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Growth Hormone Stimulates Phosphorylation and Activation of Elk-1 and Expression of c-*fos*, *egr-1*, and *junB* through Activation of Extracellular Signal-regulated Kinases 1 and 2*

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Growth hormone (GH), a major regulator of normal body growth and metabolism, regulates cellular gene expression. The transcription factors Elk-1 and Serum Response Factor are necessary for GH-stimulated transcription of c-*fos* through the Serum Response Element (SRE). GH stimulates the serine phosphorylation of Elk-1, thereby enabling Elk-1 to mediate transcriptional activation. The contribution of the Ras/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway to Elk-1-mediated transcriptional activation of the c-*fos* SRE in response to GH was examined. The MEK inhibitor PD098059 attenuated GH-induced expression of the endogenous SRE-regulated genes c-*fos*, *egr-1*, and *junB* as well as transcriptional activation mediated by the c-*fos* promoter. The MEK inhibitor blocked GH-stimulated activation of MEK, phosphorylation of ERK1/ERK2, and MAP kinase activity in 3T3-F442A cells. Blocking MEK activation prevented GH-induced phosphorylation of Elk-1, as well as the ability of Elk-1 to mediate transcriptional activation in response to GH. Overexpression of dominant-negative Ras or the ERK-specific phosphatase, mitogen-activated protein kinase phosphatase-1, blocked the Ras/MEK/ERK pathway and abrogated GH-induced phosphorylation of Elk-1. GH failed to stimulate phosphorylation or activation of Jun N-terminal kinase under the conditions used. GH slightly increased p38-mediated mitogen-activated protein kinase-activated protein (MAP-KAP) kinase-2 activity, but the p38 inhibitor SB203580 did not attenuate GH-promoted Elk-1 phosphorylation. Wortmannin, which inhibited GH-induced ERK phosphorylation, also attenuated transcriptional activation of c-*fos* by GH. Taken together, these data suggest that GH-dependent activation of the Ras/MEK/ERK pathway and subsequent serine phosphorylation of Elk-1 contribute to GH-stimulated c-*fos* expression through the SRE.

Expression of the c-*fos* proto-oncogene is rapidly induced by growth hormone (GH)[1] (1, 2). The Serum Response Element (SRE), an enhancer sequence upstream of the c-*fos* gene, can mediate transcriptional activation in response to GH (3, 4). Transcriptional activation via the SRE in response to GH requires the presence of the transcription factors Ternary Complex Factor (TCF) and Serum Response Factor (SRF) at the SRE (4).

Elk-1 is a member of the TCF family of transcription factors, which also includes SAP-1 and SAP-2/ERP/Net (5, 6). GH was recently found to stimulate serine phosphorylation of Elk-1 and to stimulate Elk-1-dependent transcriptional activation (4). Phosphorylation and activation of Elk-1 in response to agents such as serum and ultraviolet light are reported to be mediated by mitogen-activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinases (ERKs) 1 and 2, as well as the stress-induced kinases Jun N-terminal kinase (JNK) and p38 (7–12).

The binding of GH to its receptor results in the activation of JAK2 (13), initiating several signaling cascades, including one in which a SHC-Grb2-SOS complex activates Ras (14). These events correlate in time with GH-induced activation of Raf, MAPK/ERK kinase (MEK), and ERK1 and -2 (14–17). Activation of the ERK pathway by GH raises the possibility that a MAPK pathway may be involved in the regulation of GH-promoted Elk-1 activity. GH has also been documented to stimulate tyrosine phosphorylation of insulin receptor substrate-1 and -2 and concomitant association of both insulin receptor substrate proteins with the 85-kDa regulatory subunit of phosphatidylinositol (PI) 3-kinase (18–21). Studies in which the PI 3-kinase inhibitor, wortmannin, interferes with ERK activation suggest a link between PI 3-kinase activation and activation of ERKs (22–25). Thus, signaling pathways involving PI 3-kinase may modulate the ERK pathway. This study examines the involvement of Ras, MEK, and ERK1 and -2 in Elk-1-mediated transcriptional activation of c-*fos* in response to GH and possible sensitivity of this pathway to the PI 3-kinase inhibitor wortmannin. Potential roles for JNK and p38 in mediating GH-induced Elk-1 phosphorylation were also investigated.

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[1] The abbreviations used are: GH, growth hormone; GHR, GH receptor; SRE, Serum Response Element; SRF, Serum Response Factor; TCF, Ternary Complex Factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; MKP-1, MAPK phosphatase-1; MAPKAP kinase, MAPK-activated protein kinase; PI, phosphatidylinositol; SAP, SRE-associated protein; STAT, signal transducer and activator of transcription; EGFR, epidermal growth factor receptor; CHO, Chinese hamster ovary; RSV, Rous sarcoma virus; CMV, cytomegalovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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EXPERIMENTAL PROCEDURES

Materials—Stocks of 3T3-F442A cells were provided by Dr. H. Green (Harvard University) and Dr. M. Sonenberg (Sloan-Kettering, New York, NY). Chinese hamster ovary (CHO) cells expressing full-length rat GH receptor (GHR-(1–638)) or GHR lacking the C-terminal half of the cytoplasmic domain (GHR-(1–454)) were provided by Drs. Gunnar Norstedt (Karolinska, Stockholm, Sweden) and Nils Billestrup (Hagedorn Laboratory, Gentofte, Denmark) (26). Recombinant human GH was provided by Lilly. The MEK inhibitor PD098059 was a gift from Dr. A. Saltiel (Parke Davis, Ann Arbor, MI). Wortmannin, the p38 inhibitor SB203580, and anisomycin were purchased from Calbiochem. Sorbitol and phenylmethylsulfonyl fluoride were from Sigma. Leupeptin, aprotinin, and pepstatin were purchased from Boehringer Mannheim, bovine serum albumin (CRG7) from Intergen, and radioisotopes from NEN Life Science Products. Lipofectamine was purchased from Life Technologies, Inc., and luciferin was from Promega. The ECL detection system and Rediprime™ kit were purchased from Amersham Pharmacia Biotech.

Cell Culture and Treatment—3T3-F442A fibroblasts were grown to confluence in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose and 8–9% calf serum in an atmosphere of 10% CO_2/90% air at 37 °C. CHO-GHR cells were grown in Ham's-F12 medium containing 0.5 mg/ml G418 and 10% fetal calf serum in an atmosphere of 5% CO_2, 95% air. All media were supplemented with 1 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin. Prior to treatment, cells were deprived of serum for 16–18 h in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose or Ham's F-12 medium containing 1% bovine serum albumin. Cells were preincubated with inhibitor or vehicle (Me_2SO, 0.2% final concentration, or ethanol) for 1 h prior to treatment with GH or other agents. Unless otherwise indicated, the final concentration of the MEK inhibitor was 100 μM, and GH was used at a concentration of 500 ng/ml (22 nM). This concentration of GH is within the physiological range for circulating rodent GH (27, 28).

Northern Blot Analysis—Total RNA was isolated as described (29) and subjected to Northern blot analysis using ExpressHyb (CLONTECH). Prehybridization was performed at 55 °C, and hybridization was performed at 68 °C. The membranes were probed with cDNAs for mouse c-*fos* (2), *egr-1* (from Dr. L. Lau, University of Illinois, Chicago, IL (30)), *junB* (from Dr. D. Nathans, John Hopkins, Baltimore MD (31)), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, from Dr. M. Alexander-Bridges, Harvard University), which were labeled with ^{32}P using a Rediprime kit from Amersham Pharmacia Biotech. Membranes were subjected to autoradiography and/or phosphor imaging, and quantification was performed using Multi-Analyst/PC Molecular Imager software (Bio-Rad).

