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A Role for Mitogen-Activated Protein Kinase in Mediating Activation of the Glycoprotein Hormone α-Subunit Promoter by Gonadotropin-Releasing Hormone

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Gonadotropin-releasing hormone (GnRH) interacts with a G protein-coupled receptor and increases the transcription of the glycoprotein hormone α-subunit gene. We have explored the possibility that mitogen-activated protein kinase (MAPK) plays a role in mediating GnRH effects on transcription. Activation of the MAPK cascade by an expression vector for a constitutively active form of the Raf-1 kinase led to stimulation of the α-subunit promoter in a concentration-dependent manner. GnRH treatment was found to increase the phosphorylation of tyrosine residues of MAPK and to increase MAPK activity, as determined by an immune complex kinase assay. A reporter gene assay using the MAPK-responsive, carboxy-terminal domain of the Elk1 transcription factor was also consistent with GnRH-induced activation of MAPK. Interference with the MAPK pathway by expression vectors for kinase-defective MAPKs or vectors encoding MAPK phosphatases reduced the transcription-stimulating effects of GnRH. The DNA sequences which are required for responses to GnRH include an Ets factor-binding site. An expression vector for a dominant negative form of Ets-2 was able to reduce GnRH effects on expression of the α-subunit gene. These findings provide evidence that GnRH treatment leads to activation of the MAPK cascade in gonadotropes and that activation of MAPK contributes to stimulation of the α-subunit promoter. It is likely that an Ets factor serves as a downstream transcriptional effector of MAPK in this system.

The ability of the pituitary to synthesize and secrete the gonadotropic hormones luteinizing hormone and follicle-stimulating hormone is essential for normal reproductive function. The gonadotropins are subunit hormones, each containing a noncovalently linked α and β subunit (37). Within a species, the α-subunits are identical, while the β-subunits differ and confer the physiological specificity of the heterodimeric hormone. The synthesis and secretion of gonadotropins are regulated by a hypothalamic peptide hormone, gonadotropin-releasing hormone (GnRH). The GnRH receptor is a member of the family of receptors which contain seven membrane-spanning regions (56). Binding of GnRH to its receptor has been shown to activate phospholipase C in a G-protein-dependent manner, leading to activation of protein kinase C (PKC) and increases in intracellular Ca^{2+} concentrations (3, 22, 23, 40). GnRH stimulates gonadotropin synthesis and increases gonadotropin subunit mRNA levels (1, 14, 20, 27, 31, 36, 50, 60). In addition, GnRH effects on expression of gonadotropin subunit genes involve changes at the transcriptional level (7, 48).

Recent studies in this laboratory have provided evidence that transcriptional regulation of the α-subunit gene by GnRH requires at least two unrelated DNA elements (46, 47). One of these elements functions as a tissue-specific enhancer and has been designated the pituitary glycoprotein hormone basal element (PGBE). The PGBE appears to function permissively to allow GnRH effects at the transcriptional level. The other element is sufficient to permit GnRH responses when present as a multimer and is therefore designated the GnRH-responsive element (GnRH-RE).

The signal transduction pathways which mediate GnRH effects at the transcriptional level have not been fully defined. The observation that the DNA sequences of the α-subunit gene which are required for responses to GnRH are also required for responses to phorbol esters (47) is consistent with a role for PKC in mediating the response. The initial steps mediating GnRH effects on transcription would thus involve activation of phospholipase C, leading to production of diacyl glycerol and activation of PKC. However, the steps downstream of activation of PKC are not clear. Conceivably, PKC could directly phosphorylate a transcription factor and alter the subcellular location, DNA-binding activity, or transcriptional activity of the factor. Alternatively, other protein kinases may lie downstream of PKC. For instance, phorbol ester-induced activation of the polyomavirus enhancer requires Raf-1 kinase (5). Similarly, mitogen-activated protein kinases (MAPKs), also known as extracellular signal-regulated kinases (ERKs), have been shown to be important for phorbol ester-induced activation of AP-1 (16). Thus, there is evidence that Raf-1 and the MAPK pathway appear to be important for mediating phorbol ester effects on the activation of at least some genes. These studies led us to investigate a possible role for the MAPK pathway in mediating GnRH effects on activation of the gonadotropin α-subunit promoter. We find that GnRH treatment of a gonadotrope-derived pituitary cell line leads to activation of MAPK and this response appears to be necessary for maximal induction of α-subunit promoter activity after GnRH stimulation.

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MATERIALS AND METHODS

Preparation of expression vectors and reporter genes. Reporter genes containing the mouse glycoprotein hormone α-subunit promoter linked to luciferase as well as a construct containing three copies of the GnRH response element upstream of a minimal promoter from the rat prolactin gene (positions −33 to +34) linked to luciferase (3×GnRH-RE-luciferase) have been described previously (46, 47). A fusion gene containing the DNA-binding domain of the yeast transcription factor GAL4 (residues 1 to 147) linked to the transcriptional activation domain of Elk1 (residues 307 to 428) was prepared by PCR amplification of the Elk1 coding sequence (38). A *Bgl*II site overlapping codon 307 of Elk1 was used to prepare the fusion of the Elk1 transcriptional activation domain to the GAL4 DNA-binding domain. The GAL4-Elk1 fusion was then subcloned downstream of the cytomegalovirus (CMV) promoter in pcDNA3 to generate CMV-GAL4-Elk1. A reporter gene containing five GAL4 binding sites upstream of the E1B minimal promoter linked to luciferase (5×GAL4-E1B-Luc) has been described previously (54). An expression vector for MAP kinase phosphatase 2 (MKP2) was prepared by subcloning the MKP2 coding sequence (32a) into an *Eco*RV site in pcDNA3. The MKP2 coding sequences were obtained by PCR amplification from a PC12 cell cDNA library with degenerate oligonucleotide primers corresponding to the amino acid sequences WFNEAI (5′ termini) and NFSFMG (3′ termini). The resulting MKP2 fragment was then used to obtain full-length coding sequences for MKP2 by screening a PC12 cell cDNA library by hybridization (32a).

