Cooperative regulation of endogenous cAMP-response element binding protein and CCAAT/enhancer-binding protein β in GH-stimulated *c-fos* expression

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Abstract

GH activates the *c-fos* promoter by regulating multiple transcription factors. This study adds to our understanding of GH-regulated transcription by demonstrating that GH regulates the *c-fos* cAMP-response element (CRE) and its binding protein, CREB. Activation of the *c-fos* promoter by GH is impaired by expression of dominant-negative A-CREB. GH stimulates rapid and transient phosphorylation of CREB at Ser 133 (P-CREB), a critical site for transactivation by CREB, in 3T3-F442A preadipocytes. Mutation of this residue impairs GH-induced *c-fos* expression, suggesting that phosphorylation of CREB at Ser 133 contributes to GH-induced *c-fos* activation. The MEK inhibitor UO126 impaired the phosphorylation of CREB and that of C/EBP β , suggesting that ERKs mediate the phosphorylation of both proteins. UO126, but not the protein kinase A inhibitor H89, blocked GH-induced *c-fos* mRNA

expression. A combination of CREB and C/EBP β enhanced *c-fos* promoter activation, and mutation of the CRE impaired the enhancement, as well as GH-stimulated *c-fos* activation. GH treatment increased the occupancy of both endogenous phospho-CREB and phospho-C/EBP β on the *c-fos* promoter. The increases were impaired by UO126. The active P-CREB and P-C/EBP β are induced by GH to occupy the same *c-fos* promoter DNA, suggesting that they may participate in a GH-regulated complex on *c-fos*. These findings suggest that coordinated phosphorylation of CREB and C/EBP β in response to GH is mediated by ERK1/2, and that the phosphorylated proteins are part of a regulatory complex that occupies *c-fos* *in vivo* to regulate *c-fos* transcription cooperatively in response to GH.

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Introduction

Growth hormone (GH) is a major regulator of growth and metabolism and regulates the transcription of numerous genes (Tollet-Egnell *et al.* 2004, Rowland *et al.* 2005, Huo *et al.* 2006). The proto-oncogene *c-fos*, which participates in cellular proliferation and differentiation (Holt *et al.* 1986, Nishikura & Murray 1987), is rapidly and transiently induced by GH (Doglio *et al.* 1989, Gurland *et al.* 1990) as well as by other stimuli. Analysis of *c-fos* provides a model for understanding mechanisms of GH-regulated gene transcription (Cesena *et al.* 2007). The *c-fos* promoter contains multiple GH-responsive enhancer elements (Fig. 1A), including the sis-inducible element (SIE), serum-response element (SRE) and an adjacent CCAAT/enhancer-binding protein (C/EBP) site, and an activator protein-1 (AP-1) site; these elements can be bound by cognate transcription factors to regulate *c-fos* transcription in response to GH (Meyer *et al.* 1993, 1994, Gronowski & Rotwein 1994, Chen *et al.* 1995, Clarkson *et al.* 1995, Gronowski *et al.* 1995, 1996, Liao *et al.* 1997, 1999, Hodge *et al.* 1998).

A highly conserved cAMP-response element (CRE) located ~60 bps upstream of the transcription start site of *c-fos* (Fig. 1A)

binds the CRE-binding protein (CREB), and related activating transcription factor (ATF) family proteins (Sheng *et al.* 1988, Fisch *et al.* 1989); mutation of the CRE prevents CREB binding (Berkowitz *et al.* 1989). CREB occupies the *c-fos* promoter constitutively, as detected by chromatin immunoprecipitation (ChIP; Fass *et al.* 2003, Cha-Molstad *et al.* 2004). Transactivation by CREB generally depends upon its phosphorylation at the critical Ser 133 (P-CREB; Shaywitz & Greenberg 1999, Mayr & Montminy 2001, Quinn 2002, Servillo *et al.* 2002). Protein kinase A (PKA) mediates Ser 133 phosphorylation of CREB which then binds to the CRE to mediate *c-fos* promoter activation (Gonzalez & Montminy 1989, Boutillier *et al.* 1992). Numerous other kinases can also promote phosphorylation of CREB at Ser 133, including calcium-calmodulin-dependent kinases, protein kinase C, and MAP kinase (MAPK) pathway members, such as pp90^{Rsk}, mitogen- and stress-activated protein kinase, and p38 (Shaywitz & Greenberg 1999, Mayr & Montminy 2001).

To date, the CRE and CREB have not been implicated in GH-induced *c-fos* expression. However, GH has been reported to induce the phosphorylation of CREB at S133 in 3T3-F442A preadipocytes (Yarwood *et al.* 1998).

Analysis of *c-Fos* expression *in vivo* indicated that the CRE acts interdependently with the SIE, SRE, and AP-1 sites to mediate its endogenous expression (Robertson *et al.* 1995). These observations suggest that the CRE and the CREB contribute to GH-stimulated *c-fos* expression; such a role is documented herein.