Luciferase Assay—3T3-F442A cells were stably transfected with a GH-responsive luciferase reporter plasmid driven by the region of the c-*fos* promoter from −379 to +1 with a mutation in the CCAAT/enhancer-binding protein binding site (mC/E) (32) (provided by Dr. W. Walton, Moffitt Cancer Center, Tampa, FL) and RSV-neo (from Dr. Nils Billestrup, Hagedorn Laboratory). Pooled clones were generated and maintained as described previously (4). Cells were deprived of serum overnight and incubated with GH for 4 h. Cell lysates were prepared in reporter lysis buffer (100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100, 1 mM dithiothreitol), and luciferase (Promega) or β-galactosidase (Tropix) activity was measured using a Wallac-Berthold Autolumat luminometer or an MGM Opticomp luminometer. Analysis of variance with factorial Scheffe F-test was used to analyze data. A value of $p < 0.05$ was considered significant. For control experiments, the plasmids Spi tkluc and RSV-β-gal were transiently co-transfected into CHO cells expressing GHR-(1–454), and luciferase values were measured as above. Spi tkluc containing eight copies of the *spi* 2.1 gene (−147/−102) upstream of a luciferase reporter gene was constructed as follows: the CAT gene was excised from Spi tkCATAn (from S. Berry, University of Minnesota, Minneapolis, MN (33)) with *Xho*I/*Sss*T1, leaving the Spi tk backbone. A 2.7-kilobase pair *Xho*I/*Sst*I fragment containing the luciferase gene was excised from tkluc and then ligated into the Spi tk vector backbone to create Spi tkluc.

Elk-1-mediated Reporter Activation—An expression-reporter system was used to measure Elk-1-mediated transcriptional activation. The expression plasmid Gal4/ElkC (34) encodes a fusion protein containing the Elk-1 transactivation domain fused to the Gal4 DNA binding domain. The control plasmid Gal4 (1–147) (35, 36) encodes amino acids 1–147 of the Gal4 DNA binding domain without an activation domain. The reporter plasmid, 5× Gal/Luc (37) contains five copies of the Gal4 binding site upstream of luciferase cDNA. The plasmids Gal4/ElkC and 5× Gal/Luc were provided by Dr. C. Der (University of North Carolina), and Gal4 (1–147) was received from Dr. M. Ptashne (Harvard University). CHO cells expressing GHR-(1–454) were plated on 35-mm plates and transiently co-transfected using calcium phosphate with 1 μg of Gal4/ElkC or Gal4(1–147) DNA, 1 μg of Gal/Luc DNA, and 0.1 μg of RSV β-galactosidase DNA (provided by Dr. M. Uhler, University of Michigan). At 44–48 h after transfection, cells were deprived of serum for 16–18 h and treated as indicated. Luciferase assays were performed as described above, and luciferase values from experiments evaluating transcriptional activation were normalized to β-galactosidase activity.

Elk-1 Phosphorylation—The cDNA for Elk-1 (from Dr. R. Treisman, Imperial Cancer Research Fund, London) was inserted into the vector pcDNA3 downstream of a CMV promoter to generate CMV-Elk-1 (38). In experiments evaluating Elk-1 phosphorylation, CMV-Elk-1 or pcDNA3 was transiently transfected into CHO cells expressing GHR-(1–454) using calcium phosphate. Other plasmids were co-transfected with CMV-Elk-1 as follows. The plasmids pZIP-ras(WT), pZIP-ras(15A), and the vector pZIP-NeoSV(x)1 (39, 40) provided by Dr. C. Der (University of North Carolina) were each used at 30 μg/100-mm plate. The plasmid encoding MAP kinase phosphatase-1 (MKP-1) (41) was from Dr. J. Pessin (University of Iowa). The CLDN vector, used as a control, was recovered by excising the *Eco*RI/*Bam*HI fragment containing the MKP-1 sequence. Klenow was then used to generate blunt ends, which were ligated with T4 ligase. Both MKP-1 and CLDN were used at 5 μg/100-mm plate. 26 h after transfection, the cells were incubated in medium containing 0.5% fetal calf serum for 16 h and were then incubated in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose and 1% bovine serum albumin for 5 h prior to treatment as indicated. Cells were lysed and immunoblotted as described.

Antibodies—Polyclonal antibodies against a peptide corresponding to residues 379–392 of Elk-1 phosphorylated on Ser-383 (α-P-Elk-1), and polyclonal antibodies against the corresponding nonphosphorylated Elk-1 peptide (αElk-1) were purchased from New England Biolabs. Antibodies to dually phosphorylated ERK1 and ERK2 (α-P-ERK), and dually phosphorylated JNK (α-P-JNK) were purchased from Promega. According to the manufacturer, the α-P-JNK antibodies cross-react with phosphorylated ERK1 and -2. Antibodies to JNK and p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies recognizing MEK1 and MAPKAP kinase-2 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies to nonphosphorylated ERK1/ERK2 and to MEK1/MEK2 were made as described (42).

Immunoblotting—Following treatment, cells were washed three times with PBSV buffer (10 mM Tris, 150 mM sodium phosphate, 100 μM Na_3VO_4, pH 7.4). For analysis of ERKs and Elk-1, cells were scraped in 0.5 ml of 50 mM Hepes, pH 7.0, containing 250 mM NaCl, 0.1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each aprotinin and leupeptin. In assays for JNK phosphorylation, cells were lysed in 50 mM Hepes, pH 7.5 containing 150 mM NaCl, 1.5 mM EGTA, 10% glycerol, and 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg each of aprotinin and leupeptin, 100 mM NaF, and 0.5 mM $Na_4P_2O_7$. Cell lysates were analyzed on 7.5% polyacrylamide gels for Elk-1, 12% polyacrylamide gels for JNK, and 5–12% gradient polyacrylamide gels for ERK1 and -2. Western blot analysis was performed as described (43) with the indicated antibodies. Enhanced chemiluminescence was used for detection. The apparent M_r indicated are based on the migration of prestained molecular weight standards (Life Technologies, Inc.). Membranes were stripped by submerging them in 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7 at 50 °C for 30 min with shaking.

Kinase Assays—MAP kinase assays were performed with myelin basic protein as substrate as described previously (16). MEK activity was measured as described previously (14). Briefly, cell lysates were incubated for 2 h with an antibody recognizing both MEK1 and -2 (1:1000 dilution). Immune complexes were collected on protein A-agarose during a 1-h incubation with rotation at 4 °C; washed three times with 50 mM Tris, pH 7.5, 0.1% Triton X-100, 137 mM NaCl, and 2 mM EGTA; and washed once with 25 mM HEPES, pH 8.0, 0.5 mM EDTA, 0.25% β-mercaptoethanol. The immobilized MEK preparation was then incubated with 0.3 μg of ERK/sample in kinase buffer (90 mM HEPES, pH 7.5, 5 mM magnesium acetate, and 250 μM ATP) for 30 min at 30 °C. Triplicate aliquots (10 μl) of each sample were incubated with 30 μl of kinase buffer containing 20 μg of myelin basic protein and 2 μCi of [γ-^{32}P]ATP for 30 min. Samples were spotted on p81 Whatman filter paper, washed with 1% phosphoric acid, and counted. Assays for p38 utilized its substrate MAPKAP kinase-2. Antibody to MAPKAP kinase-2 (5 μg/sample) was used for immunoprecipitation, and MAPKAP kinase-2 activity was measured in an *in vitro* kinase assay using MAP-

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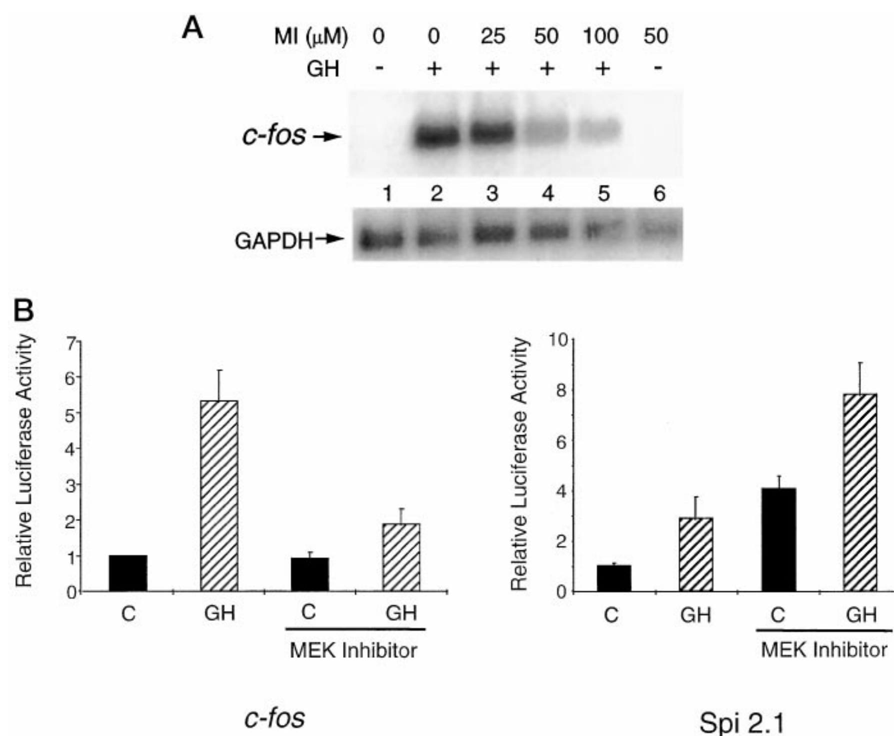