Expression vectors for kinase-defective ERK1 and ERK2 (16) were generously provided by Melanie Cobb. An expression vector for a constitutively active form of c-*Raf-1* kinase (RSV-Raf-BXB) has been described previously (5) and was a gift of Ulf Rapp. A carboxy-terminal portion of the human Ets-2 coding sequence containing the DNA-binding domain (amino acid residues 333 to 469) was prepared by PCR with pAPrEts-Z (29) as the template. The amplified Ets-2 DNA-binding domain was subcloned so that an initiation codon was added in frame and then inserted into the pcDNA3 vector. In addition, the Ets-2 DNA-binding domain coding sequences were subcloned in frame and downstream of the polyhistidine segment of the pET16b bacterial expression vector.

Cell culture and transfections. αT3-1 cells (61) were maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. For transient-transfection experiments, cells were grown to approximately 60% confluence prior to transfection. Cells were transfected by a single electrical pulse at 220 V and 960 μF as described previously (46). Cells were treated with 10 nM Buserelin ([D-Ser(tBu)^6, Pro^9-ethylamide]GnRH [63]), a GnRH agonist, for 6 h before collection. All transient-transfection experiments were conducted in the presence of medium containing 10% fetal bovine serum. Cell lysates were prepared 20 to 24 h following transfection by three freeze-thaw cycles, and luciferase activity was determined in samples standardized for protein content (15). All transfections were conducted at least twice, with triplicate observations within each experiment, and the data are presented as mean ± standard error of the mean.

Immunoprecipitation of MAPK, phosphotyrosine Western blotting (immunoblotting), and MAPK assay. αT3-1 cells were cultured in 100-mm plates to approximately 50% confluence in the presence of serum-containing medium. The cells were then serum starved (DMEM alone) for 12 to 16 h prior to administration of hormones and antagonists. The cells were treated with 10 nM GnRH agonist (Buserelin) for 0, 5, or 15 min before being collected. Some cells were treated with 10 nM GnRH agonist plus 1 μM GnRH antagonist Antide, [*N*-Ac-D-Nal(2)^1-pCl-D-Phe^2-D-Pal(3)^3-Lys(Nic)^5-D-Lys(Nic)^6-Lys(iPr)^8-D-Ala^{10}]GnRH (30) (BaChem). Antide was administered 30 min prior to treatment with Buserelin and remained in the culture medium with Buserelin. Following exposure to hormones for the various time periods, cells were washed three times with ice-cold buffer containing 0.15 M NaCl and 10 mM *N*-2-hydroxyethylpiperzine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.5, and lysed in lysis buffer (70 mM β glycerolphosphate [pH 7.2], 2 mM sodium vanadate, 2 mM $MgCl_2$, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.5% Triton X-100, 2.5 μg of leupeptin per ml, 2.5 μg of pepstatin per ml, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 1 mM dithiothreitol) with gentle agitation at 4°C (17). Cell debris was removed by centrifugation, and 1 μg of antiserum to ERK1 or ERK2 (Santa Cruz Biotechnology Inc.) was added to supernatants standardized for protein content (approximately 1 to 1.5 mg of protein). After 1 h of gentle mixing at 4°C, protein A-agarose was added and mixed for an additional 3 h. Immune complexes were then washed four times with 1 ml of lysis buffer. For identification of phosphotyrosine-containing ERK1 and ERK2, immune complexes were resolved by denaturing polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane by electroblotting, and then phosphotyrosine-containing proteins were visualized by immunostaining with a mouse anti-phosphotyrosine monoclonal antibody (35) with the enhanced chemiluminescence reagents and protocols described by the supplier (Amersham). For an immune complex assay of MAP kinase activity, the protein A-agarose beads were resuspended in 100 μl of a buffer containing 50 mM HEPES (pH 7.5), 1 mg of bovine serum albumin per ml, 10 mM $MgCl_2$, 150 mM NaCl, 2 mM sodium vanadate, 0.1% β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 12.5 μg of myelin basic protein (Sigma), and 100 μM [γ-^{32}P]ATP (460 mCi/mmol). The reaction mixture was incubated at 30°C for 30 min, with frequent mixing. Phosphorylated proteins were then resolved by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography.

Expression of Ets-2 DNA-binding domain and DNase protection studies. The DNA-binding domain of human Ets-2 (residues 333 to 469) was fused to a polyhistidine segment and expressed in bacteria by using the bacterial expression vector pET16b (Novagen). Growth of bacteria, induction of protein expression, and collection of bacterial cells were performed as described before (51). Purification of the polyhistidine-tagged Ets-2 DNA-binding domain by nickel-chelate chromatography was done as described previously (42). Interaction of the Ets-2 DNA-binding domain with a fragment of the mouse glycoprotein hormone α-subunit promoter was assessed by DNase I protection analysis. As a probe for these studies, a DNA fragment representing positions −457 to −351 of the mouse α promoter, which includes the GnRH-RE (47), was radiolabeled at one terminus with [α-^{32}P]dATP and *Escherichia coli* DNA polymerase I. A probe in which the GnRH-RE was disrupted by a block mutation of positions −406 to −399 was also prepared. DNase I protection reaction mixes contained 15,000 to 20,000 cpm of ^{32}P-labeled DNA probes, various amounts of Ets-2 DNA-binding domain, 30 μg of bovine serum albumin, 400 ng of salmon sperm DNA, 10 mM Tris (pH 7.5), 5% glycerol, 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol in a total volume of 25 μl. Reaction mixes were incubated for 5 min in the absence of radiolabeled probe and then for 20 min in the presence of probe at room temperature. Then 0.075 U of RQ1 DNase (Promega) in 100 mM Tris (pH 7.5)–35 mM $MgCl_2$ was added, and incubation was continued for an additional 8 min. The reaction was terminated by the addition of 3 μl of 0.5 M EDTA, and the mixture was extracted with phenol and precipitated with ethanol. The precipitated products were collected by centrifugation and analyzed by denaturing polyacrylamide gel electrophoresis.

