the maximum stimulation seen following 2 min of treatment. By 30 min, phosphorylated levels had returned to those seen in control cells (Fig. 3). A different pattern of MAPK stimulation was seen in cells treated with PMA, where a sustained increase in phosphorylated p44/p42 levels was observed for up to 1 h. When cells were treated with GHRH at 10 n*M*, p44/p42 phosphorylation was similar to that seen with the higher dose (data not shown). In these experiments, GHRH-RP and p75-92NH2 had no effect on phosphokinase levels.

To confirm these MAPK results, two experimental approaches were used. First, cells were transfected, described above, with the Elk-1 reporter system, and then pretreated for 2 h with PD98059 a specific inhibitor of MEK1 *(14)*. Following PD98059 treatment, cells were incubated with GHRH, p75-92NH2 (negative control) or PMA (positive control), for the times indicated. PD98059 treatment decreased luciferase activity in GHRH-treated cells by approx 60%, indicating that GHRH signaling, at least in part, involves the p44/p42 MAPK pathway (Fig. 4). In PMA-treated cells, PD98059 decreased the PMA-induced Elk-1 activity by 30%. Second, Western blots were performed using protein extracts prepared from GH3 cells that were pretreated with PD98059. Similar to the results seen in the transfection studies, pretreatment of cells with the inhibitor reduced the levels of phosphorylated p44/p42 by approx 60% (Fig. 5) and confirmed the participation of MAPK in the GHRH stimulation of Elk-1.

PKC Pathway Is Not Involved in GHRH-Induced Elk-1 Activation

Details of the mechanisms by which GHRH activates MAPK are not entirely understood. To assess whether GHRH requires PKC for activation of Elk-1, GH3 cells were pretreated with staurosporin, and Elk-1 luciferase activity was monitored. While staurosporin significantly reduced the PMA-induced activation of Elk-1, it had no effect on that induced by GHRH (Fig. 6, top panel). To confirm this, cells were also pretreated for 18 h with PMA in order to deplete intracellular PKC stores and then treated with GHRH for varying times, as before. Western blot analysis determined that similar to staurosporin, pretreatment of the cells with PMA had no effect on phospho-p44/p42 levels (Fig. 6, bottom panel).

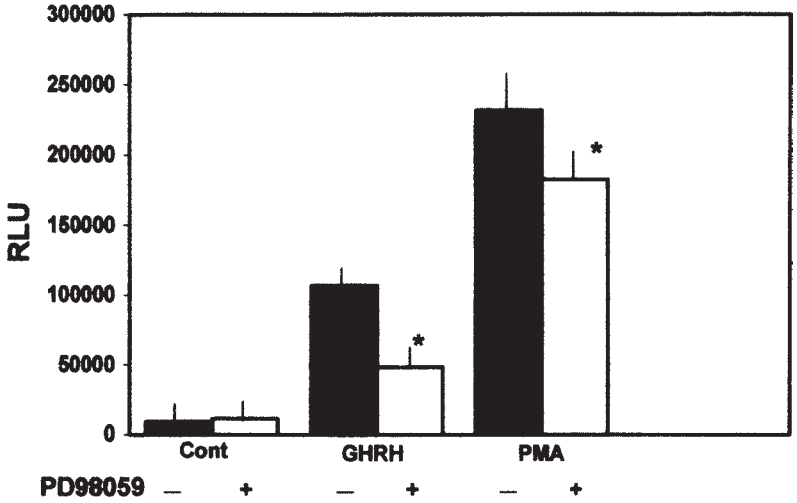

Fig. 4. Inhibition of GHRH-induced Elk-1 activation by PD98059. GH3 cells (3.5×10^5 cells/well) were cotransfected (5 h at 37ºC) with the pFA/Elk-1 and pFR/luc plasmids, preincubated for 1 h with (+) or without (–) the specific MEK1 inhibitor PD98059 (25 μ*M*), and then treated with GHRH. Results shown are the mean ± SEM of luciferase activity calculated from three separate experiments each containing three replicates per treatment. Luciferase activity was normalized to total protein per well. *$p < 0.05$ for cells pretreated with PD98059 vs cells treated with GHRH. RLU, relative light units.