FIG. 1. **The MEK inhibitor PD098059 attenuates GH-stimulated expression and promoter activation of c-*fos*.** *A*, 3T3-F442A fibroblasts were incubated for 1 h with the indicated concentrations of PD098059 in Me_2SO (*MI*, *lanes 3–6*) or Me_2SO alone (*lanes 1* and *2*). Then GH (*lanes 2–5*) or vehicle (*lanes 1* and *6*) were added for an additional 30 min. Total RNA was isolated from cells and assayed for endogenous c-*fos* (*top*) and GAPDH mRNA levels (*bottom*) by Northern blot analysis. Similar results were obtained in three independent experiments. *B*, *left*, 3T3-F442A fibroblasts stably transfected with a plasmid (mC/E) containing a c-*fos* promoter region upstream of a luciferase reporter gene were treated with PD098059 (100 μM) or Me_2SO. GH (*hatched bars*) or vehicle (*C*, *solid bars*) was added for 5 h. Luciferase activity in cell lysates is normalized to the activity in untreated control cells (control = 1). Results show the mean + S.E. for three independent experiments, each assayed in triplicate. Luciferase activity was significantly different ($p < 0.005$) between control and GH-treated cells and between GH-treated cells in the presence and absence of PD098059. There was not a significant difference between vehicle-treated control cells and PD098059-treated control or GH-treated cells. c-*fos* promoter-mediated luciferase values from control and GH-treated cells incubated with PD098059 were not significantly different from those from Me_2SO-treated control cells. *Right*, CHO cells stably expressing GHR-(1–454) were transiently co-transfected with a *spi* 2.1 luciferase reporter and RSV-βgal. The results are representative of two independent experiments.

KAP kinase-2 substrate peptide (250 μmol/sample) as described (44). JNK activity was assayed using lysates from 3T3-F442A cells that were incubated with glutathione *S*-transferase-Jun (from B. Margolis, University of Michigan) complexed to glutathione beads for 3 h. Beads were washed twice with wash buffer (140 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA) and 1% Triton-100 and twice with wash buffer without Triton X-100. Beads were incubated for 20 min at 30 °C with 10 μCi/sample [γ-^{32}P]ATP in 50 mM Tris, pH 7.4, 10 mM $MgCl$, 1 mM EDTA, 40 μM ATP. They were then washed and boiled, and eluted proteins were analyzed by SDS-polyacrylamide gel electrophoresis using a 12% polyacrylamide gel.

RESULTS

Inhibition of the MEK/ERK Pathway Impairs GH-stimulated Transcriptional Activation Mediated by the c-fos Promoter—GH has been shown to promote c-*fos* gene expression (2, 45) as well as activation of Ras, Raf, MEK, and ERK1 and -2 in 3T3-F442A fibroblasts (14–16). To determine whether the MEK/ERK pathway contributes to GH-stimulated c-*fos* expression, the MEK inhibitor PD098059 was used to block activation of the MEK/ERK pathway. PD098059 suppresses MEK activation by binding inactive MEK and preventing its phosphorylation by upstream kinases (46).

The GH-promoted increase in endogenous c-*fos* expression detected after 30 min (Fig. 1*A*, *lane 2*, *top*) was progressively inhibited by increasing concentrations of MEK inhibitor (Fig. 1*A*, *lanes 3–5*). When normalized to GAPDH expression, which is unaffected by GH or PD098059 treatment, expression of c-*fos* was attenuated an average of 31, 69, and 84% with 25, 50, and 100 μM PD098059, respectively ($n = 2$).

To determine whether blocking MEK activation decreases GH-induced activation of the c-*fos* promoter, PD098050 was added to 3T3-F442A fibroblasts stably transfected with a plasmid (mC/E) containing a GH-responsive c-*fos* promoter fragment (−379/+1) upstream of a luciferase reporter gene (Fig. 1*B*). GH produced a greater than 4-fold stimulation of reporter expression (Fig. 1*B*). Pretreatment of cells with 100 μM MEK inhibitor resulted in an 80% reduction in GH-promoted transcriptional activation through the c-*fos* promoter (Fig. 1*B*, *left*). This degree of reduction corresponds well with the 84% decrease in endogenous c-*fos* expression observed with 100 μM PD098059 (Fig. 1*A*). In contrast, PD098059 did not reduce reporter expression mediated by eight tandem copies of the GH-responsive promoter of the *spi* 2.1 gene (Fig. 1*B*, *right*), indicating some specificity of the MEK inhibitor for the pathway leading to expression of the c-*fos* promoter.

The SRE, which mediates GH-promoted expression of c-*fos* (3, 4) is also present in the promoters of the GH-responsive genes *egr-1* (29) and *junB* (47). To assess whether MEK is involved in the expression of SRE-regulated genes other than c-*fos*, the effect of PD098059 on the expression of *egr-1* and *junB* was examined (Fig. 2). In 3T3-F442A cells, GH stimulated expression of both *egr-1* and *junB* (Fig. 2, *A* and *B*, *top*, *lane 2*). In two experiments in which mRNA levels were normalized to GAPDH mRNA levels, expression of *egr-1* and *junB* was inhibited by pretreatment with PD098059 (Fig. 2, *lanes 3–5*). Pretreatment of cells with 50 μM PD098059 decreased GH-promoted expression of *egr-1* by 35% and *junB* by 43%, and 100 μM PD098059 decreased the expression of *egr-1* by 61% and *junB* by 55%.

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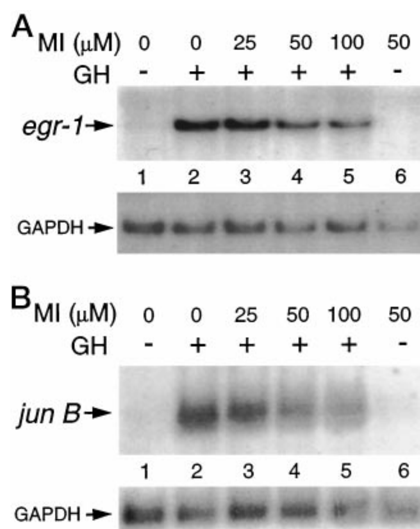

FIG. 2. **The MEK inhibitor PD098059 attenuates GH-induced expression of *egr-1* and *junB*.** 3T3-F442A fibroblasts were incubated for 1 h with Me_2SO (*lanes 1* and *2*) or the indicated concentrations of PD098059 (*MI*, *lanes 3–6*) and then with GH (*lanes 2–5*) or vehicle (*lanes 1* and *6*) for an additional 30 min. Total RNA was assayed by Northern blot analysis for endogenous GAPDH expression (*lower panels*) and either endogenous *egr-1* expression (*A*) or endogenous *junB* expression (*B*) (*upper panels*). Similar results were obtained in two experiments.

To verify that PD098059 effectively blocks GH-stimulated activation of MEK and ERK, 3T3-F442A fibroblasts were preincubated with PD098059 for 1 h prior to GH addition (Fig. 3). The activity of ERK1 and -2 was assessed by Western blot analysis with an antibody (α-P-ERK) specific for dually phosphorylated, activated ERK1 and ERK2. GH treatment for 5 min increased levels of phosphorylated ERK1 and -2 (Fig. 3*A*, *top*, *lanes 4* and *5 versus lanes 2* and *3*). The addition of increasing concentrations of PD098059 progressively lowered levels of dually phosphorylated ERK1 and ERK2 in GH-treated cells (Fig. 3*A*, *top*, *lanes 6–11*). In five separate experiments, ERK phosphorylation was inhibited 47% with 25 μM, 77% with 50 μM, and 101% with 100 μM PD098059. Levels of nonphosphorylated ERK were not affected by treatment with PD098059 (*lane 1 versus lane 2*, *bottom*).