Analysis of MKP1 and MKP2 mRNA in αT3-1 cells. αT3-1 cells were incubated in the absence of serum overnight and then treated with the GnRH agonist Buserelin for 0, 0.25, 0.5, 1, 2, or 4 h. Total cellular RNA was isolated by solubilizing the cells in guanidine HCl and sedimentation through cesium chloride as described before (19). RNA (10 μg) was electrophoresed through an agarose gel containing formaldehyde (55) and transferred by blotting to a Magna NT nylon filter (MSI, Westboro, Mass.) in 6× SSC (0.9 M NaCl, 0.09 M sodium citrate). Filters were incubated in a buffer containing 5% sodium dodecyl sulfate (SDS), 0.4 M $NaPO_4$ (pH 7.2), 1 mM EDTA, 1 mg of bovine serum albumin per ml, and 50% formamide for 4 h at 65°C before addition of the radioactive probe. MKP1 and MKP2 mRNAs were identified by hybridization to specific, radiolabeled antisense RNA probes. A 204-bp cDNA fragment of MKP1 (representing amino acids 237 to 305) was obtained by PCR amplification with oligonucleotide primers corresponding to the amino acid sequences WFNEAI (5′ primer) and NFSFMG (3′ primer) with total PC12 cell cDNA as a template (32a). For the MKP2 probe, a 336-bp cDNA fragment (representing amino acids 190 to 301) was obtained by PCR amplification with oligonucleotide primers corresponding to the amino acid sequences YDQGGP (5′ primer) and MKKRVR (3′ primer) with total PC12 cell cDNA as a template (32a). Both cDNA fragments were subcloned into pBluescript (SK−) and linearized with *Sal*I. Specific antisense RNA probes were prepared in a reaction mix containing 1 μg of linearized DNA as template, 40 mM Tris (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 20 U of RNasin, 0.5 mM each ATP, rGTP, and CTP, 12 μM UTP, 50 μM [α-^{32}P]UTP (800 Ci/mmol), and 15 U of T3 RNA polymerase. The reaction was stopped by digesting the DNA template with 2 U of DNase, followed by phenol extraction and ethanol precipitation. Antisense riboprobe (2×10^7 to 5×10^7 cpm/ml) was then added to the hybridization buffer and incubated for 24 h at 65°C. Following hybridization, filters were washed in 1× SSC at room temperature for 15 min and then in 0.05× SSC–0.1% SDS–5 mM EDTA for 3 to 4 h at 70°C. Specific bands were visualized by autoradiography. The initial hybridization was done to assay for MKP2 mRNA. The filter was then boiled to remove hybridized probe and incubated with a probe for MKP1. All lanes contained similar amounts of rRNA, as determined by ethidium bromide staining.

RESULTS

Raf kinase is sufficient to activate the α-subunit gene. We initially examined the ability of a number of different kinases to activate the glycoprotein hormone α-subunit promoter. From studies implicating the Raf-1 kinase in the activation of the phorbol ester-responsive polyomavirus enhancer (5), we tested the role of a constitutively active form of Raf kinase in the activation of the α-subunit promoter. These studies were performed with αT3-1 cells, a gonadotrope-derived cell line (61) which supports transcriptional responses to GnRH (22, 46, 47). In addition, as GnRH treatment results in elevated concentrations of intracellular Ca^{2+} (22), we tested the ability of two multifunctional Ca^{2+}/calmodulin-dependent protein kinases to activate the α-subunit promoter. An expression vector for the catalytic subunit of the cyclic AMP (cAMP)-dependent protein

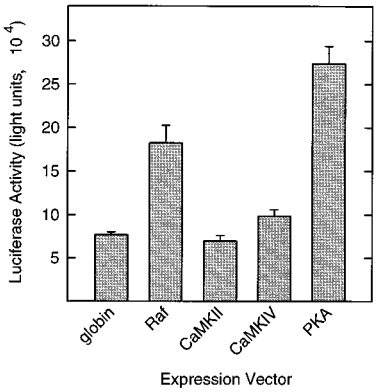

FIG. 1. Constitutively active form of Raf kinase is sufficient to activate the mouse α-subunit promoter. αT3-1 cells were cotransfected with 3 μg of a reporter gene containing the −507 to +42 region of the mouse α-subunit gene linked to luciferase. The cells were also transfected with 3 μg of either an expression vector for globin, as a control, a constitutively active form of Raf kinase (Raf), constitutively active forms of Ca^{2+}/calmodulin-dependent protein kinase type II (CaMKII) or type IV (CaMKIV), or the catalytic subunit of the cAMP-dependent protein kinase (PKA). Luciferase activity was assayed 20 to 24 h after transfection. Values are means ± standard errors for three separate transfections.

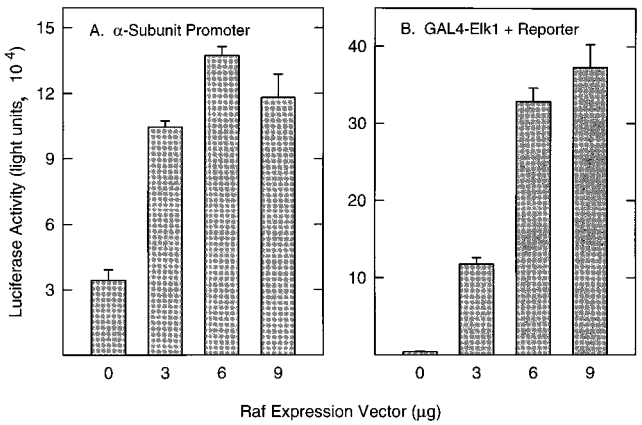

FIG. 2. Expression vector for a constitutively active Raf kinase activates the α-subunit promoter and the Elk1 carboxy-terminal transcriptional activation domain in a dose-dependent manner. αT3-1 cells were cotransfected with either 3 μg of the α-subunit reporter gene or 3 μg of an expression vector for the DNA-binding domain of GAL4 fused with the carboxy-terminal transcriptional activation domain of Elk1 (CMV-GAL4-Elk1) and 3 μg of a reporter construct containing five copies of a GAL4 DNA-binding site upstream of the E1B TATA box linked to luciferase, as indicated. Cells were also transfected with either an expression vector for globin (9 μg; control) or 3, 6, or 9 μg of a constitutively active form of Raf kinase (Raf). The total amount of expression vector was maintained at a constant value (9 μg) for each transfection by adding appropriate amounts of the globin expression vector. Luciferase activity was assayed 20 to 24 h after transfection. Values are means ± standard errors for three separate transfections.

kinase was also used, as previous studies had demonstrated that the α-subunit promoter is responsive to cAMP (47). We found that an expression vector for a constitutively active form of Raf-1 was able to induce expression of the α-subunit reporter gene (Fig. 1). In contrast to the effects of the Raf-1 vector, expression vectors for Ca^{2+}/calmodulin-dependent protein kinases type II or type IV were unable to stimulate expression of the α-subunit reporter. This finding suggests that neither of these multifunctional Ca^{2+}/calmodulin-dependent protein kinases likely plays a major role in mediating the transcriptional response to GnRH. As expected from previous studies (46), the α-subunit reporter gene was responsive to the catalytic subunit of the cAMP-dependent protein kinase.