Among the transcription factors that mediate GH-stimulated *c-fos* expression, C/EBP β appears to be critical (Clarkson *et al.* 1995, Liao *et al.* 1999). Depletion of C/EBP β by RNA interference abolishes the ability of GH to stimulate expression of endogenous *c-fos* mRNA and *c-fos* promoter activation (Cui *et al.* 2005). The phosphorylation of C/EBP β , like that of CREB, contributes to its ability to activate transcription. For C/EBP β , an activating phosphorylation is mediated by a mechanism involving activation of the MAPK extracellular signal-regulated kinases 1 and 2 (ERK1/2), which leads to phosphorylation of murine C/EBP β at Thr 188 (equivalent to T235 in human C/EBP β). C/EBP β phosphorylation at T188 (P-C/EBP β) is required for activation of *c-fos* in response to GH (Piwien-Pilipuk *et al.* 2002, 2003). Since MAPKs can also phosphorylate and activate CREB (Shaywitz & Greenberg 1999), the activation of ERK1/2 by GH suggested that their ability to mediate phosphorylation of CREB and C/EBP β might play a role in coordinating their participation in *c-fos* activation by GH.

Gene regulation is recognized to involve regulatory complexes containing multiple factors that occupy target gene promoters and cooperatively regulate gene transcription (Merika & Thanos 2001, Cosma 2002). C/EBP β associates with other GH-regulated nuclear proteins, such as serum-response factor and Elk-1 that bind to the *c-fos* SRE; this association can increase *c-fos* promoter activation in a Ras-dependent manner (Treisman 1994, Hanlon *et al.* 2000, 2001). C/EBP β has also been identified to be part of a complex containing the co-activator p300 that occupies *c-fos* *in vivo*, in response to GH, and p300 enhances C/EBP-mediated *c-fos* expression (Cui *et al.* 2005). Thus, GH-regulated *c-fos* transcription appears to involve the formation of complexes containing C/EBP β and other factors on the *c-fos* promoter. Although CREB has not previously been implicated in GH-regulated transcription, it is well established that CREB participates in transcription complexes which often contain CREB-binding protein (CBP), and are detected on sequences throughout the genome (Impey *et al.* 2004, Zhang *et al.* 2005).

The present study demonstrates that CREB contributes to GH-regulated *c-fos* activation. CREB can cooperate with C/EBP β , another GH-regulated transcription factor which activates *c-fos* to enhance transcription. In response to GH, both CREB and C/EBP β are phosphorylated by mechanisms involving activation of ERK1/2. The endogenous activated P-CREB and P-C/EBP β are induced to occupy the same *c-fos* promoter DNA in response to GH. These findings suggest that phosphorylated CREB and C/EBP β may be part of a regulatory complex, coordinated by activation of

ERK1/2, and that they occupy *c-fos* and regulate its transcription cooperatively in response to GH.

Materials and Methods

Materials

Murine 3T3-F442A preadipocytes were provided by H Green (Harvard University) and M Sonenberg (Sloan-Kettering, NY, USA). Chinese hamster ovary (CHO) cells stably expressing a truncated GH receptor (GHR1-454) were provided by G Norstedt (Karolinska Inst., Sweden) and N Billestrup (Novo Nordisk, Gentofte, Denmark; Moller *et al.* 1992); they are designated CHO-GHR cells and were used as described (Gong *et al.* 1998, Piwien-Pilipuk *et al.* 2001, Cui *et al.* 2005). Human GH was generously provided by Eli Lilly Inc. Culture media, fetal calf serum, L-glutamine, and antibiotic-antimycotic were purchased from Invitrogen, and calf serum from Invitrogen or from Atlanta Biologicals. BSA (CRG7) was purchased from Serologicals Corp., MEK inhibitor UO126 from Promega, and PKA inhibitor H89 from Calbiochem. Protease inhibitor cocktail tablets (EDTA free) and the protease inhibitors leupeptin and aprotinin were purchased from Roche, phenylmethylsulfonyl fluoride (PMSF) from Mallinckrodt, sodium orthovanadate and formaldehyde from Sigma, immobilized protein A from Repligen, protein G beads from Amersham, sonicated salmon sperm DNA from Stratagene, and the PCR Purification Kit from Qiagen. Immobilon-P transfer membrane (pore size 0.45 μ m) was purchased from Millipore, the ECL Western Blotting Detection Reagents from Amersham, protein molecular weight standards from Invitrogen and Santa Cruz Biotechnology (Santa Cruz, CA, USA), RNA STAT-60 from Tel-Test B Inc., TaqMan Reverse Transcription Kit from Applied Biosystems (Foster City, CA, USA), SYBR Green I from Sigma, luciferin from Promega, and β -galactosidase chemiluminescence reagents from Tropix.

Plasmids and antibodies

The expression plasmids for an N-terminally FLAG-tagged CREB and for a similarly tagged mutant CREB with Ser 133 mutated to Ala (S133A) were described previously (Chrivia *et al.* 1993). The expression plasmid for A-CREB was kindly provided by C Vinson (NCI; Ahn *et al.* 1998). The expression plasmid for rat C/EBP β was a gift from U Schibler (University of Geneva, Geneva, Switzerland). The reporter plasmids *fos*-Luc wild-type (WT) containing the mouse *c-fos* enhancer (−379 to +1, referred to as ‘promoter’ throughout) upstream of the luciferase gene, and *fos*-Luc mutated at the CRE (mCRE: CTCAGCTAG mutated residues underlined, Fig. 1A; Berkowitz *et al.* 1989) were generously provided by B Cochran (Tufts University). The vector pcDNA3 (Invitrogen) was used as a control. The plasmid CMV- β -galactosidase (β -gal) was provided by M Uhler (University of Michigan).