To provide additional evidence that the MEK inhibitor attenuates GH-promoted MAP kinase activity, MAP kinase activity was directly examined in 3T3-F442A lysates using an *in vitro* kinase assay with myelin basic protein as substrate. GH increased MAP kinase activity almost 2-fold (Fig. 3*B*). PD098059 reduced GH-stimulated MAP kinase activity at concentrations (25, 50, and 100 μM) that inhibit GH-induced phosphorylation of ERK1 and ERK2 (Fig. 3*B*). PD098059 decreased basal MAP kinase activity slightly (8%, data not shown). MEK activity was measured using MEK that was immunoprecipitated from 3T3-F442A cell lysates with antibodies that recognize MEK1 and MEK2 (Fig. 3*C*). MEK immunoprecipitates were used in an *in vitro* kinase assay containing a glutathione *S*-transferase-ERK fusion protein (14) and myelin basic protein. GH stimulated an 11-fold increase in MEK activity, which was reduced approximately 75% when cells were pretreated with 50 or 100 μM MEK inhibitor (Fig. 3*C*). In Fig. 3*C*, the MEK inhibitor failed to abrogate MEK activity completely. It seemed possible that MEK2 activity might account for the residual activity, although 10-fold higher concentrations of PD098059 are needed to inhibit MEK2 than MEK1 (46). However, results similar to those shown in Fig. 3*C* were obtained using antibodies specific for MEK1, suggesting that the residual MEK activity is not due to MEK2.

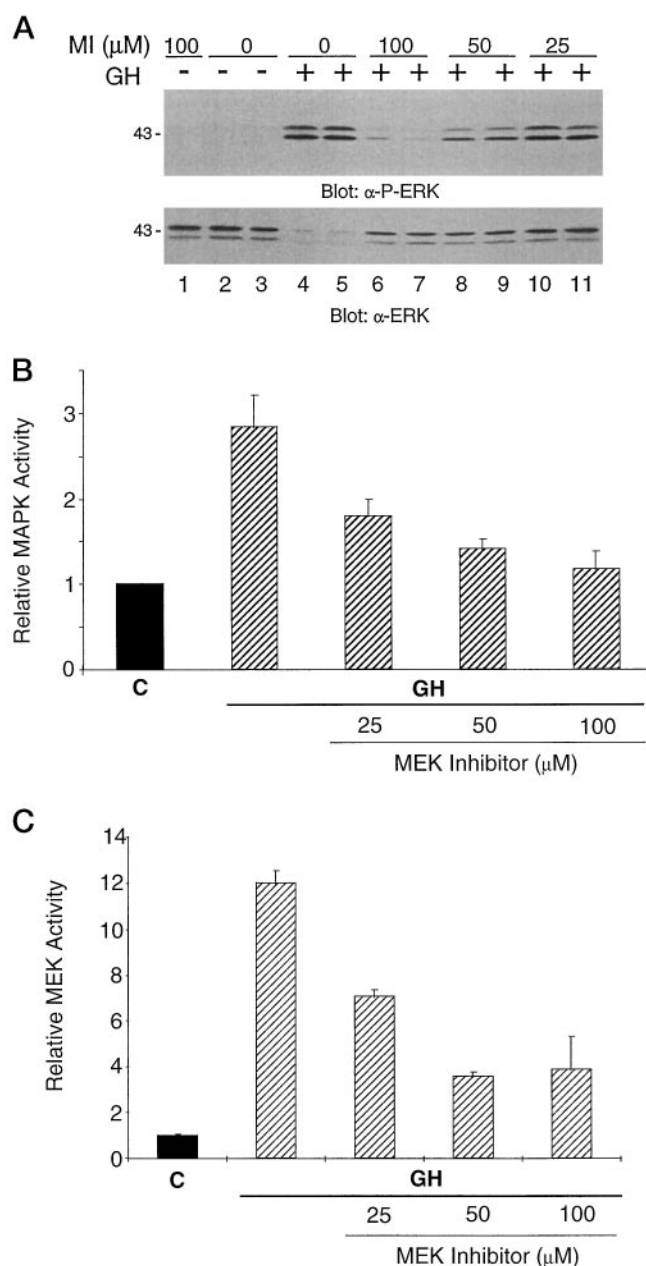

FIG. 3. **The MEK inhibitor PD098059 attenuates GH-induced activation of ERK1 and ERK2.** *A*, 3T3-F442A fibroblasts were preincubated for 1 h with the indicated concentrations of PD098059 (*lanes 1* and *6–11*) or Me_2SO alone (*lanes 2–5*). Cells were then treated with GH (*lanes 4–11*) or vehicle (*lanes 1–3*) for an additional 5 min. Duplicate aliquots of whole cell lysates were immunoblotted with α-P-ERK (1:20,000, *top*). Blots were stripped and reprobed with α-ERK (1:2000, *bottom*). When aliquots of the same lysates were initially blotted with α-ERK, constant levels of ERK protein were observed in all conditions (data not shown). Apparent M_r values × 1000 are shown on the *left* in this and subsequent figures. Similar results were obtained in five independent experiments. *B*, 3T3-F442A fibroblasts were preincubated with the indicated concentrations of PD098059 or Me_2SO for 1 h, and vehicle (*solid bar*) or GH (*hatched bars*) was added for an additional 5 min. Extracts were prepared and assayed for MAPK activity. *Bars* represent the means + S.E. from three independent experiments, each assayed in triplicate. Stimulation of MAPK activity by GH was significant ($p < 0.005$). GH-stimulated MAPK activity in the presence of 50 or 100 μM PD098059 was significantly different from that with GH alone ($p < 0.02$) and was not different from untreated control ($p > 0.05$). *C*, 3T3-F442A fibroblasts were preincubated with the indicated concentrations of PD098059 or Me_2SO and treated with GH (*hatched bars*) or vehicle (*solid bar*) for 5 min. Extracts were prepared and assayed in triplicate for MEK activity. Similar results were obtained in two experiments.

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Wortmannin Impairs GH-stimulated Expression and Promoter Activation of c-fos—Wortmannin, a potent PI 3-kinase inhibitor, has been demonstrated to interfere with GH-induced ERK activation, presumably via a mechanism involving PI 3-kinase (25, 48). If ERKs mediate GH-promoted c-*fos* expression, then wortmannin would also be expected to inhibit GH-induced c-*fos* expression. In 3T3-F442A cells, expression of c-*fos* mRNA (normalized to GAPDH mRNA levels) was reduced by 41% with 100 nM wortmannin and by 84% with 500 nM wortmannin (Fig. 4*A*, *lanes 3* and *4 versus lane 2*). Wortmannin inhibited GH-induced c-*fos* promoter-mediated reporter expression by 64% (250 nM) and 86% (500 nM) (Fig. 4*B*). Concentrations of wortmannin of 100 and 500 nM inhibited GH-induced ERK phosphorylation (Fig. 4*C*, *top*, *lanes 4* and *5 versus lane 2*) in the absence of a change in ERK expression. Thus, wortmannin effectively inhibits ERK phosphorylation and also decreases expression of c-*fos* mRNA and c-*fos* promoter activity in response to GH. Taken together, the inhibition by wortmannin is consistent with ERKs mediating GH-dependent activation of c-*fos*. Furthermore, a wortmannin-sensitive molecule, possibly PI 3-kinase, may participate in ERK-dependent activation of gene expression in response to GH.

Inhibition of the MEK/ERK Pathway Blocks GH-stimulated Activation of Elk-1—Previous studies (4) demonstrated that GH stimulates Elk-1-mediated transcriptional activation of a luciferase reporter gene in CHO cells stably expressing full-length GHR-(1–638) or truncated GHR-(1–454). GH induces c-*fos* mRNA and stimulates transcriptional activation via the SRE to comparable extents in both cell lines (29). Unless otherwise indicated, CHO cells expressing GHR-(1–454) (referred to hereafter as CHO-GHR cells) were used in the present study. To examine whether blocking ERK activity alters the ability of Elk-1 to mediate transcriptional activation in response to GH, CHO-GHR cells transfected with plasmids encoding a Gal-Elk/Gal-luciferase expression-reporter system (see "Experimental Procedures") were treated with PD098059 prior to GH (Fig. 5, *left panel*). GH alone stimulated Elk-1-mediated transcriptional activation in CHO-GHR cells, as expected. Incubation with PD098059 prior to GH treatment blocked GH-stimulated luciferase activity (Fig. 5, *left panel*), indicating that GH-stimulated Elk-1-dependent gene expression requires MEK activation. Similar results were observed in CHO cells stably expressing full-length GHR-(1–638) (data not shown). In control experiments, GH failed to stimulate Elk-1-mediated reporter expression in cells transfected with a plasmid (Gal4 (1–147)) encoding the Gal4 DNA binding domain lacking an activation domain (Fig. 5, *right panel*), indicating that the Elk-1 transcriptional activation domain is required for GH-stimulated reporter expression.