We then examined a dose response for Raf effects on the α-subunit promoter. For these studies, we compared the ability of Raf to activate the α-subunit reporter and the Ets-related protein Elk1. Several studies have shown that Elk1 is important for mediating growth factor effects on the transcription of the c-*fos* proto-oncogene (24, 32). The Elk1 transactivation domain contains several MAPK phosphorylation sites, and these sites are inducibly phosphorylated in vivo. The carboxy-terminal transcriptional activation domain of Elk1 has been shown to be sufficient to permit transcriptional responses to growth factors, and the MAPK phosphorylation sites are crucial for this response (21, 25). For our studies, we used a fusion containing the DNA-binding domain of the yeast transcription factor GAL4 linked to the carboxy-terminal transcriptional activation domain of Elk1. A reporter gene containing five GAL4 binding sites upstream of a minimal promoter linked to luciferase was used to assess activation of the GAL4-Elk1 fusion protein. Transfection studies with various amounts of Raf expression vector demonstrated concentration-dependent activation of both the α-subunit reporter gene and the GAL4-Elk1 transactivation domain (Fig. 2). Although GAL4-Elk1 permitted greater inductions than the α-subunit promoter, both reporter genes demonstrated activation over a similar range of Raf expression vector concentrations.

GnRH treatment increases MAPK activity. The preceding experiments demonstrated that Raf-1 is sufficient to activate the α-subunit promoter in a dose-dependent fashion. As the Raf-1 expression vector would be expected to increase the activity of the MAPK pathway, this finding suggested a possible role for the MAPK signal transduction cascade in mediating responses to GnRH. Of course, if the MAPK pathway mediates GnRH effects, then GnRH should alter the activity of this protein kinase cascade. Activation of MAPK has been shown to be accompanied by phosphorylation of the kinase on both threonine and tyrosine residues (39). To determine if GnRH treatment leads to increased phosphorylation of tyrosine residues of MAPK, we immunoprecipitated ERK1 and ERK2 proteins from αT3-1 cell lysates, resolved the proteins by denaturing gel electrophoresis, and then identified phosphorylated proteins by immunoblotting with a monoclonal anti-phosphotyrosine antibody (Fig. 3A). GnRH treatment led to substantial increases in the phosphotyrosine content of both p44 ERK1 and p42 ERK2, suggesting activation of these kinases.

The effects of GnRH on the activity of ERK1 and ERK2 were determined by an immune complex assay (Fig. 3B). ERK1 and ERK2 were immunoprecipitated, and the immune complexes were incubated with myelin basic protein in the presence of [γ-^{32}P]ATP. In this assay, GnRH treatment resulted in a rapid stimulation of MAPK activity. Quantitation by PhosphorImager indicated a three- to fourfold induction of activity for both ERK1 and ERK2 after 15 min of GnRH treatment. Activation of ERK1 and ERK2 was specifically blocked by treatment of αT3-1 cells with a GnRH receptor antagonist (Antide), demonstrating that activation of the MAPK signal transduction cascade is receptor mediated. Immunoblotting demonstrated that similar amounts of ERK1 and ERK2 were present in all samples (data not shown).

We next examined the ability of GnRH and phorbol esters to activate a downstream effector of the MAPK pathway. We used the ability of Elk1 to respond to the MAPK pathway as an additional assay to determine if GnRH can increase the activity of a nuclear MAPK. These studies demonstrated that treat-

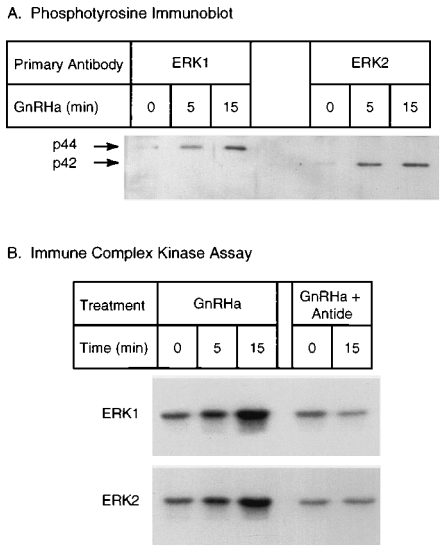

FIG. 3. GnRH induces increased tyrosine phosphorylation and activation of ERK1 and ERK2 in αT3-1 cells. (A) For determination of the phosphotyrosine content of ERK1 and ERK2, αT3-1 cells were maintained in serum-free medium overnight and then treated with 10 nM GnRH agonist (GnRHa; Buserelin) for 0, 5, or 15 min. ERK1 and ERK2 were isolated from cell lysates by immunoprecipitation, separated by denaturing electrophoresis, and transferred to a polyvinylidene difluoride membrane, and then proteins containing phosphotyrosine were visualized by immunostaining with a monoclonal antibody. Arrows identify phosphotyrosine immunoreactivity detected for ERK1 (p44) and ERK2 (p42). (B) For immune complex kinase assays, αT3-1 cells were treated with GnRH agonist as indicated or with the GnRH agonist plus a GnRH antagonist, Antide. ERK1 and ERK2 were immunoprecipitated, and the kinase activity of the immune complexes was assayed with [γ-^{32}P]ATP and myelin basic protein as the substrate. Kinase reactions were resolved by denaturing gel electrophoresis, and phosphorylation of myelin basic protein was visualized by autoradiography.

ment of αT3-1 cells with either GnRH or phorbol esters resulted in substantial activation of the GAL4-Elk1 fusion protein (Fig. 4). The finding that GnRH can activate Elk1 is consistent with the biochemical analysis of MAPK activation, and taken together, the findings strongly suggest a role for GnRH in stimulating the activity of MAPK.