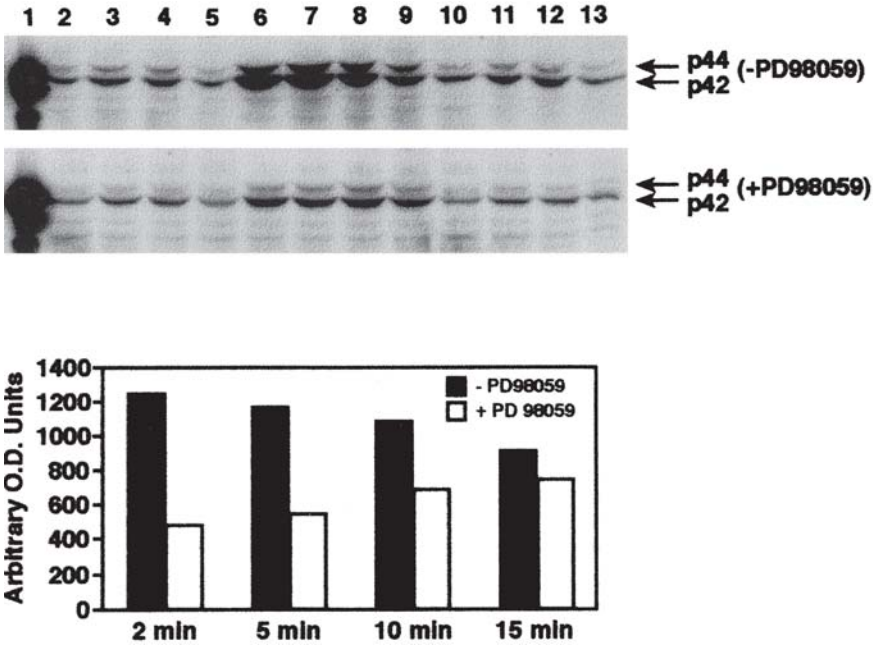

Fig. 5. MEK1 inhibitor PD98059 decreases the levels of GHRH-induced phosphorylated p44/p42 MAPK. (**Top**) Western blot of phosphorylated p44/p42 MAPK in GH3 cells not pretreated with PD98059 (–PD98059). Lane 1: positive kit control; lanes 2, 3, 4, and 5: vehicle-treated cells at 2, 5, 10, and 15 min, respectively; lanes 6, 7, 8, and 9: GHRH treated cells at 2, 5, 10, and 15 min, respectively; lanes 10–13: GHRH-RP (negative control) treated for the same times. (**Bottom**) Quantification of the decrease in the above GHRH-induced phospho-p44/42 MAPK levels in GH3 cells pretreated with PD98059 (+PD98059) compared with cells without pretreatment (–PD98059). Levels were measured at 2, 5, 10, and 15 min and bands quantified using imaging densitometry. The results shown are representative of two individual Western blot experiments.

Inhibition of PKA Increases GHRH-Induced Elk-1 Activation

We also examined whether inhibition of PKA results in alteration of GHRH-stimulated Elk-1 activation. GH3 cells were transfected, as previously described, pretreated for 1 h with the PKA inhibitor, and then treated with GHRH. In contrast to the results seen with PD98059, the PKA inhibitor not only failed to block GHRH, but resulted in a greater than twofold increase in GHRH-stimulated Elk-1 activity (Fig. 7). Pretreatment of GH3 cells with the PKA inhibitor had no effect on Elk-1 activity after the addition of either GHRH-RP or p75-92NH2. Treatment of cells with the PKA inhibitor, alone, had no effect on basal Elk-1 activity (data not shown).

