An Early Growth Response Protein (Egr) 1 cis-Element Is Required for Gonadotropin-releasing Hormone-induced Mitogen-activated Protein Kinase Phosphatase 2 Gene Expression*

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Tong Zhang‡, Michael W. Wolfe§, and Mark S. Roberson‡†

From the ‡Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853 and the §Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas 66160-7401

In pituitary gonadotropes, gonadotropin-releasing hormone (GnRH) activates all three major mitogen-activated protein kinase (MAPK) cascades. The MAPKs play key roles in transcriptional activation of GnRH-responsive genes. MAPK phosphatases (MKPs) are dual specificity protein phosphatases involved in feedback regulation of MAPK activity. Previous studies indicate that GnRH activates MKP-2 expression in gonadotropes, dependent upon activation of multiple MAPKs and discrete Ca2+ signals. To further understand the transcriptional mechanism(s) of MKP-2 induction by GnRH, we studied the activity of a 198-nucleotide MKP-2 proximal promoter region that supports GnRH responsiveness in reporter gene assays. Functional analysis of the MKP-2 promoter confirmed a requirement for the protein kinase C-extracellular signal-regulated kinase (ERK) pathway and VGCC-derived Ca2+ signals in transcriptional activation of the MKP-2 gene. However, the inhibitory effect of thapsigargin on MKP-2 protein expression previously identified was not mediated at the level of promoter activation, suggesting a distinct mechanism for the action of thapsigargin-sensitive Ca2+ signals. MGRE (MKP-2 GnRH response element) within the MKP-2 promoter mediated promoter activation through the protein kinase C-ERK pathway. The zinc finger transcription factor Egr-1 was identified in the MGRE-binding complex. Egr-1/MGRE binding was induced by GnRH in an ERK-dependent manner. Transcriptional activity of Egr-1 protein was enhanced by GnRH treatment. In addition, overexpression of the Egr-interacting protein, NAB1, resulted in increased GnRH-stimulated MKP-2 gene transcription. Consistent with the putative role of Egr-1 in MKP-2 promoter regulation, Egr-1 protein expression closely correlated with the expression of MKP-2 protein in αT3–1 cells. Together, these data suggest that Egr-1 may be a key factor in mediating GnRH-dependent transcriptional activation of the MKP-2 gene.

The hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH),1 controls synthesis and secretion of pituitary gonadotropic hormones follicle-stimulating hormone and LH. Follicle-stimulating hormone and LH are heterodimeric glycoprotein hormones essential for reproductive function in mammals (1). The GnRH receptor is coupled to a G-protein in the Gq/11 family (2, 3). Activation of the GnRH receptor results in elevation of intracellular calcium levels, a requirement for gonadotropin secretion (for review, see Ref. 4). In addition, GnRH activates PKC isozymes (5–7) and all three major subfamilies of MAPKs, including ERK, c-Jun N-terminal kinase, and p38 MAPK (8–15). The MKP-2 cascade mediates cell signaling from the GnRH receptor to the nucleus and are critical for transcriptional activation of many GnRH-responsive genes, including the glycoprotein hormone α subunit gene (9, 12), the LHβ subunit gene (16), the GnRH receptor gene (17), and the MKP-2 gene (18). These MAPK-dependent transcriptional activation events are mediated by activation of transcription factors such as c-Fos, c-Jun (19), Elk1 (9), and Egr-1 (16).

MAPKs are activated by dual phosphorylation of the Thr and Tyr residues in a conserved TXY motif (for review, see Refs. 20 and 21). Dephosphorylation of either residue inactivates MAPKs. Members of a unique family of dual specificity protein phosphatases, the MKPs, specifically inactivate MAPKs and therefore serve as important intracellular negative regulators of MAPK signaling cascades (for review, see Refs. 22–25). Ten MKPs have been identified in mammalian tissues (25, 26). Each MKP has distinct binding affinity toward different MAPKs that defines the substrate specificity of the MKP. Binding of a specific substrate to MKPs induces conformational changes in the MKPs (27, 28), leading to catalytic activation of these phosphatases (26, 29–32). In addition to the post-translational regulation of MAPK catalytic activity by MAPKs, MKP expression is also tightly controlled both temporally and spatially, suggesting that each MKP has a distinct physiological function. Several MKPs, including MKP-1 (33, 34), PAC-1 (35), and hVH5 (36), are induced as immediate early genes in various cell types. The responses of other MKPs, such as MKP-2 (37, 38) and MKP-3 (39, 40), can be either immediate or delayed, depending on cell type and stimuli. In many cases MKP expression is induced by MAPKs (18, 41–46), suggesting that MKPs form a negative feedback loop to control intracellular MAPK activity.