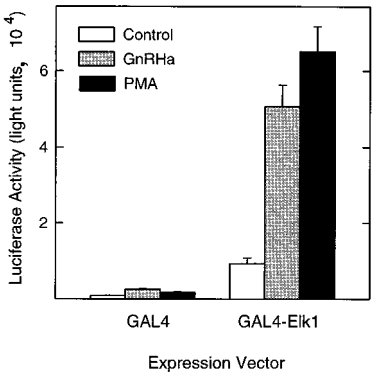

FIG. 4. Activation of the Elk1 transcriptional activation domain by treatment with GnRH agonist (GnRHa) or phorbol esters. Expression vectors (3 μg) for the DNA-binding domain of GAL4 (CMV-GAL4) or GAL4 linked to the carboxy-terminal transcriptional activation domain of Elk1 (CMV-GAL4-Elk1) were transfected into αT3-1 cells with 3 μg of the 5×GAL4-E1B-luciferase reporter gene. Cells were treated with vehicle alone (control), 10 nM GnRH agonist (Buserelin), or 1 μM phorbol myristate acetate (PMA) 18 h after transfection. Cell were collected 24 h after transfection (6 h after treatment), and luciferase activity was determined. Data are reported as means ± standard error.

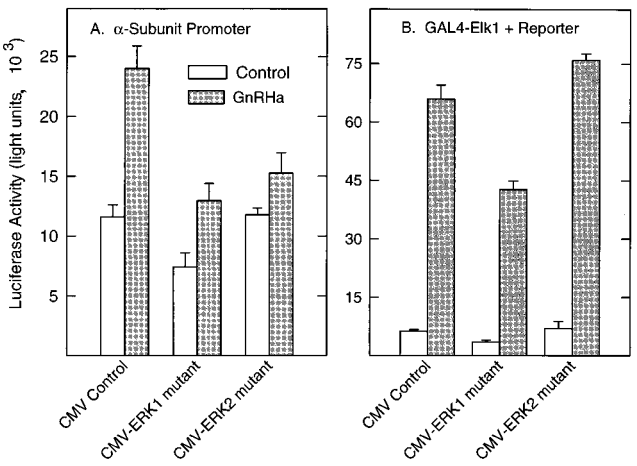

FIG. 5. Expression vectors for kinase-defective ERK1 and ERK2 reduce GnRH-induced activation of an α-subunit reporter gene and a GAL4-Elk1 fusion protein. αT3-1 cells were transfected with 30 μg of either an empty expression vector as a control (CMV control), an expression vector for kinase-defective ERK1 (CMV-ERK1 mutant), or an expression vector for kinase-defective ERK2 (CMV-ERK2 mutant). Cells also received either 3 μg of the α-subunit reporter gene or 3 μg of CMV-GAL4 Elk1 expression vector plus 3 μg of 5×GAL4-E1B-luciferase reporter, as indicated. The cells were treated with vehicle alone (control) or 10 nM GnRH agonist Buserelin (GnRHa) 18 h after transfection. Cells were collected 24 h after transfection (6 h after treatment), and luciferase activity was determined. Data are reported as means ± standard error.

Inhibition of MAPK/ERK activity reduces transcriptional responses to GnRH. To determine if MAPKs are necessary for mediating the transcriptional responses to GnRH, we used expression vectors for inhibitors of the kinases. Initially we tested the ability of expression vectors for kinase-defective mutants of ERK1 and ERK2 to function as inhibitors of GnRH-mediated activation of the α-subunit reporter gene. Although these mutant ERKs can be phosphorylated on activating sites, they possess less than 5% of the wild-type activity (41) and, as expected, they appear to interfere with endogenous ERK1 and ERK2 activity (16, 49). Expression vectors for kinase-defective ERK1 and ERK2 reduced the ability of GnRH to activate the α-subunit reporter gene (Fig. 5A). The relative ability of the mutant ERKs to inhibit GnRH effects on α-subunit expression is similar to that obtained in studies of the activation of AP-1 (16) and the prolactin promoter (12). Kinase-defective ERK1 but not ERK2 was able to attenuate the ability of the GnRH agonist to activate the GAL4-Elk1 fusion protein (Fig. 5B). The effects of kinase-defective ERK1 on activation of GAL4-Elk1 were quite similar to the effects on the α-subunit reporter gene. The observation that kinase-defective ERK2 has little effect on activation of GAL4-Elk1 is somewhat surprising, as ERK2 has been shown to phosphorylate Elk1 in vitro (32). However, in αT3-1 cells, it appears likely that ERK1 rather than ERK2 is more important for activation of Elk1. In any case, the ability of expression vectors for mutant ERK1 and ERK2 to reduce GnRH-induced activation of the α-subunit reporter gene is consistent with a role for MAPK in mediating GnRH effects on the α-subunit gene.

We also examined the ability of expression vectors for MAPK phosphatase 2 (MKP2) to alter GnRH-induced activation of the α-subunit reporter gene (Fig. 6). MKPs are dual-specificity phosphatases which can dephosphorylate MAPK on threonine and tyrosine residues both in vitro and in vivo, leading to decreased kinase activity (9, 32a, 33, 52, 53). An expression vector for MKP2 was effective in attenuating GnRH-