GH-stimulated Phosphorylation of Elk-1 Is Blocked by the MEK Inhibitor, Dominant Negative Ras, and MAPK Phosphatase-1—Since phosphorylation of Elk-1 is thought to be important in Elk-1 activation, the ability of the MEK inhibitor to block GH-stimulated Elk-1 phosphorylation was examined. CHO-GHR cells were transiently transfected with a plasmid (CMV-Elk-1) encoding Elk-1, and phosphorylated Elk-1 was detected by immunoblotting with antibodies against a synthetic Elk-1 peptide phosphorylated on serine 383 (Fig. 6). The stimulation of Elk-1 phosphorylation by GH was concentration-dependent from 5 to 500 ng/ml (0.22–22 nM). The appearance of multiple bands with decreased mobility in the lysates of GH-treated cells is consistent with Elk-1 being phosphorylated on multiple sites (*upper panel*). Pretreatment of cells with PD098059 (100 μM) obliterated all bands recognized by the α-P-Elk-1 antibodies at all concentrations of GH (Fig. 6, *lanes 5–8*), consistent with a role for ERKs in GH-stimulated Elk-1 phosphorylation. Nonphosphorylated Elk-1 protein levels were comparable under all conditions (Fig. 6, *lower panel*).

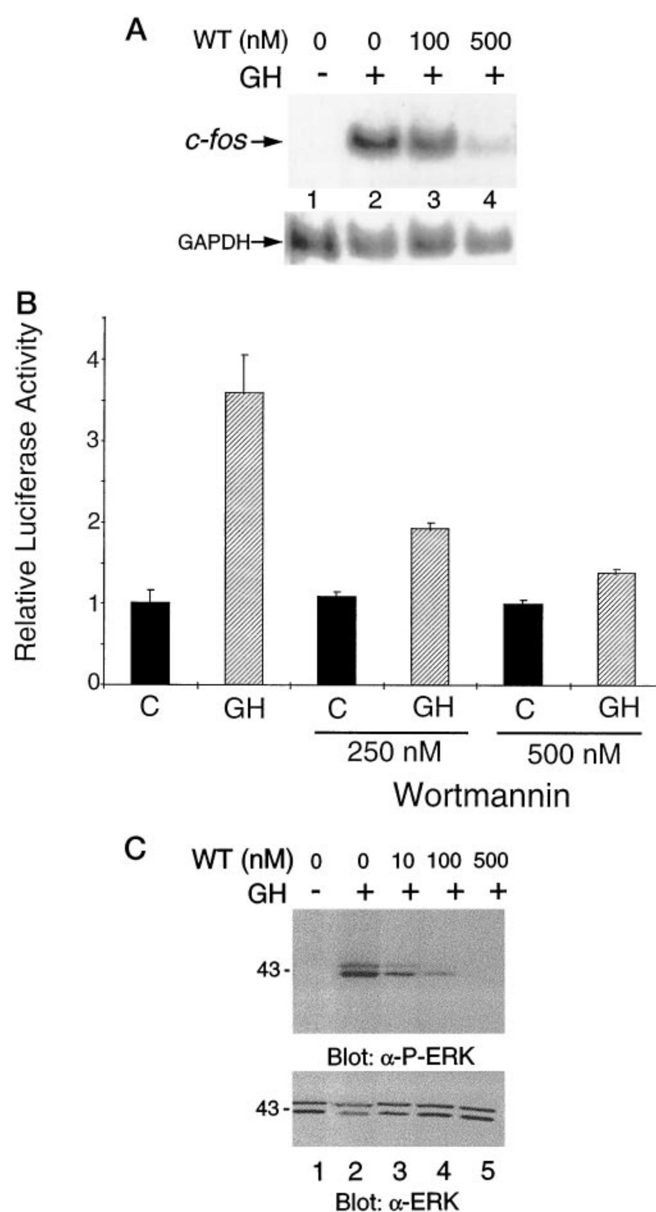

FIG. 4. **Wortmannin impairs GH-stimulated c-*fos* expression and promoter activation of c-*fos*.** *A*, 3T3-F442A fibroblasts were preincubated for 1 h with the indicated concentrations of wortmannin in Me_2SO (*WT*, *lanes 3* and *4*) or Me_2SO alone (*lanes 1* and *2*) and treated with GH (*lanes 2–4*) or vehicle (*lane 1*) for an additional 30 min. Total RNA from cells was assayed by Northern blot analysis for c-*fos* expression (*upper panel*) or GAPDH expression (*lower panel*). Similar results were obtained in three experiments. *B*, 3T3-F442A fibroblasts stably transfected with a plasmid (mC/E) containing the c-*fos* promoter upstream of a luciferase reporter gene were pretreated with Me_2SO or the indicated concentrations of wortmannin and then treated with GH (*hatched bars*) or vehicle (*C*, *solid bars*) for an additional 5 h. Luciferase activity in cell lysates is normalized to the activity in untreated control cells (control = 1). *Bars* represent the means + S.E. of triplicate determinations from one experiment. Similar results were obtained in three experiments. *C*, 3T3-F442A cells were preincubated for 1 h with wortmannin (*lanes 3–5*) or Me_2SO (*lanes 1* and *2*) prior to the addition of GH for 15 min. ERK phosphorylation was measured in whole cell lysates by immunoblotting as described for Fig. 3.

To demonstrate further the role of the signaling pathway involving Ras, MEK, and ERK in GH-stimulated Elk-1 phosphorylation, additional steps of this MAPK pathway were modulated. Interfering with MAPK activation by co-expressing MKP-1 with CMV-Elk-1 prevented the appearance of the bands

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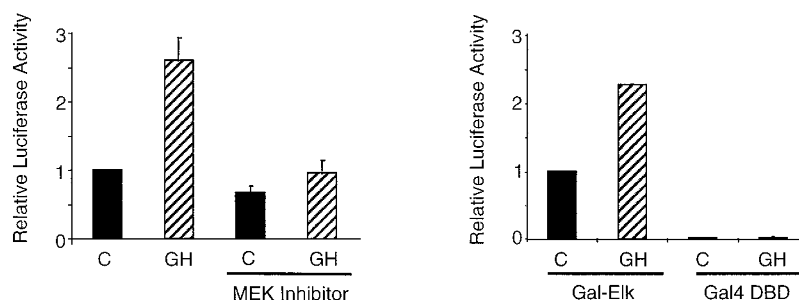

FIG. 5. **The MEK inhibitor PD098059 attenuates GH-induced Elk-1-mediated transcriptional activation.** *Left*, CHO-GHR cells were transiently co-transfected with plasmids Gal4/ElkC, 5× Gal/Luc, and RSV-βgal. Cells were treated with or without MEK inhibitor (100 μM) for 1 h and subsequently incubated with GH (*hatched bars*) or vehicle (*C*, *solid bars*) for an additional 4 h. Luciferase activity, normalized for β-galactosidase activity, is expressed relative to that in control cells treated with Me_2SO (control = 1). *Bars* represent the means + S.E. of three independent experiments, each measured in triplicate. Stimulation of Gal4/ElkC-mediated luciferase activity by GH is significant ($p < 0.005$). The values from cells treated with GH and PD098059 are statistically different from those in cells treated with GH in the absence of PD098059 ($p < 0.005$). CHO-GHR cells were transfected with the plasmids for Gal4 DNA binding domain alone, 5× Gal/Luc, and RSV-βgal (*Gal4 DBD*, *right panel*). Cells were treated with vehicle (*solid bars*) or GH (*hatched bars*) as above. *Bars* represent the means + S.E. for two independent experiments, each measured in triplicate.