The antibody against CREB (06-863) was purchased from Upstate. Antibody against CREB phosphorylated at Ser 133 (anti-P-CREB) of rabbit (06-519) or mouse origin was from Upstate or Cell Signaling respectively, an antibody against the C-terminus of C/EBP β was from Santa Cruz, the anti-P-C/EBP β antibody against murine C/EBP β phosphorylated at T188 (equivalent to T235 of human C/EBP β) was from Cell Signaling, antibody against Fos, NF κ B, and normal rabbit IgG were from Santa Cruz, antibody against RNA polymerase II phosphorylated at Ser 5 in the C-terminal domain (YSPT[pS]PS; anti-P-Pol II, 05-623) was from Upstate, the antibodies against Thr 202/Tyr 204 of human p44/42 MAP kinase (ERK1/2) and total p44/42 MAP kinases (ERK1/2) were from Cell Signaling, anti-FLAG antibody was from Sigma, and horseradish peroxidase-conjugated secondary antibodies for western blots were from Amersham or Santa Cruz. The secondary antibody conjugated with IRDye800, which was used in western blots scanned by Odyssey, was obtained from Rockland Inc.

Cell culture and treatment

3T3-F442A preadipocytes expressing endogenous GHR and CHO-GHR cells expressing exogenous GHR were grown and maintained as described previously (Cui *et al.* 2005). Cells were treated without or with human GH (500 ng/ml = 22 nM) or with forskolin (30 μ M) for the times indicated. In some experiments, 3T3-F442A cells were pre-treated with UO126 (10 or 20 μ M) for 30 min, or H89 (20 μ M) for 4 h. Controls were treated with equivalent amounts of dimethyl sulfoxide (DMSO) vehicle (0.1% v/v) for equivalent times. Prior to treatments, cells were deprived of serum for 18 h in the appropriate medium containing 1% BSA instead of serum.

Gene transcription assay

Activation of the *c-fos* promoter was tested in GH-responsive CHO-GHR cells, in which the truncated GHR mediates *c-fos* transcription as efficiently as full-length GHR (Gong *et al.* 1998). The cells were transiently transfected using calcium phosphate co-precipitation as described (Piwien-Pilipuk *et al.* 2001, Cui *et al.* 2005) along with the *fos*-Luc reporter plasmids either wild-type or mCRE mutant (0.5 μ g each), and with plasmids for A-CREB or control vector (5 μ g each). CMV- β -gal (10 ng) was co-expressed as a control for transfection efficiency. After 24 h, cells were incubated in F12 medium containing 1% BSA for 18 h and then treated with GH or vehicle for 4 h. Cells were lysed and the luciferase and β -galactosidase activities in the cell lysates were measured using an Opticomp Luminometer as described previously (Liao *et al.* 1999, Piwien-Pilipuk *et al.* 2001). Luciferase activity, which is normalized using β -galactosidase activity, is shown in relative luciferase units (RLUs). The basal *c-fos* promoter activation (set to 1) was measured in the absence of GH and/or in the presence of vector DNA. Luciferase activity is presented as mean $\pm$ S.E.M. for at least three independent experiments, each performed in triplicate. Statistical analysis of

increases due to either GH treatment or overexpressed gene was performed using one-way ANOVA with Bonferroni's multiple comparison test (Prism version 3, www.GraphPad.com). Statistical significance ($P < 0.05$) is indicated in figures and figure legends by asterisks.

Quantitative real-time PCR (QT-PCR)

QT-PCR was performed on total RNA isolated from 3T3-F442A or CHO-GHR cells and analyzed using iCycler iQ real-time detection system software (Bio-Rad Laboratories) as described to amplify mouse *c-fos* and control *GAPDH* genes (Cui *et al.* 2005, Huo *et al.* 2006). Values for *c-fos* were normalized to *GAPDH*. When inhibitors were used, basal *c-fos* expression (set to 1) was measured in cells treated with DMSO. In some experiments, CHO-GHR cells were transiently transfected with plasmids for CREB, S133A-CREB, or pcDNA3 vector (5 μ g each). After 24 h, cells were incubated in F12 medium containing 1% BSA for 18 h, then treated without or with GH for 30 min, and total RNA was prepared for QT-PCR. Statistical analysis was performed using one-way ANOVA with Bonferroni's multiple comparison test as described.

Immunoblotting analysis

Nuclear extracts from 3T3-F442A or CHO-GHR cells were prepared as described (Sadowski & Gilman 1993, Liao *et al.* 1997) with several modifications. In some experiments, the plasmid DNAs for WT-CREB, A-CREB, S133A-CREB, or pcDNA3 (10 μ g each) were expressed in CHO-GHR cells. To prepare the nuclear extracts, cells were lysed in buffer A (20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA, and 0.2% NP-40) containing protease inhibitor cocktail for 10 min on ice followed by centrifugation (16 000 *g*, 30 s at 4 °C). Then, the nuclear pellet was dissolved in buffer B (60 mM Tris-HCl, pH 6.8, 1% SDS) and was boiled for 10 min. The nuclear extracts (30 μ g each) were separated by SDS-PAGE (10%) and transferred to PVDF membrane. Immunoblotting for endogenous or expressed protein was performed at 4 °C overnight as described (Liao *et al.* 1997) with the following antibodies: anti-P-CREB (1:1000), anti-CREB (1:1000), anti-P-C/EBP β (T188; 1:1000), anti-C/EBP β (1:1000), anti-NF κ B (1:1000), anti-*fos* (1:1000), anti-P-ERK1/2 (1:2000), anti-ERK1/2 (1:1000), or anti-FLAG (1:1000). The proteins were visualized using ECL (Amersham) or the Odyssey scanning system (LI-COR Biosciences; Figs 1B and C, 2C, D and F; Cui *et al.* 2005). Molecular weight was estimated using MagicMark Western Standard (Invitrogen) or Cruz Marker Molecular Weight Standard (Santa Cruz).