Because the physiological functions of MKPs are largely determined by their expression patterns, it is important to...
understand the mechanisms of regulation of MKP expression. Studies of the MKP-1 gene have identified an E-box and two cAMP response elements in the proximal promoter region as important elements for activation of the gene (47–49), whereas an E-box and an AP-2-like site are critical for PAC-1 activation in hematopoietic tissues (44). MKP-2 is a nuclear MKP closely related to MKP-1 and PAC-1 (37, 38, 50). It is expressed in a broad range of tissues and is induced by various extracellular stimuli. GnRH stimulation of pituitary gonadotropes results in marked induction of MKP-2 expression (9, 18). This induction is mediated through MAPK-dependent and independent pathways (18). Activation of both the ERK and c-Jun N-terminal kinase pathways, but not the p38 MAPK cascade, is required for MKP-2 expression. Discrete Ca2+ signals also contribute to MKP-2 expression through modulation of MAPK-dependent and -independent pathways. To better understand the mechanism underlying MKP-2 induction by GnRH, we conducted functional analysis of the 5'-flanking region of the MKP-2 gene. A DNA element, MGRE (MKP-2 GnR response element), was identified within the proximal promoter region of the MKP-2 gene that mediates GnRH responsiveness. Egr-1, a zinc finger transcription factor essential for pituitary LHβ expression and normal reproductive function (51, 52), was identified in the MGRE-binding complex. GnRH induced binding of Egr-1 to the MGRE in an ERK-dependent manner and enhanced transcriptional activity of Egr-1. Egr-1 protein expression closely correlated with the expression of MKP-2 protein in αT3-1 cells, suggesting a role of Egr-1 in transcriptional regulation of the MKP-2 gene.

MATERIALS AND METHODS

Preparation of Reporter Constructs and Expression Vectors—A luciferase reporter construct MKP-2-Luc containing nucleotides −1664 to +123 of MKP-2 5'-flanking region in pGL3-basic vector (Promega) was prepared as described previously (53). Successive deletions of the 5’ end of the MKP-2 sequence were made by PCR. The forward primers used in these reactions were: fw −545, 5’-TCCGGGGAATCCGATT-3’, fw −198, 5’-CTCTAGAATCCGGATTTTTTTT-3’, fw −169, 5’-GGGAGCAGCCACCCGG-3’, fw −115, 5’-AGCCGGCTGCGAGGG-3’, fw −89, 5’-GTCAGTCTAGATCTTTTTAAATTAT-3’; and MGRErev oligonucleotides were annealed and radiolabeled with 32P using T4 polynucleotide kinase. The consensus Sp1- and Egr-1-binding sites are in bold: MUT, 5’-GACCGAGGAGGAAACTCTGGCT-3’; and fw 193, 5’-CACCGAGC-3’.

Mutated nucleotides are underlined. Wild type MGRE sequences are described previously (9, 57, 58).

Expression vectors for Gal4-Elk1, Gal4-Egr-1, and NAB1 have been prepared as described previously (53). Successive deletions of the 5’-flanking region in pGL3-basic vector (Promega) were resuspended in an annealing buffer and incubated at 94 °C for 2 min. The molecules were then ligated into pGL3-basic vector. The products from these two reactions were gel-purified and combined together to be used as template for another PCR reaction using primer pair fw −198/rev +193. The product of this PCR reaction was gel-purified and the nucleotide sequence was verified by nucleotide sequence analysis. The mutant sequence was cloned into the pGL3 basic vector as described above. The resulting construct containing MKP-2 sequence from nucleotides −198 to +123, with partial block substitution of MGRE, is designated MUT-198-Luc.

Cell Culture, Transient Transfection, and Luciferase Assay—The mouse gonadotrope cell line, αT3-1 (generously provided by Dr. Pamela Mellon, University of California, San Diego), was cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum and 5% horse serum. For transient transfection studies, the cells were grown to ~50% confluence in 60-mm culture dishes. pGL3-basic vector or luciferase reporter constructs containing MKP-2 5'-flanking sequence were transfected into αT3-1 cells using the calcium phosphate precipitation method. Plasmid DNA-calcium phosphate precipitate was added to the cell culture medium for 4 h. At the end of the incubation, cells were washed and placed into fresh serum-containing medium. The cells were then stimulated with or without 10 nM GnRHs for 4 h prior to preparation for luciferase assay. In overexpression studies using NAB1, a 4-h incubation period was used between the end of transfection and the initiation of agonist treatment to allow for accumulation of overexpressed NAB1 protein in transfected cells. When fetal bovine serum was used as an agonist, the cells were serum-starved for 4 h following transfection and then stimulated with or without 10 nM GnRHs for 4 h. For transient transfection studies using 5xGal4-E1B-Luc reporter, αT3-1 cells were transfected with LipofectAMINE (Life Technologies, Inc.) overnight. The cells were then washed with serum-free medium and cultured with or without GnRHs for 8 h. For both transfection methods, cell lysates were prepared by three freeze-thaw cycles and clarified by centrifugation. Luciferase activity was determined in samples containing similar amounts of total cellular protein as described previously (9). The transfection studies were conducted in triplicate on at least three separate occasions. The data shown are from a representative experiment and reported as the means ± S.E. (n = 3).

Preparation of Nuclear Extracts—αT3-1 cells were cultured in 150-mm culture dishes to ~50% confluence. Prior to hormone stimulation, the cells were serum starved for 2 h followed by pretreatment with or without PD98059 (50 μM) for 30 min. The cells were then treated with or without GnRHs for 30 min. At the end of the hormone treatment, nuclear extracts were collected as described previously (9). Briefly, after one wash with ice-cold HEPES-buffered saline (20 mM HEPES, pH 7.5, 137 mM NaCl, 5 mM KCl, 1 mM Na2HPO4, and 0.1% dextrose), the cells were scraped in HEPES-buffered saline and collected by centrifugation. The cells were placed in hypotonic buffer and lysed in a Dounce homogenizer, and the nuclei were isolated by centrifugation. Nuclei were resuspended in an EMSA binding buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerc, 1 mM EDTA, 1 mM diithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 25 mM β-glyceral phosphate, 1 mM disodium pyrophosphate and 5 mM sodium vanadate. Nuclear proteins were extracted by adding additional NaCl to a final concentration of 450 mM and incubating at 4 °C for 30 min with constant rocking. Nuclear debris was removed by centrifugation at 75,000 rpm for 30 min, and protein concentration of the samples was determined by Bradford assay. Nuclear extracts were aliquoted and stored at ~80 °C until use.