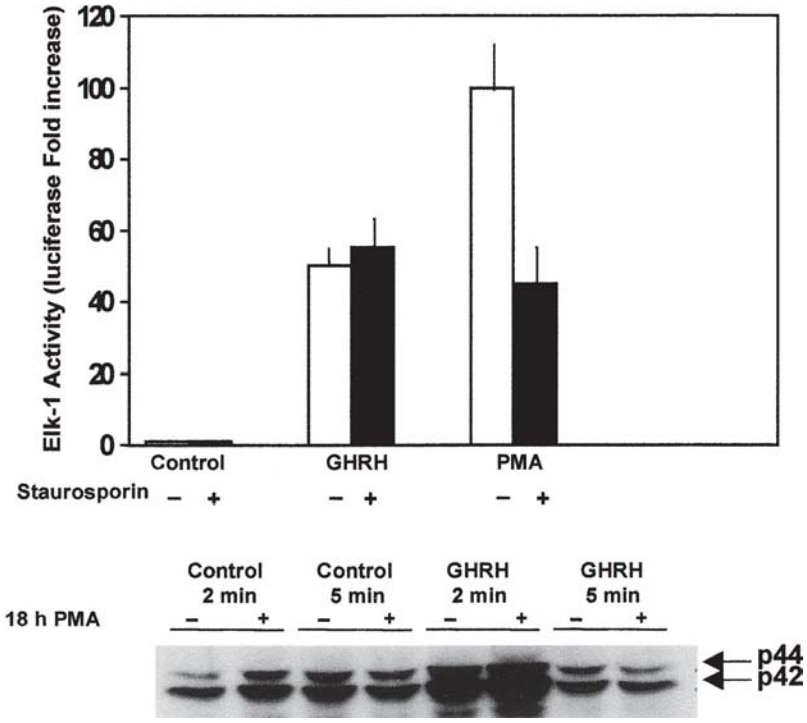

Fig. 6. PKC is not required for the GHRH activation of either Elk-1 or p44/p42 MAPK. (**Top**) Comparison of the effect of staurosporin (+, 10 m*M*, for 1 h) on GHRH and PMA-stimulated Elk-1 activity to cells not pretreated (–). (**Bottom**) Western blot showing the effect of pretreating GH3 cells with (+) or without (–) PMA (1 μ*M* for 18 h) on GHRH-stimulated phospho-p44/p42 MAPK levels at 2 and 5 min. Control cells at the same times and pretreated with PMA alone served as controls. Results of the luciferase assays are the mean ± SEM calculated from three separate experiments.