ChIP and re-ChIP

ChIP was carried out as described (Cui *et al.* 2005). In some experiments, 3T3-F442A preadipocytes were pre-treated with UO126 (20 μ M) or with an equivalent amount of DMSO vehicle (0.1%, v/v) for 30 min and were then treated with GH

Figure 1 Phosphorylated CREB participates in GH-stimulated *c-fos* expression. (A) Schematic of the murine *c-fos* enhancer (not drawn to scale) shows multiple elements regulated by GH, including a CRE and a C/EBP site. Sequences of wild-type (WT) CRE or mutant CRE (mCRE) are shown with mutations underlined. The arrows P1 and P2 indicate the primers that were used in ChIP PCR to amplify a single fragment of the murine *c-fos* promoter (−364 to −34 bp). (B) A-CREB or pcDNA3 (control; 10 μ g each) was co-expressed with *fos*-Luc (0.5 μ g) and β -gal (0.1 μ g) in CHO-GHR cells. Cells were then treated with GH for 4 h, and luciferase activity was measured ($n=3$ independent experiments). Luciferase activity in cell lysates (RLU) is presented as mean $\pm$ S.E.M. in this and subsequent figures. The increase in *c-fos* promoter activation due to GH, which is highly reproducible although somewhat modest relative to serum (Gurland *et al.* 1990, Ashcom *et al.* 1992, Meyer *et al.* 1993), is significantly ($P<0.05$) reduced when A-CREB is expressed. Lower panel: immunoblotting indicates the expression of FLAG-tagged A-CREB in CHO-GHR cells (arrowhead). Tubulin indicates loading. Similar results were obtained in two experiments. (C) Plasmids for WT CREB or S133A-CREB (1 μ g each) were expressed in CHO-GHR cells. Cells were treated without (open bars) or with GH (black bars) for 30 min. Total RNA was prepared and analyzed by QT-PCR for *c-fos* expression. Results are expressed as mean $\pm$ S.E.M. relative to vector alone (set to 1, not shown; $n=3$). GH significantly ($P<0.05$) increases *c-fos* mRNA expression in the presence of wild-type CREB. However, GH-stimulated *c-fos* mRNA expression was significantly ($P<0.05$) reduced in the presence of the non-phosphorylatable mutant S133A-CREB. Lower panel: the same plasmids for FLAG-tagged WT CREB, FLAG-tagged S133A-CREB, or pcDNA3 (control) were expressed in CHO-GHR cells (10 μ g each). Nuclear extracts were analyzed by immunoblotting with anti-FLAG. The arrowhead indicates the FLAG-tagged CREB or S133A-CREB. A faster migrating non-specific band indicates loading. (D and E) Murine 3T3-F442A preadipocytes or E. CHO-GHR cells were treated with GH for the times indicated. Nuclear extracts were analyzed by immunoblotting with anti-P-CREB (top) or anti-CREB (middle). The bottom panel shows NF κ B (p65) to indicate loading. Similar experiments were obtained in at least two experiments.

for 15 min. The nuclear extracts prepared from the cells were used for ChIP. In each immunoprecipitation, 100 μ g nuclear protein were incubated overnight at 4 °C with 4 μ g of one of the following antibodies: anti-CREB, anti-P-CREB, anti-C/EBP β , anti-P-C/EBP β , or anti-P-Pol II. Samples incubated without antibody or normal rabbit IgG served as a negative control. One percentage of input was used to indicate the relative amount of each sample used for individual ChIP analysis. A single 330 bp fragment (–364 to –34 bp) of the mouse *c-fos* promoter encompassing the SIE, SRE, C/EBP site, and CRE (Fig. 1A, P1, and P2) was amplified as described (Cui *et al.* 2005). The 5' distal region of mouse *c-fos* promoter (–1692 to –1436 bp) was amplified as a negative control for the *c-fos* promoter using the primers 5'-CAGGGAGGCAGAGGTTCTCTAG-3' and 5'-CATTCCAAGACGGACTAGAGAG-3'. Transcription factors CREB, C/EBP β , and P-Pol II, which can be immunoprecipitated with *c-fos* promoter DNA but not with DNA from the 5' distal region of *c-fos* (see Supplementary Fig. S1 in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol96/issue1/>), supporting the specificity of the occupancy on the *c-fos* promoter by these proteins. In each experiment, all of the immunoprecipitated samples were analyzed using the same PCR conditions to evaluate relative amounts of each protein associated with the promoter. PCR products were separated on 2% agarose gels and stained with ethidium bromide. Bands were analyzed using Quantity One and Multi-Analyst software (Bio-Rad). Data, expressed as ratios of GH/control (C), for multiple experiments were presented as mean $\pm$ S.E.M. and were analyzed using Student's *t*-test (Prism version 3). The reduction by UO126 relative to DMSO was calculated from the ratio UO126/DMSO.

re-ChIP was performed as described (Cui *et al.* 2005) with the following modifications: after the first ChIP, the beads were eluted with 100 μ l ChIP SDS lysis buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, and 1% SDS) containing protease inhibitors (1 mM each of sodium orthovanadate, PMSF, sodium pyrophosphate, and 10 μ g/ml each of aprotinin and leupeptin) and 10 mM DTT (Argetsinger *et al.* 1993), and were rotated at room temperature for 15 min with vortexing. Eluates were diluted (1:10) with ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, and 1.1% Triton X-100) containing protease inhibitors as described above. Each eluted sample was divided into two for the second immunoprecipitation (re-ChIP) with 4 μ g of either anti-P-CREB (rabbit), anti-CREB, anti-P-C/EBP β , anti-C/EBP β , no antibody (control), or normal rabbit IgG as control.