EMSA—EMSA was conducted as described previously (56). MGREFw and MRGRrev oligonucleotides were annealed and radiolabeled with polyadenylate kinase nucleotide and (γ-32P)ATP. αT3-1 cell nuclear extracts were incubated with 1 μg of poly(dI-dC) in EMSA binding buffer and incubated at room temperature for 30 min. Labeled MGRE probe (~20,000 cpm) was added, and the binding reactions were incubated for another 30 min at room temperature. The reactions were resolved on 4% native polyacrylamide gels in 0.25% TBE (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM EDTA) at 4 °C. The gels were dried, and DNA-protein binding complexes were visualized by autoradiography. For competition EMSA studies, the labeled oligonucleotides were incubated with nuclear extract for 30 min before the addition of the labeled MGRE probe. The competitors include MGRE, MUT, Sp1, Sp1, and eGGR. The sequences of MUT, Sp1, and eGGR are indicated below. The consensus Sp1- and Egr-1-binding sites are in bold: MUT, 5’-ATGGACTGTAGCTATCTTAAAAATTAT-3’; Sp1, 5’-CGTGAGGCCGCCGGGCCTGCG-3’; and 3’-CGAGGGGGCGCCGCGGCGTGGTATGTTATCTGGGTTGCGAGGGG-5’.
RESULTS

A 198-Base pair 5′-Flanking Sequence of the MKP-2 Gene Supports GnRH Inducibility—We have previously isolated the 5′-flanking region (−1664 to +123) of the rat MKP-2 gene (53). This MKP-2 sequence supports both basal and PKC-induced expression of the luciferase reporter gene in the rat somatolactotrope cell line, GH3. In the present study, we first examined the expression of the same MKP-2 reporter construct (MKP-2-Luc) in the αT3-1 gonadotrope cell line. Transient transfection studies indicate that the basal expression level of MKP-2-Luc was higher than the expression level of pGL3 basic vector or mα-Luc (Fig. 1A). GnRH markedly induced MKP-2-Luc expression, suggesting that the MKP-2−1664 to +123 sequence can support both basal and GnRH-induced expression of the MKP-2 gene in gonadotropes. The expression level of MKP-2-Luc was comparable with that of the mouse glycoprotein hormone α subunit reporter (mα-Luc).

To identify regions of the MKP-2 5′-flanking sequence that mediate GnRH responsiveness of the gene, luciferase reporter constructs bearing 5′ deletions of the MKP-2 sequence were examined in αT3-1 cells. The cells were transiently transfected with 1 μg of the reporter construct using the calcium phosphate precipitation method. 3 h after transfection, the cells were washed with fresh serum-containing medium and cultured with or without GnRH (10 nM) for 4 h.

The expression levels of MKP-2-Luc were compared with those of the empty luciferase vector (pGL3 basic), a mouse glycoprotein hormone α subunit reporter gene (mα-Luc), and a reporter gene containing the minimal thymidine kinase promoter (TK-Luc). The GnRH-dependent fold induction of pGL3 basic, MKP-2-Luc, and mα-Luc is indicated by the numbers to the left, B, reporter genes bearing 5′ deletions in the MKP-2 5′-flanking region were prepared by PCR and tested in transient transfection studies. The lines and numbers on the left side indicate the MKP-2 sequence contained in each reporter construct. The arrows indicate the position of the most 5′ transcription start site of the MKP-2 gene (53). The construct containing MKP-2 sequence from nucleotide −198 to +123 was designated MKP-2-198-Luc. All transfection studies were conducted in triplicate on three separate occasions with similar results. The data shown are from a representative experiment reported as the means ± S.E. (n = 3).

FIG. 1. Expression of MKP-2 reporter genes in αT3-1 cells. A, basal and GnRH-induced expression of a MKP-2 reporter gene (MKP-2-Luc) containing the rat MKP-2 sequence from nucleotides −1664 to +123 was examined in αT3-1 cells. The cells were transiently transfected with 1 μg of the reporter construct using the calcium phosphate precipitation method. 3 h after transfection, the cells were washed with fresh serum-containing medium and cultured with or without GnRH (10 nM) for 4 h. The expression levels of MKP-2-Luc were compared with those of the empty luciferase vector (pGL3 basic), a mouse glycoprotein hormone α subunit reporter gene (mα-Luc), and a reporter gene containing the minimal thymidine kinase promoter (TK-Luc). The GnRH-dependent fold induction of pGL3 basic, MKP-2-Luc, and mα-Luc is indicated by the numbers to the left, B, reporter genes bearing 5′ deletions in the MKP-2 5′-flanking region were prepared by PCR and tested in transient transfection studies. The lines and numbers on the left side indicate the MKP-2 sequence contained in each reporter construct. The arrows indicate the position of the most 5′ transcription start site of the MKP-2 gene (53). The construct containing MKP-2 sequence from nucleotide −198 to +123 was designated MKP-2-198-Luc. All transfection studies were conducted in triplicate on three separate occasions with similar results. The data shown are from a representative experiment reported as the means ± S.E. (n = 3).