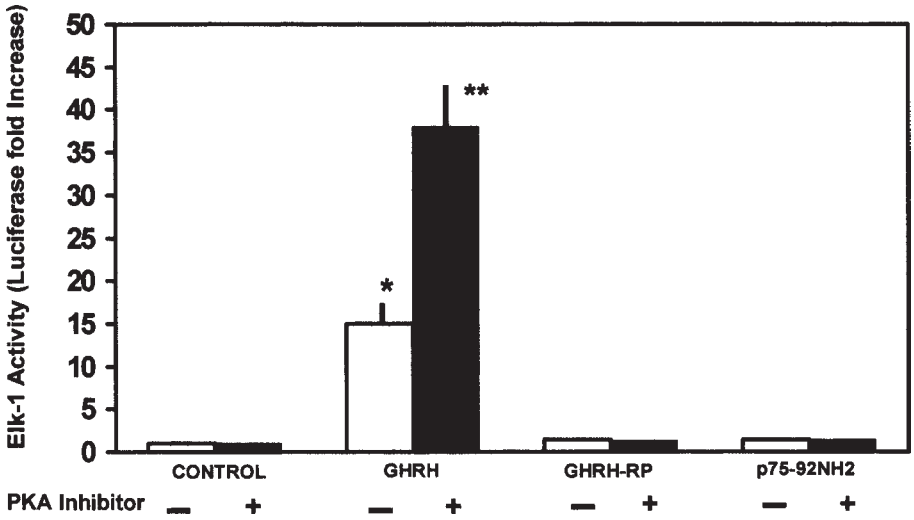

Fig. 7. Inhibition of PKA potentiates the effect of GHRH on Elk-1 activation. GH3 cells were plated and transfected to monitor Elk-1 activation (as before) and then pretreated with a specific PKA inhibitor (PKA amide fragment, 20 μ*M* for 1 h) before the addition of either GHRH, or GHRH-RP or p75-92NH2 (negative controls). Results shown are the fold increase in luciferase activity in cells pretreated (+) or not pretreated (–) with the PKA inhibitor. Results were calculated from six separate transfections performed in two separate experiments. *$p < 0.05$ for control vs GHRH-treated cells; **$p < 0.05$ for cells pretreated with the PKA inhibitor (+) vs cells not pretreated with the inhibitor (–) and control cells.

GHRH, GHRH-RP, and p75-92NH2 Stimulate p38 MAPK and the Transcription Factor CHOP

Regulation of gene transcription by MAPKs can involve the activation of transcription factors other than Elk-1. It was therefore determined whether GHRH increases the activity of additional nuclear factors associated with MAPK activation. In GH3 cells, GHRH stimulated the activation of CHOP 2-fold (Fig. 8), while GHRH-RP and p75-92NH2 increased CHOP activity by 2.5- and 2.2-fold respectively. In PMA-treated cells, CHOP activation increased approx