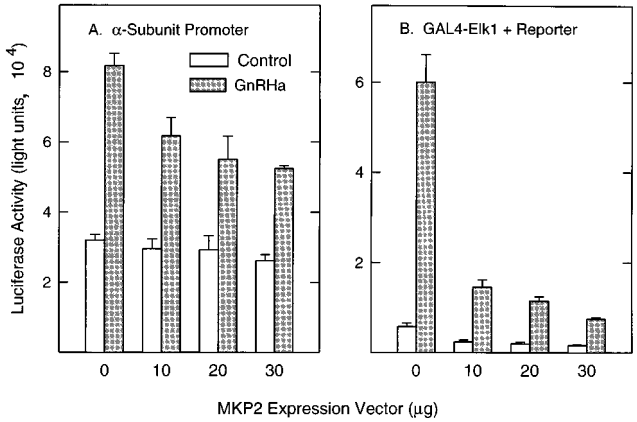

FIG. 6. Expression vector for MKP2 reduces GnRH-induced activation of the α-subunit reporter gene and a GAL4-Elk1 fusion protein in a dose-dependent manner. αT3-1 cells were transfected with either 30 μg of an empty expression vector as a control (CMV control) or 10, 20, or 30 μg of expression vector for MKP2 (CMV-MKP2). The total amount of expression vector in each transfection was maintained at a constant amount (30 μg) by adding the appropriate amount of the CMV control vector. Cells also received either 3 μg of the α-subunit reporter gene or 3 μg of CMV-GAL4 Elk1 expression vector plus 3 μg of 5×GAL4-E1B-luciferase reporter, as indicated. Cells were treated with vehicle alone (control) or 10 nM GnRH agonist Buserelin (GnRHa) 18 h following cotransfection. Cells were collected 24 h after transfection (6 h after treatment), and luciferase activity was determined. Values are means ± standard errors.

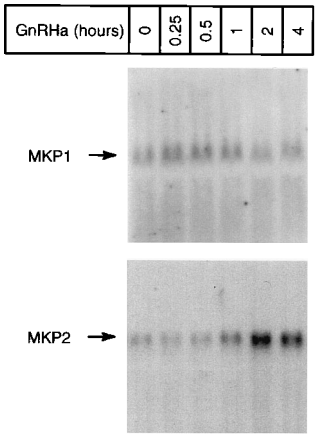

FIG. 7. GnRH increases the concentration of MKP2 mRNA. αT3-1 cells were incubated in serum-free medium overnight and treated with 10 nM GnRH agonist Buserelin (GnRHa) for 0, 0.25, 0.5, 1, 2, or 4 h, and then total cellular RNA was isolated. RNA (10 μg) was electrophoresed under denaturing conditions and then transferred to nylon membranes. MKP1 and MKP2 mRNAs were identified by hybridization to specific, radiolabeled antisense RNA probes.

induced activation of the α-subunit promoter or GAL4-Elk1 in a concentration-dependent manner (Fig. 6). Both MKP1 and MKP2 expression vectors were able to reduce the ability of GnRH to stimulate expression of the α-subunit promoter, and the combination of MKP1 and MKP2 did not appear to have additive effects (data not shown), suggesting that both phosphatases are capable of blocking the transcriptional effects of GnRH in a similar fashion. These findings provide further evidence that inhibition of the MAPK signal transduction cascade can diminish the ability of GnRH to maximally activate α-subunit expression.

GnRH increases the concentration of MKP2 mRNA. MKP1 was originally identified as the product of an immediate-early gene which is rapidly induced by growth factors and serum (8). This led to the hypothesis that growth factor-induced expression of MKP1 may serve to attenuate activation of MAPK and terminate the effects of growth factors. It seemed possible that GnRH might also induce expression of MKPs and that this would contribute to termination of the effects of GnRH on transcription. Therefore, we examined the effects of GnRH on regulation of MKP1 and MKP2 mRNA concentrations (Fig. 7). Transcripts which hybridize to probes for MKP1 and MKP2 are present in αT3-1 cells, and MKP2 mRNA levels were substantially increased after 1 to 4 h of GnRH treatment. Surprisingly, GnRH treatment had little effect on the concentration of MKP1 mRNA. These findings suggest that GnRH-induced expression of MKP2 plays a physiological role in regulating the effects of GnRH on glycoprotein hormone gene expression in vivo.

Ets binding site within the GnRH response element is essential for responses to the MAPK cascade. To further explore the mechanisms which permit GnRH-mediated regulation of the α-subunit promoter, we examined the DNA elements which are necessary for this response. Previously we found that two different DNA elements, designated PGBE and GnRH-RE, are required for regulation of the α-subunit promoter by GnRH (47). We therefore determined if these same DNA elements are also required for responding to a constitutively active form of Raf-1 which would lead to activation of the MAPK cascade (Fig. 8). Consistent with our previous studies of GnRH responsiveness, mutations within either the PGBE or the GnRH-RE substantially reduced Raf-induced activation of the α-subunit reporter gene. We also previously found that a multimer of the GnRH-RE placed upstream of a minimal promoter was sufficient to permit a response to GnRH, while a similar construct containing a multimer of the PGBE did not support a response to GnRH (47). This led us to test the response of these constructs for responsiveness to the Raf-1 expression vector. We find that, as was observed for responses to GnRH, the multimer of the GnRH-RE permits a response to Raf-1, but the PGBE multimer is essentially unresponsive (Fig. 9). The finding that the GnRH-RE is sufficient to permit a transcriptional response to activation of the MAPK pathway led us to focus our attention on this DNA element.

The GnRH-RE contains a site which matches a core Ets binding site, GGAA (Fig. 10A), and this Ets binding site lies within the mutation which inhibits GnRH responsiveness.

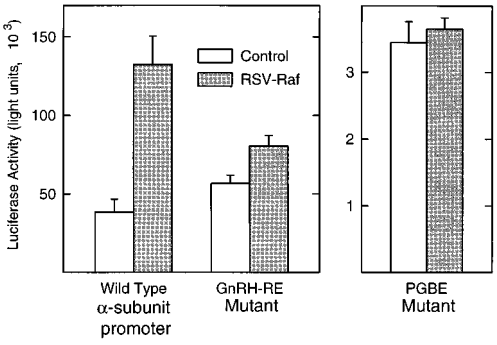

FIG. 8. Analysis of DNA sequences required to mediate Raf kinase-induced expression of the mouse α-promoter. αT3-1 cells were transfected with 3 μg of either a wild-type α-subunit reporter gene described in the legend to Fig. 1 or variants containing mutations within the PGBE or the GnRH-RE in the absence (control) or presence of 6 μg of an expression vector encoding a constitutively active form of Raf kinase (RSV-Raf). Cells were collected 24 h after transfection, and luciferase activity was determined. Values are means ± standard errors.