DNA Pull-down Assay—The MGREfw oligonucleotide was biotinylated at the 5′ terminus during the synthesis reaction (Life Technologies, Inc.). Equal amounts of the biotin-labeled MGREfw and the unlabeled MGRErev were annealed in binding buffer (20 mM HEPES, pH 7.9, 20 mM NaF, 1 mM EGTA, 1 mM EDTA, 0.2% Nonidet P-40, 5% glycerol, 100 mM NaCl, 1 mM dithiothreitol, 1 mM disodium pyrophosphate and 0.5 mM phenylmethylsulfonyl fluoride). MGRE pull-down was performed as described previously (54). Briefly, annealed biotinylated MGRE oligonucleotides were bound to streptavidin-agarose (SA) beads (Sigma) in binding buffer at 4 °C. Approximately 5 pmol of MGRE oligonucleotides were added for each μl of SA beads. Bound MGRE-SA beads were washed four times in the binding buffer and resuspended to the original volume of the SA beads. MGRE pull-down reactions were carried out in the binding buffer containing 20 μl of MGRE-SA beads and 85 μg of nuclear protein in a total volume of 0.5 ml. In some reactions nonbiotinylated competitor oligonucleotides were included. These included MGRE, MUT, and cEgr oligonucleotides. The binding reactions were incubated for 4 h at 4 °C with constant rocking. At the end of the incubation, the SA beads were pelleted by low speed centrifugation and washed six times in the binding buffer. The binding complexes were denatured by boiling and resolved on SDS-polycrylamide gels. The proteins were transferred to polyvinylidene difluoride membrane (PerkinElmer Life Sciences) and subjected to Western blot analysis. Egr-1 and Sp1 antibodies were purchased from Santa Cruz Biotechnology.

Preparation of Whole Cell Lysates and Western Blot Analysis—αT3-1 cells were cultured in 60-mm dishes to ~50% confluence. The cells were serum-starved for 4 h before GnRH or serum stimulation. At the end of the experiment, whole cell lysates were collected for Western blot analysis as described previously (18). ERK2, MKP-1, MKP-2, and Egr-1 antibodies (Santa Cruz Biotechnology), anti-active ERK antibody (Promega), and herringdiah peroxidase-conjugated secondary antibody (Bio-Rad) were used according to the manufacturers’ instruction. Immunostained signals were detected using enhanced chemiluminescence reagents (PerkinElmer Life Sciences).
were generated by PCR and tested in transient transfection studies in αT3–1 cells. Basal activity of the MKP-2 promoter progressively declined with successive 5′ deletions (Fig. 1B). However, in these deletion mutants, GnRH inducibility was not abolished until deletion to nucleotide +35 downstream of the putative transcription start site. The shortest MKP-2 fragment identified in this deletion series that retained full GnRH responsiveness was nucleotides −198 to +123. The reporter construct containing this fragment was designated MKP-2–198-Luc.

**Regulation of MKP-2–198-Luc Expression by PKC, MAPK, and Calcium Signals—**GnRH-induced MKP-2 expression requires activation of the ERK cascade (18). PKC activation and VGCC-derived Ca²⁺ signals are essential for GnRH-induced ERK activation (13) and are therefore also required for MKP-2 induction. In addition, thapsigargin-sensitive intracellular Ca²⁺ signals regulate MKP-2 protein expression independent of MAPK activation. To understand how these pathways regulate MKP-2 expression at the transcriptional level, we examined their roles in GnRH-dependent MKP-2–198-Luc activation in transient transfection studies. Inhibition of PKC isoforms by staurosporine or chronic phorbol 12-myristate 13-acetate treatment blocked both basal and GnRH-induced expression of MKP-2–198-Luc (Fig. 2). Similar results were observed when the ERK pathway was inhibited by PD98059. In contrast, the specific p38 inhibitor SB203580 had no effect on the fold induction of MKP-2–198-Luc by GnRH. These studies suggest that the PKC-ERK pathway regulates MKP-2 expression through up-regulation of promoter activation. Blockade of extracellular Ca²⁺ influx through VGCCs by nifedipine inhibited GnRH-induced MKP-2–198-Luc expression. In contrast, disruption of intracellular Ca²⁺ stores by thapsigargin reduced basal luciferase expression by 50% but did not alter the fold induction by GnRH. The effect of nifedipine is highly correlated with blockade of GnRH-induced ERK activation (13). However, previous studies have shown that thapsigargin completely blocked GnRH-induced MKP-2 protein accumulation (18). The data presented here suggest that the thapsigargin-sensitive Ca²⁺ signal is not required for MKP-2 promoter activation but rather functions through distinct mechanisms.