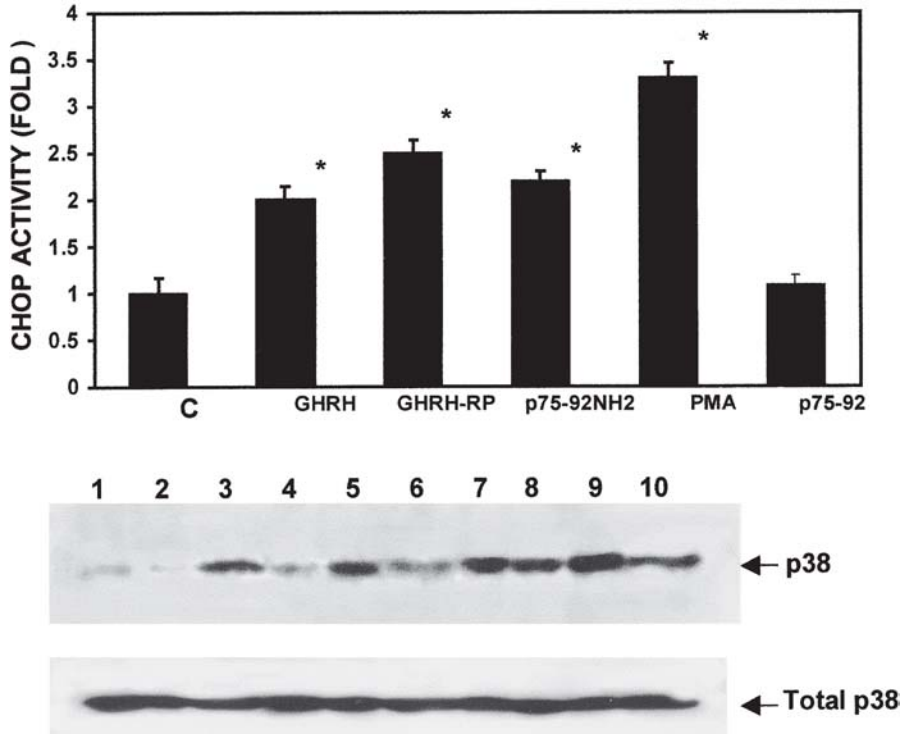

Fig. 8. Activation of the transcription factor CHOP and p38 MAPK by GHRH, GHRH-RP, and p75-92NH2 peptides. (**Top**) GH3 cells plated and cotransfected with the pFA/CHOP reporter system, and treated with GHRH, GHRH-RP, p75-92NH2, PMA (positive control), or p75-83 (negative control). Results are presented as the mean ± SEM in luciferase activity of six individual transfections. *$p < 0.05$. (**Bottom**) Western blot of phosphorylated p38 MAPK activity in GH3 cells treated with vehicle (lane 1), GHRH (lane 3), GHRH-RP (lane 5), or p75-92NH2 (lane 7) (10 n*M* each), and PMA (lane 9). Lanes 2, 4, 6, 8, and 10 show p38 MAPK activity following pretreatment of the cells for 1 h with SB203580 in cells treated with vehicle, GHRH, GHRH-RP, p75-92NH2, and PMA, respectively.

3.5-fold. To determine specificity, cells were also treated with a control peptide, p75-83, which is composed of the first 18 amino acids of GHRH-RP. Cells treated with this peptide failed to show CHOP activation. Western blot analysis of protein extracts from similarly treated GH3 cells were analyzed to detect increases in phosphorylation of p38 MAPK, which normally is responsible for CHOP activation. In untreated control GH3 cells there was a low level of phospho-p38 activity. GHRH, GHRH-RP, and p75-92NH2 (10 n*M* each) increased the basal levels of phosphorylated p38 MAPK, as did treatment of the cells with PMA. Preincubation of cells with MKK3/6 inhibitor SB203580 almost completely blocked p38 phosphorylation in control and GHRH-treated cells. SB203580 treatment of the cells partially blocked activation by GHRH-RP but failed to blunt the effect of p75-92NH2. SB203580 also partially inhibited the effect of PMA (Fig. 8).

Discussion

The data reported here provide evidence that GHRH and the peptides GHRH-RP and p75-92NH2 differentially stimulate multiple MAPK pathways in pituitary cells. GHRH induced the phosphorylation of p44/p42 MAPK in GH3 cells in a PKC-independent manner and activated Elk-1, a nuclear factor belonging to the Ets family of transcriptional regulators, with the latter activity negatively regulated by PKA. GHRH-RP and p75-92NH2 peptides also significantly stimulated CREB, but in contrast to GHRH, they had no effect on p44/p42 MAPK activation. Furthermore, this is the first report showing that GHRH as well as GHRH-RP and p75-92NH2 promote the phosphorylation of p38 MAPK and the activation of the transcription factor CHOP.

GHRH is a member of a family of neuroendocrine hormones that includes secretin, VIP, glucagon, PACAP, and GIP. GHRH regulates the synthesis and release of GH *(1)* and induces somatotroph proliferation both in vitro *(15)* and in vivo *(16,17)*. A common characteristic of the peptide products in this family is that they are derived from a larger precursor molecule that is posttranslationally processed to form multiple bioactive peptide products *(18)*.

We have previously reported that posttranslational processing of proGHRH results in the production of several peptides, in addition to GHRH. One of these is GHRH-RP, a 30 amino acid peptide derived from the C-terminus of the proGHRH molecule. The second peptide, which we have designated p75-92NH2, is composed of the first 18 amino acids of GHRH-RP. Identification of these additional peptides was demonstrated in hypothalamic neuronal cell cultures using immunocytochemistry and pulse-chase analysis *(2)*. Prior reports have focused on characterizing the actions of these peptides in the testis and hematopoietic system. This

is the first study to suggest that GHRH-RP and p75-92NH2 may also have biologic actions in the pituitary based on their ability to stimulate multiple intracellular signaling pathways in GH3 cells.