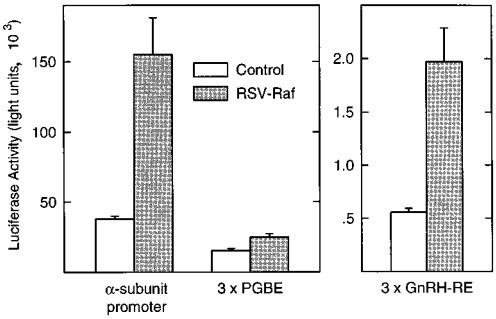

FIG. 9. Multimers of the GnRH-RE can function as a Raf-1-responsive enhancer. αT3-1 cells were transfected with 3 μg of a reporter gene containing the α-subunit promoter, a reporter containing three copies of the PGBE upstream of a minimal promoter linked to luciferase, or a similar reporter gene containing three copies of the GnRH-RE in the absence (control) or presence of 6 μg of an expression vector encoding a constitutively active form of Raf kinase (RSV-Raf). Cells were collected 24 h after transfection, and luciferase activity was determined. Values are means ± standard errors.

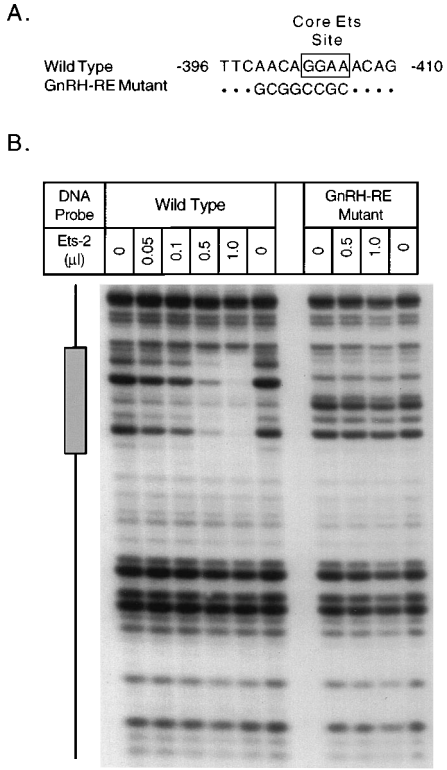

FIG. 10. DNA-binding domain of an Ets transcription factor binds to a specific site within the GnRH-RE of the α-subunit gene. (A) The sequence of a portion of the α-subunit gene is indicated, and below the sequence, a clustered point mutation which diminishes responses to GnRH (GnRH-RE mutant) is also shown. DNase protection studies were used to examine the sequence specificity of Ets binding to this region of the α-subunit gene. Recombinant Ets-2 DNA-binding domain was expressed in *E. coli* as a fusion protein containing a polyhistidine amino-terminal extension. The recombinant Ets-2 DNA-binding domain was purified by chromatography over nickel chelate-agarose. DNA probes representing either wild-type GnRH-RE (−457 to −351) or a GnRH-RE mutant of the mouse α-subunit gene were radiolabeled at their 5′ termini and incubated with increasing amounts of recombinant Ets-2 DNA-binding domain. The DNA was partially digested with DNase I, and the fragments were analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. The protected region of wild-type DNA is indicated by the shaded box.

There are a number of examples of Ets family members which participate in transcriptional responses to the MAPK pathway. For instance, the Ets family member Elk1 is important for mediating growth factor- and serum-induced activation through the serum response element (21, 24). Several other members of the Ets family have been implicated in transcriptional responses to the MAPK signal transduction cascade (6, 10, 12, 18, 35, 57, 59). This led us to consider the possibility that the putative Ets factor-binding site in the GnRH-RE might play a role in mediating transcriptional regulation of the α-subunit gene. A DNase protection assay was used to determine if an Ets factor could interact with the GnRH-RE in a sequence-specific manner (Fig. 10B). These studies revealed that the DNA-binding domain of Ets-2 was capable of binding to a specific site in the 5′-flanking region of the α-subunit gene and that a mutation within the GnRH-RE blocked this protein-DNA interaction. The DNA-binding domain of ER81, another Ets factor (4), was also capable of binding to the α-subunit promoter in a similar sequence-specific manner (data not shown).

To test the functional role of Ets factors in regulation of the α-subunit promoter, we transfected an expression vector for the DNA-binding domain of Ets-2 into αT3-1 cells. This approach is based on the anticipation that the DNA-binding domain of Ets-2 would lack transcription-stimulating activity and that it would function as a competitive inhibitor of an endogenous Ets factor. Transfection of the expression vector for the DNA-binding domain of Ets-2 reduced the ability of the GnRH agonist to stimulate expression of the α-subunit reporter gene (Fig. 11A). The Ets-2 DNA-binding domain was also able to reduce the GnRH responsiveness of a enhancer construct containing three copies of the GnRH-RE upstream of a minimal promoter (Fig. 11B). These findings suggest that the Ets factor-binding site in the GnRH-RE likely plays a role in mediating transcriptional responses to GnRH.

DISCUSSION

These studies provide evidence that MAPKs participate in mediating GnRH effects on the transcription of the glycoprotein hormone α-subunit gene. Several different lines of evidence are consistent with this conclusion. One approach has involved transfection of an expression vector for a constitutively active form of Raf which would be expected to activate MAPK kinase and MAPK. The constitutively active form of Raf was able to increase expression of α-subunit reporter genes in a concentration-dependent manner. Thus, activation of the MAPK pathway is sufficient to stimulate transcription of the α-subunit gene. We found that GnRH treatment resulted in activation of MAPK, as determined by analysis of the phosphotyrosine content of ERK1 and ERK2. Similarly, an immune complex assay indicated that GnRH is capable of activating ERK1 and ERK2. We also found that GnRH stimulation of gonadotropes was sufficient to activate a MAPK-regulated transcription factor, Elk1 (21, 32), again consistent with GnRH effects on activation of the MAPK pathway. Importantly, we found that expression vectors for either kinase-defective ERKs or MAPK phosphatase attenuated GnRH-induced activation of the α-subunit reporter gene. Overall, these findings suggest that GnRH activates the MAPK cascade and that activation of the pathway contributes to GnRH effects on the transcription of the α-subunit gene.