A **26-Nucleotide Element Is Required for GnRH Responsiveness of the MKP-2 Reporter Gene—**The studies on MKP-2–198-Luc indicate that the −198 to +123 region of the MKP-2 gene contains an important regulatory element(s) that mediates GnRH-dependent activation of the gene through the PKC-ERK pathway. In an effort to identify the DNA element(s), we prepared sequential deletions of 26–29 nucleotides from the 5′ end of the MKP-2 sequence. The sites of these deletions are indicated by the arrowheads in Fig. 3A. The reporter constructs bearing these 5′ deletions were tested in transient transfection studies. Although variation in GnRH inducibility was evident with two deletion mutants (−169 and −141), the largest loss in GnRH induction was revealed by the loss of the sequence between −115 and −89 (Fig. 3B), suggesting that this sequence is required for GnRH responsiveness of the MKP-2 gene. The 26-nucleotide sequence was designated MGRE. Block substitution of the MGRE sequence (shown in Fig. 3A) in the context of the −198 to +123 MKP-2 promoter (MUT-198-Luc) markedly reduced fold induction of the reporter gene by GnRH, consistent with the effect of the deletion at −89 (MKP-2–89-Luc; Fig. 3C). These data suggest that the MGRE is the key cis-element for GnRH responsiveness of the MKP-2 promoter. Further, the basal expression levels of both MUT-198-Luc and MKP-2–89-Luc were comparable with that of the wild type MKP-2–198-Luc, indicating that MGRE may not be required for basal activity of the MKP-2 promoter.
MGRE Is Sufficient to Mediate GnRH-induced Transcriptional Activation of Luciferase Reporter Gene—Our deletion and mutation analyses indicate that MGRE is required for GnRH responsiveness of the MKP-2 gene. To further test whether this DNA element is also sufficient to mediate GnRH-dependent transcriptional activation, we prepared luciferase reporter constructs containing one to three MGRE elements upstream of the PRL TATAA box. In transient transfection studies using /H9251 T3–1 cells, a single MGRE element had only a slight effect on both basal expression and GnRH induction of the luciferase reporter gene (Fig. 4A). However, two tandem MGRE elements markedly increased the GnRH responsiveness of the reporter. An additional MGRE element had little effect to further enhance GnRH fold induction of the reporter gene but dramatically increased the luciferase expression level. This reporter construct was designated 3xMGRE-Luc. These data indicate that the MGRE alone is sufficient to mediate GnRH-dependent transcriptional activation of the luciferase reporter gene. However, out of the context of the native MKP-2 promoter, multiple MGRE elements are needed to mediate optimal response to GnRH.

Pharmacological analysis revealed that the signaling requirements for GnRH-dependent 3xMGRE-Luc activation closely resembled those for MKP-2–198-Luc activation (Fig. 4B). Specifically, inhibition of the PKC pathway completely blocked GnRH-dependent expression of 3xMGRE-Luc, whereas disruption of the thapsigargin-sensitive Ca2+ signals had little effect. Together, these data indicate that MGRE mediates GnRH-dependent transcriptional activation through the PKC-ERK pathway in a manner entirely consistent with the −198 MKP-2 promoter fragment.

Binding Activity Associated with MGRE Is GnRH-inducible—To identify the transcription factors that bind to the
MGRE sequence, EMSA were performed using αT3-1 cell nuclear extract and radiolabeled MGRE oligonucleotides. Protein concentrations of all nuclear extracts were adjusted to 4 mg/ml. 4–7 μl of the nuclear extracts were used in each reaction. One predominant MGRE-binding complex was detected in the EMSA (Fig. 5). The binding intensity of this complex correlated with the amount of input αT3-1 nuclear extract. Interestingly, nuclear extracts prepared from αT3-1 cells that received 2 h of GnRH stimulation had markedly increased MGRE binding activity. When the cells were pretreated with PD98059 prior to GnRH stimulation, the MGRE binding activity from their nuclear extracts was reduced compared with the cells treated with GnRH alone. Consistent with our functional promoter analysis, the putative MGRE binding activity was induced by GnRH, and this induction was sensitive to ERK inhibition by PD98059.

Egr-1 Is Present in the MGRE-binding Complex Following GnRH Stimulation—The MGRE sequence is highly GC-rich and contains a putative STRE element (Fig. 3A) that can bind Cys2-His2 zinc finger proteins (59). A recent report indicates binding of Sp1, Sp3, and Egr-1 to a STRE-like element in the PTP1B promoter (60). Based on this observation, we sought to determine whether Egr or Sp proteins were present in the MGRE-binding complex. First, we examined the ability of consensus Sp1 or cEgr oligonucleotides to compete for MGRE binding activity in the GnRH-treated αT3-1 nuclear extract. As shown in Fig. 6, the unlabeled wild type MGRE oligonucleotide competed for DNA binding, whereas the mutant MGRE oligonucleotide (MUT) only competed with MGRE binding at 100-fold molar excess. Interestingly, when unlabeled Sp1 oligonucleotide was used as competitor, MGRE binding activity was moderately enhanced. Unlabeled cEgr oligonucleotide competed for MGRE binding to a similar extent as the wild type.

**Fig. 5.** αT3-1 cell nuclear extract formed a GnRH-inducible binding complex with the MGRE element. EMSA was performed using 32P-labeled MGRE oligonucleotide as probe. Nuclear extract (NE) from unstimulated αT3-1 cells, αT3-1 cells treated with GnRHα for 2 h, or αT3-1 cells treated with PD98059 (50 μM) and GnRHα were used in the assay. The protein concentration of the nuclear extracts was determined by the Bradford assay and adjusted to 4 mg/ml. Increasing amounts of nuclear extract (4–7 μl) were used in the assay as indicated above the gel. The arrows indicate the specific MGRE-binding complex (upper part of the gel) and the free MGRE probe (bottom part of the gel). The assay was repeated three times using different preparations of nuclear extracts. All replicates yielded similar results.
MGRE oligonucleotide. These data suggest that the specific MGRE-binding complex from GnRH-stimulated αT3–1 cells most likely contains Egr-like proteins but not Sp1. Similar results were obtained from competition EMSA studies using nuclear extracts from unstimulated αT3–1 cells (data not shown), suggesting that Egr-like proteins, but not Sp1, are also involved in MGRE binding in unstimulated αT3–1 cells.