GHRH, GHRH-RP, and p75-92NH2 have previously been shown to activate CREB in cultured Sertoli cells *(4)*. In GH3 cells, CREB activation by these peptides may or may not be mediated by PKA activation. Since we have not identified their receptor (or receptors) nor directly measured cAMP levels, it is possible that CREB is activated through a non-PKA/cAMP-dependent pathway in these cells. Our finding that these peptides increase phospho-p38 MAPK levels indicate that both CREB and CHOP could be activated through this pathway. CREB activation, by p38, has previously been demonstrated following fibroblast growth factor *(19)*, insulin-like growth factor-1 *(20)*, and nerve growth factor *(21)* treatment. It is not likely that CREB activation by GHRH-RP and p75-92NH2 is activated by p44/p42 MAPK, since neither peptide induces phosphorylation of this MAPK. Together, these findings imply that GHRH-RP and p75-92NH2 signal transduction differs from that of GHRH and leads us to speculate that these peptides possess physiologic actions that may be distinct from those of GHRH.

Prior studies examining GHRH-induction of MAPK have reported a time course similar to the one that we observed in GH3 cells *(12,13)*. Similarly, we also found that pretreatment of the cells with the MEK1 inhibitor PD98059 significantly reduced phospho-p44/p42 MAPK levels. However, in the present article, we are reporting new data that extend these findings and show that GHRH activation of p44/p42 MAPK results in the activation of the transcription factor Elk-1.

Elk-1, a member of the Ets family of transcription factors *(22)*, in combination with SAP-1 *(23)* and SAP-2 *(24–26)* comprise the ternary complex factor *(27)*. Elk-1 phosphorylation by p44/42 MAPK *(28–31)* and, to a lesser extent, by p38 MAPK *(32)* results in binding of the ternary complex factor to serum response elements found in target genes *(33)*, such as the c-*fos* gene *(34)*. GHRH has previously been shown to increase c-*fos* expression *(35)* and somatotroph proliferation both in vivo *(36,37)* and in vitro *(15)*. Based on our findings, we hypothesize that the proliferative actions of GHRH, at least in part, involve p44/p42 MAPK and Elk-1 activation. However, in our GH3 cells, GHRH induced only modest cell proliferation (data not shown).

p38 MAPK, first described by Han et al. *(38)*, phosphorylates and activates the transcription factor CHOP, leading to the alteration of cell growth and differentiation *(39, 40)*. While it is clear that GHRH increases p38 phosphorylation and activates CHOP, the physiologic consequences of these increases are less clear. It is possible that GHRH-induced p38 MAPK and CHOP activation provides a mechanism for the regulating the balance between GHRH-induced GH production and cell proliferation. Interestingly, we found that inhibition of PKA potentiated the stimulatory effect of GHRH on Elk-1, implying that cAMP exerts some level of inhibition over GHRH-induced Elk-1 stimulation. Other examples of cell type–specific PKA inhibition of MAPK activity have been reported *(41– 43)*, and it has been proposed that, when both PKA and MAPK are activated by the same receptor, it is the balance between these opposing responses that dictates the cellular response *(44)*.

We do not know the receptor (or receptors) that GHRH, GHRH-RP, or p75-92NH2 bind to in the GH3 cells used for our studies. Using reverse transcriptase polymerase chain reaction (PCR) analysis, with GHRH receptor–specific primers, we were unable to amplify a product of the predicted size (data not shown) in our GH3 cells, similar to an earlier finding in which the classic GHRH receptor was not identified in GH3 cells *(11)*. However, several other PCR bands were detected and are currently being investigated. These findings suggest that GHRH is capable of activating MAPK through a nonclassic receptor, possibly a GHRH receptor variant that is specific to GH3 cells or, alternatively, through one of the other members of the B-family of receptors to which the GHRH receptor belongs. Studies aimed at identifying the receptor responsible for these actions are in progress. Similarly, GHRH-RP and p75-92NH2 also may utilize members of this same family of G-protein-coupled receptors.

Collectively, these data provide compelling evidence that GHRH signal transduction is complex, involving multiple intracellular pathways in addition to the well-studied cAMP/PKA pathway. These data also suggest that GHRH is capable of exerting biologic responses through receptors other than the classic GHRH receptor, which may lead to very diverse biologic effects.