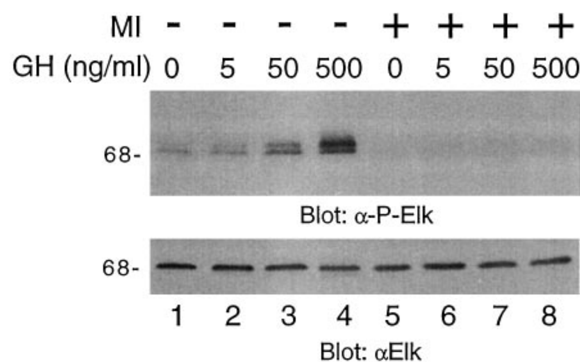

FIG. 6. **The MEK inhibitor PD098059 blocks GH-stimulated Elk-1 phosphorylation.** CHO-GHR cells were transiently transfected with the plasmid CMV-Elk-1. 36 h after transfection, cells were deprived of serum for 6 h, and PD098059 (*MI*, 100 μM, *lanes 5–8*) or Me_2SO (*lanes 1–4*) was added during the final hour of deprivation. Cells were then treated with the indicated concentrations of GH or vehicle for 30 min. Whole cell lysates were analyzed by immunoblotting with α-P-Elk-1 (1:1,000; *upper panel*) or αElk-1 (1:1,000; *lower panel*). Data are representative of three experiments.

recognized by α-P-Elk-1 in response to GH (Fig. 7*A*, *top*, *lane 4*), whereas GH clearly induced bands representing phosphorylated Elk-1 in the absence of MKP-1 (Fig. 7*A*, *top*, *lane 2*). Dominant negative Ras (15A) also decreased GH-stimulated Elk-1 phosphorylation compared with vector (pZIP-Neo) alone (Fig. 7*B*, *top*, *lane 4 versus lane 2*). Conversely, overexpression of wild-type Ras elevated levels of protein recognized by α-P-Elk-1 in both GH- and vehicle-treated cells. (Fig. 7*B*, *upper panel*, *lanes 5* and *6*). Elk-1 protein was present in all conditions (Fig. 7, *A* and *B*, *lower panels*). In lysates from GH-treated cells analyzed with αElk-1, a slower migrating band is observed that comigrates with the band recognized by α-P-Elk-1 (Fig. 7, *A* and *B*, *lane 2*, *bottom versus top*). This slower migrating band is likely to represent Elk-1 phosphorylated on serine 383 and is absent in lysates from cells transfected with MKP-1 or dominant negative Ras. These data are consistent with observations with PD098059 and together support a role for Ras and MAP kinase activation in GH-stimulated Elk-1 phosphorylation.

JNK and p38 Kinase Do Not Mediate GH-stimulated Phosphorylation and Activation of Elk-1—Although ERK1 and ERK2 appear to be the major Elk-1 kinases regulated by growth factors (10, 49), JNK and p38 kinase are also capable of activating Elk-1 in response to cellular stresses such as ultraviolet light and interleukin-1 (8, 11). To determine whether GH regulates JNK and as a step toward determining whether JNK contributes to GH-stimulated transcriptional activation via Elk-1, JNK activity was assayed in 3T3-F442A cells treated with GH. JNK phosphorylation was measured using an antibody (α-P-JNK) that recognizes dually phosphorylated, activated p54 and p46 JNK. In 3T3-F442A fibroblasts, GH treatment for 10, 20, or 30 min failed to stimulate phosphorylation of p54 or p46 JNK (Fig. 8*A*, *top*, *lanes 4–6*). GH also failed to induce JNK phosphorylation in CHO-GHR cells (data not shown). The capability of α-P-JNK to detect phosphorylation of JNK was verified using anisomycin, which stimulated phosphorylation of both p46 and p54 JNK in 3T3-F442A cells (Fig. 8*A*, *lane 7*). Due to cross-reactivity of the α-P-JNK antibody with phosphorylated ERKs, bands that co-migrate with phosphorylated ERK1 and -2 (data not shown) were observed, most notably in lysates from cells treated with GH for 10 min (Fig. 8*A*, *lane 4*). To confirm that GH did not stimulate JNK activity in 3T3-F442A cells, lysates from GH-treated cells were also tested for their ability to phosphorylate glutathione *S*-transferase-Jun. Clearly, GH failed to induce JNK activity (Fig. 8*B*, *lanes 4–6*), while anisomycin (*lane 7*) was effective. The failure of GH to stimulate JNK activity suggests that JNK is unlikely to play a role in GH-promoted Elk-1-mediated gene expression.

To investigate the possible contribution of p38 kinase activity to GH-stimulated Elk-1 phosphorylation and activation, CHO-GHR cells transfected with CMV-Elk-1 were pretreated with the specific p38 inhibitor, SB203580. The p38 inhibitor (10 μM) failed to reduce GH-stimulated Elk-1 phosphorylation when compared with vehicle-treated cells and may have increased it (Fig. 9*B*, *lane 6 versus lane 2*). In the same experiment, PD098059 blocked GH-promoted Elk-1 phosphorylation completely (*lane 4*). Furthermore, the p38 inhibitor failed to block the ability of Elk-1 to mediate transcriptional activation in response to GH (data not shown). To determine whether GH regulates p38, the activity of the p38 substrate, MAPKAP kinase-2, was measured in 3T3-F442A cells (Fig. 9*B*). GH induced a modest 0.6-fold increase in MAPKAP kinase-2 activity, compared with the more robust (4-fold) stimulation by anisomycin (data not shown), which is known to stimulate p38 kinase activation. The modest increase in GH-induced p38 kinase activity was blocked completely when cells were preincubated with 10 μM of the p38 inhibitor SB203580 (Fig. 9*B*). In contrast, the MEK inhibitor did not reduce the slight GH-promoted increase in MAPKAP kinase-2 activity in two independent experiments (data not shown). Thus, GH stimulates a modest increase in p38-dependent MAPKAP kinase-2 activity, and this activity is not sensitive to MEK inhibition. Although the p38 inhibitor blocked the modest GH-induced increase in p38 activity, it failed to attenuate GH-induced phosphorylation

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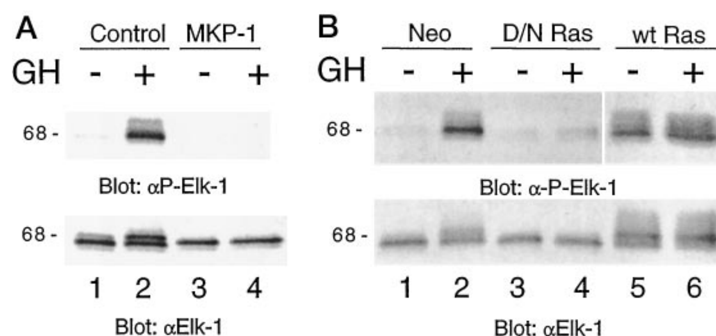

FIG. 7. **GH-stimulated Elk-1 phosphorylation is altered by MAP kinase phosphatase and Ras.** CHO-GHR cells were transiently co-transfected with CMV-Elk-1 (*all lanes*) and one of the following. *A*, irrelevent vector, pcDNA3 (*Control*, *lanes 1* and *2*) or CMV-MKP-1 (*MKP-1*, *lanes 3* and *4*); *B*, pZIP-NeoSV(x)1 (*Neo*, *lanes 1* and *2*), dominant negative Ras, pZIP-ras(15A) (*D/N Ras*, *lanes 3* and *4*), or wild-type Ras, pZIP-ras(WT) (*wt Ras*, *lanes 5* and *6*). 36 h after transfection, cells were deprived of serum for 5 h, and GH (*A*, *lanes 2* and *4*; *B*, *lanes 2*, *4*, and *6*) or vehicle (*A*, *lanes 1* and *3*; *B*, *lanes 1*, *3*, and *5*) were added for 30 min. Whole cell lysates were analyzed as described for Fig. 6. *Lanes 1–4* were exposed to x-ray film for a longer period of time than *lanes 5* and *6*. Similar results were observed in three experiments.