While these studies provide strong evidence for a role for MAPK in mediating the transcriptional effects of GnRH, several aspects of the signaling pathway have not yet been defined. Binding of GnRH to its receptor has been shown to lead to activation of phospholipase C, increases in phosphatidylinositol metabolism, and activation of PKC (3, 22, 23, 40). A num-

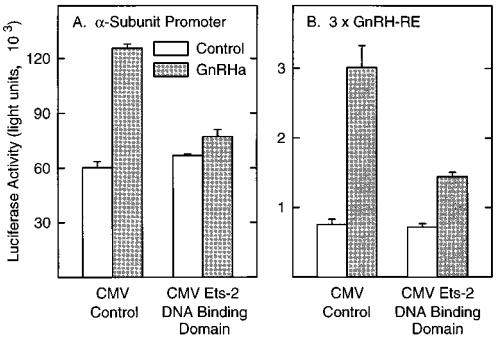

FIG. 11. Expression vector for the DNA-binding domain of Ets-2 reduces the ability of GnRH to increase transcription. αT3-1 cells were transfected with 30 μg of either an empty expression vector (CMV control) or an expression vector encoding the DNA-binding domain of Ets-2. Cells also received 3 μg of either the α-subunit reporter or a reporter gene containing three copies of the GnRH-RE upstream of a minimal promoter linked to luciferase, as indicated. The cells were treated with vehicle alone (control) or 10 nM GnRH agonist Buserelin (GnRHa) 18 h following cotransfection. Cells were collected 24 h after transfection (6 h after treatment), and luciferase activity was determined. Values are means ± standard errors for three separate transfections.

ber of studies have shown that receptors which couple to G_q and G_{i2} can activate the MAPK pathway (13, 43, 62). However, the pathways which mediate this response have not been completely determined. Possible candidates for downstream effectors of PKC which could lead to MAPK activation include Raf kinase (26) and MAPK kinase kinase (2, 28). The activation of MAPK kinase kinase by PKC has been shown to occur in both a Ras-dependent and a Ras-independent manner (28). Although we have shown that constitutively active Raf kinase can stimulate expression of the α-subunit, there is no direct evidence that Raf participates in the GnRH signal transduction pathway. In the present studies, we have used Raf expression vectors as a tool to activate the MAPK cascade. It remains to be determined if GnRH alters Raf or MAPK kinase kinase activity in αT3-1 cells.

We used expression vectors for MAPK phosphatase or kinase-defective MAPKs in an attempt to interfere with the ability of GnRH to activate the MAPK cascade. These expression vectors reduced but did not block the ability of GnRH to stimulate the α-subunit promoter. As has been suggested by others (11), the partial blocking effects of these vectors may indicate a role for signal transduction pathways other than the MAPK cascade or suggest that such reagents are not effective in disrupting activation of endogenous MAPK. At present, it is not possible to distinguish between these possibilities. A possible candidate for alternative signal transduction pathways is GnRH-induced increases in intracellular Ca^{2+} levels (3, 22, 23, 40). Presumably, Ca^{2+} mediated activation of the α-subunit promoter would involve kinases other than Ca^{2+}/calmodulin-dependent protein kinase II or IV, as we found that neither of these kinases was sufficient to activate the α-subunit promoter. While we cannot rule out a possible role for other signal transduction pathways, the present findings rather strongly suggest that GnRH-induced activation of the MAPK pathway contributes to stimulation of α-subunit promoter activity.

Our findings suggest that a member of the Ets family of transcription factors may be important for mediating the transcriptional effects of GnRH. We found that the same DNA elements which are required for GnRH-mediated activation of the α-subunit gene are also required for Raf-induced expression. The results from these and previous studies (47) strongly suggest a role for both the PGBE and the GnRH-RE in mediating responses to GnRH and activation of the MAPK pathway. While the PGBE is important for the GnRH response in the context of the normal gene structure, a reporter gene containing multiple copies of the PGBE is not capable of responding to GnRH. The interaction of the LIM homeodomain factor LH-2 with the PGBE stimulates basal expression of the α-subunit promoter (42), and it may be that this increased basal transcription is crucial for responses to GnRH. It is also possible that LH-2 physically interacts with other factors which are important for mediating responses to GnRH. This possibility is enhanced by the observation that the LIM motif can function as a protein-protein interaction domain (44, 45). In contrast to the PGBE, a multimer of the GnRH-RE is sufficient to function as a GnRH- or Raf-1-responsive enhancer. This suggests that a factor which binds to the GnRH-RE serves as an effector of the MAPK pathway. Our findings demonstrate that the GnRH-RE contains a site which can bind to Ets factors. In addition, an expression vector encoding the DNA-binding domain of Ets-2 reduced the GnRH responsiveness of the α-subunit reporter gene. These results are consistent with the involvement of a member of the Ets family of transcription factors in mediating the ability of the GnRH-RE to respond to activation of the MAPK pathway.

It is striking that Ets factors have been implicated in mediating several different transcriptional responses to the MAPK pathway. Perhaps the best characterized of these concerns the role of the Ets-related factor Elk1 in permitting responses to growth factors (21, 24, 32). In addition to Elk1 and SAP1, a new Ets factor, Net, has been shown to form a ternary complex with serum response factor and to mediate transcriptional responses to MAPK (18). A binding site for an Ets factor in the polyomavirus enhancer also appears to be important for responses to the MAPK signal cascade (5, 58). Recent studies suggest a role for Ets factors in permitting MAPK-stimulated expression of the prolactin promoter (12). In *Drosophila melanogaster*, two Ets-related transcription factors which are required for eye development, pointed and yan, are regulated by MAPK (35). Thus, there is abundant evidence that Ets factors can serve as MAPK-regulated transcription factors. We also observed that GnRH treatment was able to increase the ability of GAL4-Elk1 to activate transcription. It should be noted that although we have shown that activation of the MAPK cascade can increase the activity of GAL4-Elk1, we have no evidence that Elk1 is the endogenous Ets factor which binds to the GnRH-RE. We have simply used Elk1 as a convenient assay for nuclear effects of MAPK activation. Similarly, although we have shown that Ets-2 can bind to the GnRH-RE, we have not determined that Ets-2 is the endogenous factor which binds at this site. Further studies will be required to identify the Ets factors which interact at the GnRH-RE and which contribute to transcriptional regulation of the glycoprotein hormone α-subunit gene by the MAPK pathway.

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