Materials and Methods

Reagents and Antibodies

PKA inhibitor amide fragment 6-22, staurosporin, the MEK inhibitor PD98059, forskolin, and PMA were purchased from Sigma (St. Louis, MO). The MKK3 inhibitor SB203580 was obtained from Calbiochem (San Diego, CA). PhosphoPlus p44/42, and p38 MAPK and total MAPK antibodies were purchased from New England Biolabs (Beverly, MA). GHRH was purchased from Bachem (King of Prussia, PA), and GHRH-RP, p75-92NH2, and p75-83 peptides were synthesized by American Peptide (Sunnyvale, CA).

Plasmid DNAs

The reporter plasmid pFR/luc and the chimeric expression plasmids pFA/Elk-1, pFA/CREB, and pFA/CHOP were purchased from Stratagene (La Jolla, CA). Each plasmid encodes for a chimeric protein composed of the specific activation domain for Elk-1, CREB, or CHOP, respectively, and the yeast Gal-4-binding domain. Stimulation of appropriate intracellular signaling pathways results in phospho-

rylation (activation) of the CREB, Elk-1, or CHOP proteins, respectively. The pFR/luc plasmid contains the yeast Gal4 DNA-binding elements upstream of the luciferase gene. Phosphorylation of the pathway-specific activation sequences results in the binding of the fusion protein to the GAL4 DNA-binding sequence of the reporter construct and induction of luciferase production.

Cell Culture and Transfections

GH3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (1:1) supplemented with 15% horse serum, 2.5% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. For transfection, cells were plated at $3.5–5.0 \times 10^5$ cells per well in six-well tissue culture plates (Nunc) for 24 h in serum-free medium composed of DMEM supplemented with 1% ITS + premix (Collaborative Research) and penicillin/streptomycin as just given. Cells were transfected (5 h at 37ºC) using lipofectamine (4 µg/well) and the plus reagent (3 µL/well) with the pFR/luc plasmid (1 µg/well) and either the pFA-CREB, pFA-Elk-1, or pFA/CHOP DNA (50 ng/well). Following transfection, cells were incubated in serum-free medium for overnight and then treated with test or control peptides at final concentrations of either 10 or 100 n*M*. For inhibition studies, cells were preincubated with PD98059 (20 µ*M*) or SB203580 (1 µ*M*) for 1 h prior to the addition of other treatments. PMA (1 µ*M*) was used to pretreat cells for 18 h. The PKA inhibitor amide fragment (20-µ*M* final concentration) was added to the cell cultures 1 h prior to the addition of test peptides. Cell lysates were prepared, and luciferase activity was determined by luminometry using a Berthold model 9705 luminometer. Luciferase activity was normalized to total DNA in each sample.

Western Blot Analysis

For determination of protein expression, GH3 cells were plated and treated, for various times, with test or control peptides or with the appropriate kinase inhibitors. Following treatment, the medium was removed, the cells were washed with phosphate-buffered saline, and lysates were prepared by adding sodium dodecyl sulfate (SDS) sample buffer (62.5 m*M* Tris-HCl, 2% [w/v] SDS, 10% glycerol, 50 m*M* dithiothreitol, and 0.1% [w/v] bromophenol blue) and immediately scraping the cells. Lysates were passed through a 23-gage needle heated to 95–100ºC, cooled, and centrifuged 16,000*g*. Extracted proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose. Membranes were blocked using Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dry milk for 1 h at room temperature and incubated overnight with a rabbit polyclonal antibody that detects the phosphorylated p42/p44 or p38 MAPK (New England Biolabs). Blots were then stripped and reprobed using a polyclonal antibody that detects total p44/p42 or p38 MAPK. Immunoblotting was visualized using the LumiGLO detection kit (New England Biolabs).

Data Analyses

Statistical significance was determined using student's *t*-test or analysis of variance with post hoc testing with a probability criterion of $p < 0.05$. Results of transfections are reported as the mean ± SEM of results from at least three independent experiments containing three replicate transfections per treatment. Western blotting experiments were repeated two to three times with similar results.

Acknowledgments

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