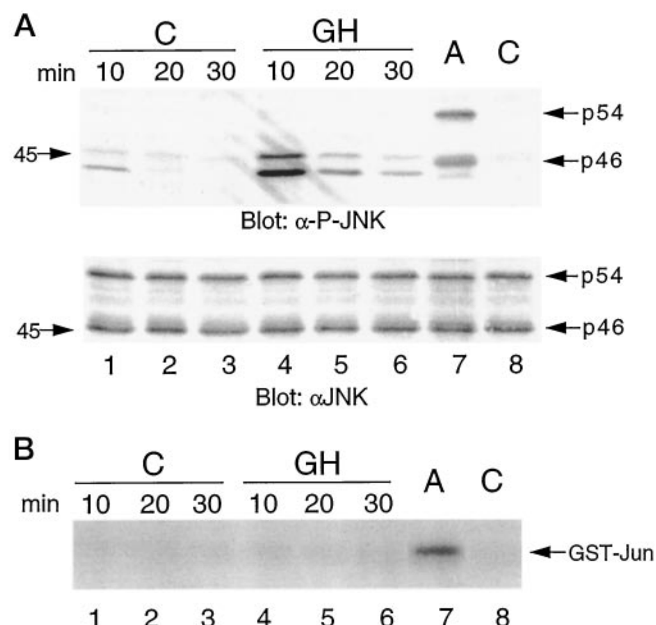

FIG. 8. **GH fails to induce JNK phosphorylation in 3T3-F442A fibroblasts.** 3T3-F442A fibroblasts were treated with vehicle (*C*, *lanes 1–3*) or 500 ng/ml GH (*lanes 4–6*) for the times indicated or for 20 min with 10 μg/ml anisomycin (*A*, *lane 7*) or with ethanol vehicle (*C*, *lane 8*). *A*, whole cell lysates were analyzed by immunoblotting with α-P-JNK (1:5000; *upper panel*) or αJNK (1:1,000; *lower panel*). The migrations of p54 JNK and p46 JNK are indicated by *arrows*. The comigration of the bands seen in *lanes 4–6* with ERK1 and -2 was determined by stripping the membrane and blotting with αERK (data not shown). *B*, glutathione *S*-transferase-Jun complexed to glutathione beads was incubated with cell lysates and used subsequently in a kinase assay. A representative experiment is shown.

of Elk-1, suggesting that GH-promoted p38 activity is not likely to play a role in GH-stimulated Elk-1 phosphorylation. These data are consistent with the hypothesis that MEK-activated ERK1 and -2 are the key MAPKs that mediate GH-induced Elk-1 phosphorylation and activation.

DISCUSSION

Activation of Ras, MEK, and ERK1 and -2 Mediates GH-stimulated Phosphorylation and Activation of Elk-1—This study demonstrates that GH-stimulated phosphorylation of Elk-1 and Elk-1-dependent gene expression are mediated by a pathway involving activation of Ras, MEK, and ERK1 and -2. Overexpression of a dominant negative Ras mutant reduces GH-stimulated Elk-1 phosphorylation, suggesting that Ras plays a role in Elk-1 phosphorylation. Conversely, overexpression of wild-type Ras elevates basal and GH-induced phosphorylation of Elk-1. Since activation of Ras leads to activation of the MEK/ERK pathway (50, 51), these data are consistent with the idea that Ras acts upstream of MEK and ERK to participate in GH-stimulated phosphorylation of Elk-1. However, treatment with GH increased the phosphorylation of Elk-1 in cells overexpressing wild-type Ras, suggesting that other signaling molecules may also contribute to activation of a MEK/ERK pathway in response to GH.

Inhibition of MEK or ERK activity blocks GH-promoted Elk-1 phosphorylation and activation completely. The MEK inhibitor PD098059 was used as one tool for examining the role of MEK and ERK in GH signaling, because it specifically inhibits the phosphorylation of inactive MEK by upstream kinases while failing to inhibit directly the activation of other signaling molecules (46) such as p38, JNK, ERK2, MAPKAP kinases 1 and 2, Raf (an activator of MEK), MKK4 (a JNK kinase), and protein kinase C (implicated in GH-stimulated c-*fos* expression) (2, 47, 52). GH-stimulated phosphorylation and activation of Elk-1 was blocked effectively by the MEK inhibitor (100 μM). Since the MEK inhibitor also abrogated GH-induced ERK activity, these data support the involvement of both MEK and ERKs in the phosphorylation and activation of Elk-1 in response to GH. Furthermore, dephosphorylation of ERKs with the ERK-specific phosphatase, MKP-1, also successfully blocks Elk-1 phosphorylation, substantiating the role of ERKs in the phosphorylation of Elk-1. Together, these data support a model in which activation by GH of a Ras/MEK/ERK cascade leads to ERK-mediated phosphorylation and activation of Elk-1.

Recent evidence suggests that the EGFR may contribute to JAK2-mediated activation of the MEK/ERK pathway in response to GH (53). The present study does not address specifically the mechanisms upstream of Ras by which GH stimulates activation of the MEK/ERK pathway. Rather, the data presented here clearly show that the MEK/ERK pathway is essential for the activation of downstream end points such as Elk-1 phosphorylation and transcriptional activation and expression of early response genes in response to GH. However, results presented here indicate that the effects of GH on Elk-1 phosphorylation and c-*fos* expression can occur independently of EGFR-mediated signaling, because CHO-GHR cells, in which these responses were studied, lack the EGFR. In 3T3-F442A cells, which have both endogenous GHR and EGFR, it is possible that EGFR-mediated signaling contributes to GH-stimulated activation of the MEK/ERK pathway. Future investigation of the varying contributions of GHR and EGFR signaling to GH-stimulated activation of the MEK/ERK pathway as

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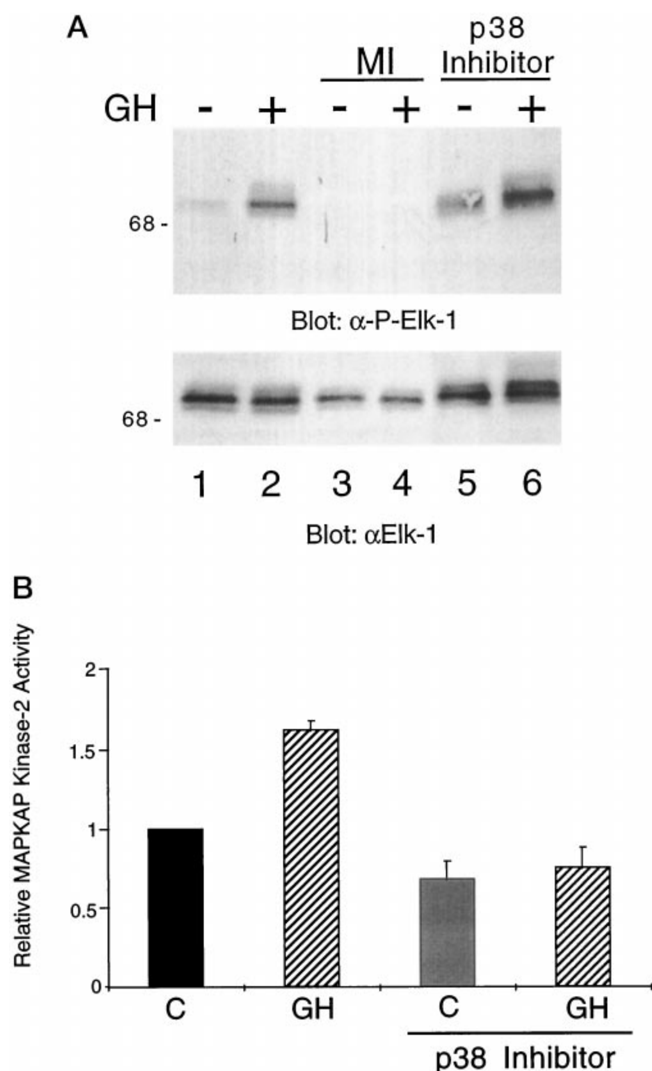

FIG. 9. **GH-induced p38 activity does not contribute to GH-stimulated Elk-1 phosphorylation.** *A*, CHO-GHR cells were transiently transfected with the plasmid CMV-Elk-1 (*all lanes*). 36 h later, cells were deprived of serum for 6 h. Me_2SO (*lanes 1* and *2*), MEK inhibitor PD098059 (*MI*, 100 μM, *lanes 3* and *4*), or p38 inhibitor SB203580 (10 μM, *lanes 5* and *6*) was added for the final hour. Cells were treated with GH or vehicle for an additional 30 min. Whole cell lysates were analyzed for the presence of phospho-Elk-1 or Elk-1 by immunoblotting as described for Fig. 6. Similar results were observed in two experiments. *B*, 3T3-F442A cells were preincubated with Me_2SO or a 10 μM concentration of the p38 inhibitor SB203580 for 1 h and then treated for 20 min with vehicle (*C*, *solid bars*) or GH (*hatched bars*). Cell lysates were then harvested and assayed for MAPKAP kinase-2 activity. MAPKAP kinase-2 activity is expressed relative to that in control cells (control = 1, *solid bar*). *Bars* show the means + S.E. for three separate experiments. The stimulation of MAPKAP kinase-2 activity by GH is significant ($p < 0.01$), and the activity observed with the p38 inhibitor in the absence and presence of GH did not differ significantly from that in control Me_2SO-treated cells.

well as downstream gene expression may yield insights into how cross-talk occurs between various signaling pathways.