Results

Phosphorylated CREB participates in GH-stimulated c-fos expression

To evaluate whether CREB plays a role in GH-stimulated *c-fos* promoter activation, the response of *c-fos* to GH was

analyzed in the presence of A-CREB, a dominant-negative form of CREB. Although A-CREB can dimerize with CREB, an acidic amphipathic extension in its basic region prevents the binding of the dimer to CRE sequences, including the *c-fos* CRE (Fig. 1A; Ahn *et al.* 1998). Activation of the *c-fos* promoter in response to GH in CHO-GHR cells is completely blocked when A-CREB is expressed (Fig. 1B). These results suggest that CREB is one of the transcription factors that participate in GH-stimulated *c-fos* expression.

P-CREB is generally regarded as the transcriptionally active form of CREB. To understand whether phosphorylation of CREB at Ser 133 contributes to the activation of *c-fos* by GH, the expression of endogenous *c-fos* mRNA was examined in the presence of WT CREB or a non-phosphorylatable mutant, S133A-CREB. GH induces its typical increase in *c-fos* mRNA levels in the presence of WT CREB in CHO-GHR cells (Fig. 1C, upper panel). However, the GH-induced increment in *c-fos* mRNA is significantly reduced in the presence of S133A-CREB, suggesting that the phosphorylation of CREB at Ser 133 contributes to GH-stimulated *c-fos* mRNA expression. Basal *c-fos* mRNA was not statistically different in cells expressing WT or mutant S133A-CREB, and the protein expression levels of CREB and S133A-CREB were comparable (Fig. 1C, lower panel). These findings support a role for phosphorylation of CREB at Ser 133 as an activating event for GH-stimulated *c-fos* transcription.

CREB phosphorylation at S133 can be induced by numerous stimuli (Shaywitz & Greenberg 1999, Mayr & Montminy 2001, Quinn 2002, Servillo *et al.* 2002). GH increases phosphorylation of endogenous CREB at Ser 133 at 10 and 30 min, detected in 3T3-F442A preadipocytes by immunoblotting with an antibody that recognizes CREB phosphorylated at S133 (anti-P-CREB; Fig. 1D, top panel, lanes 2 and 3 vs 1). The response to GH is transient, since P-CREB is substantially reduced at 60 min and returns to the basal level by 120 min. Total CREB is detected at all times (Fig. 1D, middle panel) and appears to increase transiently 30 min after GH treatment. The relatively constant level of p65 (bottom panel) indicates equal loading. Similarly, GH induces P-CREB at 10 and 30 min in CHO-GHR cells (Fig. 1E, top panel). These findings extend the results of previous report that P-CREB is detected 10 min after GH treatment of 3T3-F442A preadipocytes (Yarwood *et al.* 1998), indicating that stimulation of P-CREB by GH also occurs in other GH-responsive cells. Immunofluorescent staining of 3T3-F442A cells using anti-P-CREB similarly revealed that GH induces a rapid and transient appearance of nuclear P-CREB at 10 min which is reduced at 60 min (data not shown). Taken together, these observations suggest that the stimulation of *c-fos* by GH can be mediated by its induction of CREB phosphorylation.

ERK1 and 2 mediate GH-stimulated activation of c-fos and phosphorylation of CREB and C/EBP β

Forskolin-stimulated *c-fos* promoter activation reflects stimulation of PKA which mediates phosphorylation of CREB at S133 and binding of P-CREB to the CRE 989. To evaluate whether PKA mediates activation of *c-fos* expression by GH, *c-fos* mRNA levels in response to GH were examined in the presence of H89, an inhibitor of PKA. The PKA inhibitor H89 failed to impair GH-induced expression of endogenous *c-fos* in 3T3-F442A preadipocytes (Fig. 2A), although H89 effectively inhibited forskolin-induced *c-fos* expression (Fig. 2B). Thus, it is unlikely that PKA mediates the stimulation of *c-fos* by GH. Interestingly, when the MEK inhibitor UO126, which blocks MAPK pathway signaling, was tested in conjunction with GH, UO126 blocked GH-induced *c-fos* mRNA (Fig. 2A) and protein expression (Fig. 2C). The UO126 impaired the GH-stimulated phosphorylation of endogenous CREB (Fig. 2D); it also inhibited the phosphorylation of endogenous C/EBP β (Fig. 2E), another GH-regulated transcription factor mediating *c-fos* activation. The inhibition of phosphorylation of these proteins was accompanied by inhibition of GH-induced phosphorylation of ERK1/2 in 3T3-F442A nuclear extracts (Fig. 2F). These findings suggest that the phosphorylation of CREB at S133 in response to GH, similar to phosphorylation of C/EBP β at T188 (Piwien-Pilipuk *et al.* 2002), is mediated by activation of ERK1/2, and may contribute to GH-stimulated expression of endogenous *c-fos*.