Egr-1 is essential for LHβ gene expression in gonadotropes (51, 52) and appears to be the predominant Egr protein regulated by GnRH (61, 62). To examine whether Egr-1 is present in the MGRE-binding complex, we performed MGRE pull-down assays followed by Western blot analysis. Egr-1 was detected in the MGRE-binding complex from GnRH-treated αT3–1 nuclear extract (Fig. 7A). Binding of Egr-1 to the MGRE was blocked by the presence of excess unbound MGRE or cEgr oligonucleotides but not by excess unbound MUT oligonucleotide, suggesting that the binding between Egr-1 and MGRE was specific. Consistent with previous reports that Egr-1 protein is expressed at very low levels in unstimulated αT3–1 cells (62, 63), nontectactable levels of Egr-1 were present in the MGRE-binding complex from control cells (Fig. 7B, left panel). 2 h of GnRH stimulation of αT3–1 cells markedly increased the amount of Egr-1 bound to MGRE in this pull-down assay, and blockade of GnRH-induced ERK activation by PD98059 reduced the amount of MGRE-bound Egr-1 to near nondetectable levels. Overall, these results indicate that Egr-1 binds to MGRE and that binding of Egr-1 to the MGRE is induced by GnRH in an ERK-dependent manner. In contrast to Egr-1, no Sp1 immunoreactivity was detected in the MGRE-binding complex (Fig. 7B, right panel). The same antibody detected Sp1 immunoreactivity in αT3–1 whole cell lysate (data not shown), suggesting that the antibody was functional. These data are consistent with previous EMSA studies (Fig. 6) and suggest that Sp1 likely does not participate in the MGRE-binding complex within the MKP-2 promoter.

FIG. 6. The cEgr oligonucleotide competes for MGRE binding activity. EMSA was carried out using nuclear extract (NE) from GnRH-A-treated αT3–1 cells. Unlabeled MGRE, MUT, Sp1, and cEgr oligonucleotides were included in the reactions as competitors for MGRE binding. The number above each lane indicates the fold excess of the competitor oligonucleotide. The arrows indicate the specific MGRE-binding complex (upper part of the gel) and the free MGRE probe (bottom part of the gel). The assay was repeated three times using different preparations of nuclear extracts. All replicates yielded similar results.

FIG. 7. GnRH induced Egr-1 binding to MGRE. A, DNA pull-down assays were performed using SA-bound MGRE as probe (SA-MGRE). Nuclear extract (NE) from GnRH-A-treated αT3–1 cells was used. Unbound MGRE, MUT, and cEgr oligonucleotides were included in the binding reactions as competitors. The numbers in parentheses indicate the fold excess of the unbound competitor. At the end of the pull-down assay, Egr-1 protein in the MGRE-binding complex was detected by Western blot analysis. Molecular mass standards (in kDa) are shown to the left of the Western blot. B, nuclear extracts from unstimulated αT3–1 cells, αT3–1 cells treated with GnRH-A for 2 h, or αT3–1 cells treated with PD98059 and GnRH-A were used in the MGRE pull-down assay. The MGRE-binding complex was subjected to Western blot analysis to detect Egr-1 and Sp1 proteins. All of the MGRE pull-down assays were repeated two times with similar results. C, activation of a Gal4-Egr-1 fusion protein by GnRH. Expression vectors for the Gal4 DNA binding domain (Gal4-dbd) or the fusion proteins Gal4-Egr-1 and Gal4-Elk1 were co-transfected with the reporter gene 5xGal4-E1B-Luc into αT3–1 cells by lipofection. Following overnight transfection, the cells were stimulated with or without GnRH-A for 8 h and then collected for luciferase assay. The transfection studies were conducted in triplicate on four separate occasions with similar results. The data shown are from a representative experiment reported as the means ± S.E. (n = 3).

GnRH Enhanced the Transcriptional Activity of Egr-1 Protein—Egr-1 is an immediate early gene product induced by a broad range of stimuli. Following synthesis, phosphorylation (64) or co-repressor binding (58, 65, 66) may regulate the transcriptional activity of Egr-1. To examine the possibility that GnRH may regulate the transcriptional activity of Egr-1, we...
studied GnRH regulation of a Gal4-Egr-1 fusion protein. αT3–1 cells were transiently transfected with a luciferase reporter gene containing five Gal4-binding sites together with an expression vector for the Gal4 DNA-binding domain alone (Gal4-db), the Gal4 DNA-binding domain coupled to Egr-1 (Gal4-Egr-1), or the transcriptional activation domain of Elk1 (Gal4-Elk1). GnRH markedly enhanced the ability of Gal4-Elk1 to transactivate the reporter gene (Fig. 7C), consistent with previous observations (9). Interestingly, Gal4-Egr-1 transcriptional activity was also greatly enhanced by GnRH, and the fold activation of Gal4-Egr-1 was comparable with that of Gal4-Elk1. These results provide direct evidence that the activity of Egr-1 as a transcriptional activator is stimulated by components of the GnRH signaling pathway. Because GnRH-dependent activation of the MKP-2 promoter requires an Egr-1-binding site, the enhancement of Egr-1 transcriptional activity by GnRH likely contributes to MKP-2 gene expression.