JNK and p38 Kinase Do Not Mediate GH-stimulated Phosphorylation and Activation of Elk-1—Among the MAPKs, both JNK and p38 MAPKs are also reported to phosphorylate Elk-1 in several cell types (7, 9, 11, 12). JNK and p38 are thought to be activated primarily in response to cellular stresses such as inflammatory cytokines, heat and chemical shock, bacterial endotoxin, and ischemia (reviewed in Ref. 54). However, activation and integration of MAP kinase signaling pathways utilizing JNK, p38, and ERK are complex and dependent on many factors such as cell type and the nature of the stimulus (54). In 3T3-F442A cells, GH failed to stimulate phosphorylation of JNK or JNK activity as measured by the phosphorylation of its substrate, Jun. Although GH produced a slight (0.6-fold) increase in p38-dependent MAPKAP kinase-2 activity, the inability of the p38 inhibitor SB203580 to attenuate GH-promoted Elk-1 phosphorylation or Elk-1-mediated transcriptional activation makes it highly unlikely that p38 contributes to GH-stimulated Elk-1 activation. These data do not preclude possible involvement of p38 in other GH-dependent events. Together, the present data are consistent with the view that JNK and p38 do not contribute significantly to GH-promoted phosphorylation and activation of Elk-1. Further, because the MEK inhibitor PD098059 does not inhibit JNK or p38 activity directly *in vitro*, the complete block of GH-induced Elk-1 phosphorylation and activation observed with PD098059 suggests that ERK1 and -2 are the primary, and perhaps the sole, Elk-1 kinases activated in response to GH.

ERK1 and -2 Mediate Activation of SRE-regulated Genes in Response to GH—The present data suggest that GH-stimulated c-*fos* expression is mediated by ERK activation of transcription factors bound to the c-*fos* SRE. The c-*fos* SRE includes a CArG box, which binds SRF, and an Ets motif, which binds TCF family members (55). In GH-treated cells, Elk-1 and SRF are both present in the complex bound to the c-*fos* SRE (4). Binding of SRF to the CArG box is thought to recruit TCF family members, such as Elk-1, to the Ets motif sequence (55). Thus, GH fails to stimulate transcriptional activation via a c-*fos* promoter fragment or the SRE alone when either the SRF or TCF binding sites are mutated (4, 56). Other TCF family members such as SAP-1a and SAP-2/ERP/NET can bind to the Ets motif (6). Both of these TCFs are reported to mediate transcriptional activation when activated by ERK2, although SAP-1a has also been shown to be activated by the stress-induced MAPKs, JNK and p38 (6, 57, 58). Future investigation of the effects of GH and other agents on the binding, phosphorylation, and activation of SAP-1a and SAP-2/ERP/NET may provide additional insight into how TCF proteins regulate gene expression in a stimulus-specific manner and whether they play a role in transcriptional activation via the SRE in response to GH .

Activation of ERK1 and -2 by GH may facilitate binding of SRF to the SRE. SRF can be phosphorylated by an ERK-dependent mechanism involving $p90^{rsk}$ (59–63); SRF phosphorylation is thought to facilitate binding of SRF to the SRE (60, 61). GH stimulates activation of $p90^{rsk}$ in 3T3-F442A cells (64), but it is not currently known whether GH promotes phosphorylation of SRF. Although Elk-1 and SRF are constitutively bound to the SRE in 3T3-F442A cells, GH rapidly and transiently increases further binding of both proteins (4). More work is needed to determine whether ERK-dependent binding of SRF is necessary for transcriptional activation through the SRE in response to GH.

The model of GH-stimulated activation of SRE-containing genes mediated by ERK1 and -2 is supported by the observation that attenuation of ERK activation with the MEK inhibitor also impaired GH-stimulated expression of *egr-1* and *junB*. Two of the four SRE sequences upstream of *egr-1* mediate transcriptional activation in response to human granulocyte-macrophage colony-stimulating factor, mouse interleukin-3, urea, and antigen receptor cross-linking (65–67). The induction of *egr-1* transcription by these agents requires the Ets sequences adjacent to the CArG boxes (66, 67). Moreover, the expression of *egr-1* is thought to be ERK-dependent in response to urea and insulin (68, 69) and to involve activation of JAK2 and Ras in the case of human granulocyte-macrophage colony-stimulating factor (65). The *junB* enhancer regions also contain two SREs that mediate transcriptional activation in response

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to serum (70, 71). One of the *junB* SREs is similar to the c-*fos* SRE (70, 71), but it is not yet clear whether Ets sites play a role in transcriptional activation through the *junB* SREs. Further, Ets-independent transcriptional activation through the c-*fos* SRE can be stimulated by treatment with lysophosphatidic acid (72), indicating that multiple mechanisms exist for stimulation of c-*fos* transcription via the SRE. It will be of interest to determine whether ERK-mediated Elk-1 activation of transcription through the SRE is the only pathway by which GH induces expression of SRE-regulated genes.

The MEK inhibitor may block GH-stimulated transcriptional activation of c-*fos*, *egr-1*, and *junB* in part by inhibiting the ERK-dependent activation of transcriptional complexes other than the SRE in the promoters of these genes. For example, previous work has shown that GH induces tyrosyl phosphorylation and binding of STATs 1 and 3 to the Sis-inducible element in the c-*fos* promoter (73–75). Serine phosphorylation, possibly mediated by ERKs, is thought to contribute to STAT-mediated transcriptional activation (76–79). While these studies suggest that STAT-mediated transcriptional activation may be susceptible to MEK inhibition, the role of STATs in GH-stimulated activation of c-*fos* is still being explored.

Role of PI 3-Kinase in GH-stimulated ERK Activation—GH-induced signaling through the Ras/MEK/ERK cascade may be modulated by the activity of other signaling molecules, such as those sensitive to wortmannin. This study demonstrates that wortmannin interferes with GH-stimulated ERK activation as well as downstream transcriptional activation of c-*fos*. GH promotes phosphorylation of Insulin Receptor Substrate-1 and -2 and association of these proteins with the Src homology 2 domain of the 85-kDa regulatory subunit of PI 3-kinase, resulting in activation of PI 3-kinase (18, 19, 21). The PI 3-kinase inhibitor wortmannin has been used to implicate PI 3-kinase in GH-stimulated lipid synthesis (25) and activation of $p70^{S6K}$ (80). In addition, GH-promoted ERK activation is inhibited in wortmannin-treated cells (23, 48, 80), as observed here. The data here demonstrate that GH-stimulated expression of c-*fos* and c-*fos* promoter function is blocked by wortmannin. Such inhibition is consistent with a requirement for ERK activation in GH-promoted transcription of c-*fos* and raises the possibility that a member of the PI 3-kinase family may participate in GH-induced c-*fos* expression.

In summary, the data presented support a model in which activation by GH of the Ras/MEK/ERK cascade promotes Elk-1 phosphorylation and Elk-1-mediated transcriptional activation of gene expression. When the Ras/MEK/ERK pathway was blocked, GH failed to stimulate phosphorylation and activation of Elk-1. Since GH did not activate JNK and produced only a slight increase in p38 activity, which did not contribute significantly to GH-stimulated Elk-1 phosphorylation or activation, JNK and p38 are not likely to activate Elk-1 in response to GH. The Ras/MEK/ERK cascade was found to mediate GH-induced expression of several SRE-regulated genes, including c-*fos*, *egr-1*, and *junB*. A wortmannin-sensitive molecule, possibly PI 3-kinase, may regulate ERK activation and subsequent transcriptional activation of c-*fos* in response to GH. Knowledge of the mechanisms by which GH promotes changes in gene expression provides a foundation for exploring the many functions of this major regulator of growth and differentiation.

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