GH induces the rapid occupancy of endogenous P-CREB and P-C/EBP β on the c-fos promoter

For insight into the mechanism by which P-CREB mediates GH-stimulated *c-fos* transcription in intact cells, the occupancy of endogenous P-CREB on the *c-fos* promoter was examined by ChIP. GH significantly increases the occupancy of endogenous P-CREB on *c-fos* *in vivo* at 15 min (Fig. 3A). As reported previously (Cui *et al.* 2005), GH also increases the occupancy of P-C/EBP β on *c-fos* at 15 min (Fig. 3A) in parallel with P-CREB. The occupancy of total CREB or C/EBP β also increased after GH treatment, although such increases were somewhat inconsistent, reflecting variations in the constitutive occupancy of these transcription factors on the *c-fos* promoter in the absence of GH. Simultaneously, GH increased the occupancy of phosphorylated RNA polymerase II on *c-fos* (Fig. 3A). These changes coincide with the stimulation of *c-fos* expression induced by GH at 15–30 min (Doglio *et al.* 1989, Gurland *et al.* 1990). As controls, *c-fos* promoter DNA was not detected in the absence of antibodies (Fig. 3A), or by control primers amplifying a 5' distal region of *c-fos* (Supplementary Fig. S1). These findings indicate that GH induces the phosphorylation and/or the occupancy of P-CREB and P-C/EBP β on the *c-fos* promoter, opening the possibility that these phosphorylated transcription factors might work cooperatively in a complex with other proteins to increase *c-fos* transcription.

To ascertain whether ERK-mediated phosphorylation of CREB or C/EBP β contributes to their occupancy on *c-fos*, ChIP was performed on cells treated with the MEK inhibitor UO126, or the vehicle DMSO, for 30 min prior to GH. For P-CREB, the GH-induced increase in its occupancy (Fig. 3B, lane 2 vs 1) was almost completely blocked by UO126 (lane 4 vs 3). In multiple experiments, the reduction by UO126 in GH-induced occupancy (GH/C) for P-CREB (86% decrease) suggests a role for ERKs in the response of P-CREB to GH. The occupancy of P-C/EBP β in GH-treated cells was slightly less (15% decrease) in the presence of UO126 than DMSO in multiple experiments. Interestingly, the GH-induced occupancy of P-Pol II was also reduced by UO126, resulting in 37% reduction relative to DMSO in multiple experiments. The reduced occupancy of these factors is consistent with the observed inhibition of GH-stimulated *c-fos* mRNA in the presence of UO126 (Fig. 2A). Together, these findings suggest that the GH-induced phosphorylation of CREB and the occupancy of P-CREB on *c-fos* are dependent on ERK1/2. Since ERKs also mediate the phosphorylation of C/EBP β in response to GH, and P-C/EBP β as well as P-CREB occupies *c-fos*, these findings suggest that CREB and C/EBP β might be coordinately regulated on the *c-fos* promoter in response to GH.

c-fos CRE mediates cooperation between CREB and C/EBP β , and GH-stimulated transcription

Since GH-stimulated expression of endogenous *c-fos* is blocked in C/EBP β -deficient cells (Cui *et al.* 2005), CREB and C/EBP β were tested in combination. The co-expression of CREB and C/EBP β significantly enhances *c-fos* promoter activation compared with the activation by each factor individually (Fig. 4A, WT, black bar). A mutation in the CRE in the otherwise intact *c-fos* promoter (mCRE; Fig. 1A), which interferes with its ability to bind CREB (Berkowitz *et al.* 1989, Cui & Schwartz, data not shown), impairs the enhancement of *c-fos* activation by the combination of CREB and C/EBP β (Fig. 4A, mCRE, black bar). These observations suggest that CREB and C/EBP β can enhance *c-fos* promoter activation cooperatively, and that their cooperation requires an intact CRE. These findings complement a previous report of cooperation of CREB and C/EBP β mediated by a consensus CRE (Chen *et al.* 2003). Activation of the *c-fos* promoter by GH was also significantly impaired by mutation of the CRE (Fig. 4B). These findings indicate that the CRE can mediate GH-stimulated *c-fos* transcription, and is a site for cooperative regulation by CREB and C/EBP β .

GH promotes the occupancy of both CREB and C/EBP β on the same c-fos promoter DNA

Since GH simultaneously increases the occupancy of both P-CREB and P-C/EBP β on *c-fos*, it was of interest to test whether these two transcription factors might be present together in a regulatory complex occupying *c-fos* in response