Overexpression of NAB1 Potentiates GnRH-induced Activation of the MKP-2 Promoter—NAB1 and NAB2 specifically interact with Egr proteins resulting in modified transcriptional responses of Egr-dependent genes. In many cases, NAB overexpression results in transcriptional repression (58, 65, 66). Further, GnRH induces NAB1 expression in αT3–1 cells (62). To examine the possibility that NAB1 may modulate MKP-2 promoter activation by GnRH, we overexpressed NAB1 in αT3–1 cells. Interestingly, NAB1 overexpression moderately enhanced GnRH-dependent activation of the MKP-2 promoter in a dose-dependent manner (Fig. 8). These data suggest that NAB1 may function as a co-activator of GnRH/Egr1-dependent MKP-2 gene expression.

Expression of MKP-2 Is Correlated with Egr-1 Up-regulation in αT3–1 Cells—Many MKP genes are activated by growth factors and serum. Consistent with this, MKP-2 mRNA was up-regulated by serum and epidermal growth factor in PC12 cells (37). Based on these observations, we examined the possibility that in gonadotropes, serum and GnRH may regulate MKP-2 expression through similar mechanisms. First we compared the effect of serum and GnRH on MKP-2 promoter activity in transient transfection studies using αT3–1 cells (Fig. 9A). Surprisingly, serum only modestly increased MKP-2–198-Luc expression over basal expression, suggesting that serum is not an effective signal to activate MKP-2 promoter in αT3–1 cells. Both GnRH and serum activated the ERK pathway, as indicated by Western blot analysis of αT3–1 whole cell lysate using an anti-phospho-ERK1/2 antibody (Fig. 9B). However, the duration of ERK activation was clearly different under the two conditions. Prolonged ERK activation in GnRH-treated cells correlated with Egr-1 expression and MKP-2 protein induction in these cells. In contrast, serum stimulation resulted in transient ERK activation and only a slight induction of Egr-1 and MKP-2 proteins. Interestingly, serum was a potent activator of MKP-1 expression, suggesting that serum was an effective agonist in this system and that differential mechanisms regulate MKP-1 and MKP-2 gene activation. The highly correlative expression of Egr-1 and MKP-2 proteins in αT3–1 cells following GnRH and serum treatment suggests that optimal transcriptional activation of the endogenous MKP-2 gene may be dependent on Egr-1 expression.

DISCUSSION

In pituitary gonadotropes, GnRH activates all three major MAPK cascades as well as MKP-2 and MKP-1 (current studies and Refs. 9 and 18). MKPs are dual specificity phosphatases that specifically dephosphorylate MAPKs, suggesting a role for MKPs in intracellular negative feedback regulation of GnRH-induced MAPK activity and the subsequent MAPK-dependent gene transcription. The present study focused on transcriptional regulation of the MKP-2 gene. MKP-2 expression was
robustly induced following GnRH treatment, and the duration of MKP-2 expression was similar in clonal αT3–1 cells and rat pituitary cells in primary culture (18), indicating that the αT3–1 cell line is a suitable model for the study of MKP-2 gene regulation by GnRH. Our promoter analysis in αT3–1 cells defined a novel cis-element (MGRE) that is both required and sufficient to support GnRH-dependent transcriptional activation of the MKP-2 gene. Consistent with previous observations from our laboratory (18), pharmacological studies examining PKC and ERK inhibition confirm the requirement for the PKC-ERK cascades in GnRH-dependent activation of the MKP-2 promoter via the MGRE. Our biochemical studies support the hypothesis that Egr-1 likely plays a key role in the mechanism(s) regulating transcriptional responses of the MKP-2 gene by GnRH.

The effects of GnRH on MKP-2 protein levels were mediated by MAPK-dependent and -independent pathways that could be differentiated by disruption of discrete Ca2+ signals within the cell (18). In the current study, we confirmed that MKP-2 promoter activity was dependent upon a nifedipine-sensitive VGCC-derived Ca2+ signal. VGCC Ca2+ is necessary for GnRH-induced ERK activation (13) leading to up-regulation of the MKP-2 gene. In our previous studies (18), pretreatment with thapsigargin depleted intracellular Ca2+ stores and completely blocked MKP-2 protein expression independent of changes in MAPK activity. The functional analysis of the MKP-2 promoter presented here suggests that the effect of thapsigargin was not mediated at the level of MKP-2 promoter activity. In addition to MKP-2, thapsigargin also abolished GnRH-induced expression of MKP-1 and c-Fos proteins but had little effect on e-Jun expression.2 This suggests that thapsigargin-sensitive Ca2+ signals regulate the expression of these proteins in a gene-specific way. A recent report described a Ca2+-sensitive transcription elongation block within the first exon of the rat MKP-1 gene (67). Release of the block by Ca2+ signals plays an important role in TRH-stimulated MKP-1 expression in GH4C1 neuroendocrine cells. A similar Ca2+-sensitive elongation block located in the first intron of the c-fos gene has also been reported (68–70). It is tempting to speculate that such a mechanism may also exist for the MKP-2 gene and could be responsible for the observed thapsigargin effect. Alternatively, the thapsigargin-sensitive Ca2+ signals may regulate MKP-2 mRNA or protein stability. The precise level of thapsigargin action on MKP-2 expression is not clear.