Figure 2 ERK1 and 2 mediate GH-stimulated phosphorylation of CREB and activation of *c-fos*. (A) 3T3-F442A preadipocytes were treated with H89 (20 μ M) or an equivalent volume of DMSO vehicle for 4 h (left), or with U0126 (20 μ M) or equivalent DMSO for 30 min (right). Cells were then treated without (open bars) or with GH (black bars) for an additional 30 min. *c-fos* mRNA was analyzed by QT-PCR and is presented as mean $\pm$ S.E.M. ($n=3$). H89 failed to alter GH-induced *c-fos* expression. U0126 significantly ($P<0.05$) reduced GH-induced *c-fos* mRNA expression. (B) H89 blocks forskolin-stimulated *c-fos* mRNA expression. To verify the effectiveness of H89, 3T3-F442A cells were treated with H89 (20 μ M) or DMSO for 4 h, followed by forskolin (30 μ M) for 30 min. Total RNA was prepared and analyzed for *c-fos* mRNA by QT-PCR. H89 significantly inhibited forskolin-induced *c-fos* expression ($P<0.001$, gray bars). The results are shown as mean $\pm$ S.E.M. ($n=3$). (C) 3T3-F442A cells were treated with U0126 (20 μ M) or DMSO for 30 min, followed by GH for the times indicated. Nuclear protein was analyzed by immunoblotting with anti-Fos. Similar results were obtained in two experiments. (D) 3T3-F442A cells were treated with U0126 (20 μ M) or DMSO for 30 min, followed by GH treatment for 10 min. Nuclear extracts were used for immunoblotting with anti-P-CREB or anti-CREB. Similar results were obtained in three experiments. (E) 3T3-F442A cells were treated with U0126 for 30 min and then treated with GH for 10 min. Nuclear protein was analyzed by immunoblotting using anti-P-C/EBP β or anti-C/EBP β . Similar results were obtained in three experiments. (F) 3T3-F442A cells were treated with U0126 (10 or 20 μ M) or equivalent volumes of DMSO for 30 min, followed by GH for the times indicated. Nuclear protein was analyzed by immunoblotting with anti-P-ERK1/2 or anti-ERK1/2. Increasing amounts of the inhibitor U0126 reduced GH-stimulated ERK activation (P-ERK1/2), while total ERK1/2 was not changed. Similar results were obtained in three experiments.

Figure 3 GH induces the occupancy of P-CREB and P-C/EBPβ on *c-fos*. (A) 3T3-F442A cells were treated with GH for 15 min. Nuclear extracts were prepared for ChIP with anti-P-CREB and anti-CREB, or for ChIP with anti-P-C/EBPβ and anti-C/EBPβ, along with anti-P-Pol II or without antibody (control). One percentage of input is shown in this and subsequent figures. *c-fos* promoter DNA was amplified with ChIP PCR primers P1 and P2. Quantified results from multiple independent experiments indicate significant ($P < 0.05$) stimulation by GH of occupancy where GH/C (mean $\pm$ S.E.M.) for P-CREB was 2.2 ± 0.6 ($n = 5$), for P-C/EBPβ was 6.4 ± 0.3 ($n = 3$), and for P-Pol II was 2.2 ± 0.3 ($n = 5$). GH/C for CREB was 1.5 ± 0.5 ($n = 3$) and for C/EBPβ was 1.6 ± 0.4 ($n = 3$). (B) 3T3-F442A cells were treated with UO126 (20 μ M) or DMSO for 30 min and then with GH for an additional 15 min. Nuclear extracts were analyzed by ChIP as described. Quantified results from multiple independent experiments indicate stimulation by GH of occupancy (GH/C, mean $\pm$ S.E.M.) for P-CREB was 9.0 ± 4.4 (DMSO) and 1.2 ± 0.4 (UO126; $n = 4$); for P-C/EBPβ was 1.4 ± 0.1 (DMSO) and 1.2 ± 0.4 (UO126; $n = 3$); and for P-Pol II was 3.5 ± 0.3 (DMSO) and 2.2 ± 0.3 (UO126; $n = 5$). GH/C for CREB was 1.5 ± 0.6 (DMSO) and 1.0 ± 0.04 (UO126; $n = 4$) and for C/EBPβ was 2.8 ± 1.1 (DMSO) and 1.4 ± 0.3 (UO126; $n = 4$).

to GH. To evaluate whether CREB and C/EBPβ occupy the same *c-fos* promoter DNA, samples from GH-treated 3T3-F442A preadipocytes were subjected to sequential ChIP and re-ChIP. After the first ChIP with anti-C/EBPβ, in which the occupancy of C/EBPβ on *c-fos* was increased by GH at

15 min (Fig. 5, first IP), the *c-fos*-C/EBPβ complex was eluted from the beads, and eluates were subjected to a second immunoprecipitation using antibodies against P-CREB. Endogenous P-CREB clearly occupied the same *c-fos* DNA as C/EBPβ, and was markedly increased in response to GH

Figure 4 CREB and C/EBPβ cooperatively increase *c-fos* promoter activation. (A) Plasmids for CREB (100 ng) or C/EBPβ (25 ng) were expressed alone or in combination with WT or mutant mCRE *fos*-Luc (0.5 μg each) and β-gal (0.1 μg) in CHO-GHR cells. Luciferase activity was measured ($n=3$ independent experiments). Expression of CREB and C/EBPβ in combination (WT, black bar) significantly ($P<0.05$) enhances WT *c-fos* promoter activation compared with the activation produced by expression of CREB or C/EBPβ individually (open bars). The enhancement was blocked when the CRE in the *c-fos* promoter was mutated (mCRE versus WT, black bars). (B) Plasmids for WT or mCRE *fos*-Luc (0.5 μg each) were expressed with β-gal (0.1 μg) in CHO-GHR cells. Cells were treated without (open bars) or with (black bars) GH as described ($n=3$ independent experiments). The increase in *c-fos* promoter activation due to GH is significantly ($P<0.05$) reduced with mCRE versus WT.