Egr-1, the prototype of the Egr family, is an immediate early gene product induced by a broad range of stimuli (for review, see Ref. 71). Our studies indicate that Egr-1 binds to the MKP-2 promoter at a STRE-like sequence (MGRE), a cis-element that mediates GnRH responsiveness of the MKP-2 promoter. Mutations within the MGRE resulted in a marked loss in transcriptional activation by GnRH. A recent study revealed a similar transcriptional mechanism for the regulation of the PTP1B promoter (60). Egr-1, together with Sp1 and Sp3, bound to a STRE-like cis-element and mediated regulation of the PTP1B promoter in response to p210 bcr-abl expression. In addition to Egr-1, other proteins may be present in the MGRE-binding complex and may contribute to the MGRE binding activity and basal expression of MKP-2. For example, in other systems Sp1 can compete for Egr-1 binding in unstimulated cells and regulate basal levels of gene expression (for review, see Ref. 74). However, our EMSA competition studies and DNA pull-down assays excluded this possibility. In both control and GnRH-stimulated αT3–1 cells, Sp1 protein failed to bind to MGRE, indicating that Sp1 is not involved in MKP-2 promoter regulation at the MGRE site. Although Egr-1 is the predominant Egr protein up-regulated by GnRH in gonadotropes (61, 62), our studies do not discount a potential role for other Egr proteins, including Egr-2, Egr-3, and Egr-4, which may bind to the MGRE element.

Interestingly, knockout studies in mice revealed that Egr-1 is essential for gonadotrope function and normal reproduction (51, 52). In the absence of Egr-1, LHβ mRNA is greatly reduced, and infertility occurs, putatively because of a loss of gonadotropic stimuli. Egr-1 binds to the proximal promoter of the LHβ subunit gene and regulates GnRH-dependent LHβ subunit expression. However, LHβ subunit gene activation also requires binding of cell-specific transcription factors (such as steroidogenic factor 1 and the homeobox protein Ptx1; Refs. 61–63, 72, and 73) in concert with Egr-1, resulting in synergistic activation. It is currently not known whether cell-specific factors contribute to MKP-2 regulation within the gonadotrope. The possible involvement of Egr-1 in GnRH-dependent MKP-2 expression suggests a broader role of Egr-1 in the GnRH signaling pathway. In this case, Egr-1 may mediate intracellular negative feedback regulation of MAPK activity through transcriptional activation of MKP-2.

In αT3–1 cells, Egr-1 basal expression is very low. GnRH strongly induces Egr-1 expression through the PKC-ERK pathway (16, 62, 72, 73). The induction of Egr-1/MGRE binding by GnRH may reflect changes in total Egr-1 protein levels following GnRH treatment. In addition to the increase in Egr-1 protein, our studies suggest that GnRH can also enhance the transcriptional activity of Egr-1 protein. Both of these mechanisms may contribute to MKP-2 gene activation through the Egr-1-binding site in the proximal portion of the promoter.

Interaction between Egr-1 and NAB proteins provides yet another level of regulation of Egr-1 activity. Because expression of NAB1 is up-regulated by GnRH in αT3–1 cells (62), NAB1 may serve as a physiological regulator of GnRH-induced MKP-2 expression. Our overexpression studies suggest that NAB1 may function as a co-activator of GnRH-stimulated MKP-2 transcription. Careful examination of the LHβ promoter has revealed similar effects of NAB proteins on LHβ expression (75). Activation of Egr-dependent LHβ transcription by NAB is strictly dependent on binding of Egr proteins to the LHβ promoter and on the interaction between NAB and Egr proteins. Based on these observations, the stimulatory effect of NAB1 on GnRH-induced MKP-2 promoter activity provides indirect evidence that Egr proteins are likely involved in MKP-2 gene activation. In the studies done by Milbrandt and co-workers (75), both the number and affinity of Egr-binding sites determined whether NAB proteins functioned as transcription co-activator or co-repressor. In gene promoters bearing few or low affinity Egr-binding sites, NAB proteins generally enhanced Egr-dependent transcriptional activation. The co-activator function of NAB1 on the MKP-2 promoter supports the conclusion that the single MGRE may reflect a relatively low affinity Egr-binding site. A more detailed functional analysis of Egr-1 binding to the MGRE, as well as other potential Egr sites within the MKP-2 promoter, is necessary to understand the molecular mechanism(s) of NAB1 action in GnRH-induced MKP-2 expression.

In summary, our studies detail a molecular mechanism leading to GnRH-dependent transcriptional activation of the rat MKP-2 gene using a putative gonadotrope cell model. We provide direct evidence for a requirement for an Egr-1-binding site and the PKC-ERK signal transduction cascade in the regulation of the MKP-2 promoter by GnRH. Consistent with previous studies, Ca2+ signals derived from VGCCs are also required to support GnRH-induced ERK activation and subse-
quent up-regulation of the MKP-2 gene. Further, our studies on the MKP-2 promoter provide indirect evidence for a functional role of Egr-1 in MKP-2 promoter regulation based upon NAB1 overexpression and the correlation between in vitro expression of Egr-1 and MKP-2 proteins. These studies support the conclusion that GnRH regulation of Egr-1 plays a principal role in coordinating the expression of two prominent components (LHß and MKP-2) of the gene program induced by GnRH in the pituitary gonadotrope.

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