(Fig. 5, second IP, top panels). Controls which did not include antibodies did not precipitate *c-fos* promoter DNA. The converse experiment, in which the first IP with anti-CREB was examined for P-C/EBPβ in a second IP revealed that GH also induced the occupancy of P-C/EBPβ on the same *c-fos* DNA occupied by CREB (Fig. 5, second IP, lower panels). These observations support the presence of both P-CREB

and P-C/EBPβ on the same *c-fos* DNA in GH-treated cells. Complementary to these observations, GH-induced co-occupancy of total CREB and C/EBPβ on *c-fos* is also observed by re-ChIP with antibodies against C/EBPβ or CREB in the second IPs (Cui & Schwartz, data not shown).

These observations that endogenous P-CREB and P-C/EBPβ co-occupy the same *c-fos* promoter DNA in response to GH are consistent with the observed cooperation of CREB and C/EBPβ in activating *c-fos*, and with the increased occupancy of both P-CREB and P-C/EBPβ in response to GH. Taken together, these findings are consistent with GH coordinately stimulating the phosphorylation of CREB and C/EBPβ, possibly by activation of ERK1/2, facilitating their combined occupancy, and cooperative activation of the *c-fos* promoter.

Discussion

GH increases CREB phosphorylation and occupancy on the c-fos promoter in vivo

This study adds to our understanding of the mechanisms by which GH regulates gene transcription and by demonstrating a role for the *c-fos* CRE and CREB in GH-stimulated *c-fos* expression. Not only does expression of the dominant-negative CREB, A-CREB, prevent GH-induced *c-fos* promoter activation, but also mutation of the *c-fos* CRE impairs it. GH activates CREB by the well-established mechanism of stimulating CREB phosphorylation at Ser 133. Mutation of CREB at Ser 133 impaired GH-stimulated *c-fos* mRNA expression. Further, GH increases the occupancy of endogenous P-CREB on *c-fos* promoter DNA. The timing of the increased occupancy of P-CREB coincides with the rapid increase in CREB phosphorylation induced by GH, as well as with increased occupancy of P-Pol II and GH-stimulated *c-fos* mRNA expression. It remains to be determined whether GH stimulates the phosphorylation of unbound CREB that is newly recruited to the promoter, or whether it increases the phosphorylation of CREB already occupying *c-fos*. These findings open new possibilities for understanding cooperation among multiple GH-regulated transcription mechanisms.

Cooperativity between CREB and C/EBPβ, a transcription factor critical for GH-stimulated *c-fos* expression (Cui *et al.* 2005), is supported by demonstrating that GH stimulates the occupancy of P-CREB on the same *c-fos* DNA as C/EBPβ, and of P-C/EBPβ on the same DNA as CREB. The combination of CREB and C/EBPβ enhances *c-fos* promoter activation via the CRE relative to either transcription factor alone. Cooperation between C/EBPβ and CREB in activating CRE-Luc has also been reported in osteoblasts (Chen *et al.* 2003), indicating that such cooperation occurs in different cell types. Taken together, these findings support a model in which GH activates CREB and C/EBPβ cooperatively to increase *c-fos* transcription.

Figure 5 GH promotes occupancy of both CREB and C/EBPβ on the same *c-fos* promoter DNA. 3T3-F442A cells were treated with GH for 15 min. Nuclear extracts were subjected to ChIP (first IP) using anti-C/EBPβ, anti-CREB, anti-P-Pol II, or normal rabbit IgG as control. After the first ChIP with anti-C/EBPβ, beads were eluted as described in Materials and Methods, and eluates were immunoprecipitated using anti-P-CREB (rabbit) or no antibody as described (second IP, top panels). Conversely, after the first ChIP with anti-CREB, eluates were immunoprecipitated using anti-P-C/EBPβ (second IP, bottom panels). Similar results were obtained in three experiments.

ERKs mediate GH-induced phosphorylation of CREB to regulate c-fos expression

GH promotes the phosphorylation of CREB by a mechanism involving activation of ERK1/2. Both the stimulation by GH of the phosphorylation of CREB and the occupancy of P-CREB on *c-fos*, as well as *c-fos* mRNA and protein expression, were reversed by inhibition of MEK with U0126. GH-stimulated ERK1/2 and their downstream kinase pp90^{Rsk} are likely mediators of the phosphorylation of CREB. In contrast, inhibition of PKA by H89, an established activator of CREB phosphorylation failed to alter GH-stimulated *c-fos* expression. The mediation of GH-regulated CREB phosphorylation by MAPK rather than by PKA agrees with previous observations that the ability of GH to regulate cAMP/PKA signaling is modest (Gorin *et al.* 1976, Birnbaum & Goodman 1979, Yarwood *et al.* 1998). In contrast, the ability of GH to activate MAPK signaling pathways is well established (Moller *et al.* 1992, Winston & Bertics 1992, VanderKuur *et al.* 1995). It should be noted that an inhibitor of the MAPK p38 (SB205380) failed to impair GH-induced *c-fos* expression in the present studies (Cui & Schwartz, data not shown), suggesting that *c-fos* activation by GH is specific for ERK1/2. Since transcription factors other than CREB are phosphorylated in response to GH via activation of ERK1/2, the inhibition of GH-stimulated *c-fos* mRNA expression by U1026 may also reflect impaired phosphorylation of C/EBP β , Elk-1, or other factors (Hodge *et al.* 1998, Zhu & Lobie 2000, Piwien-Pilipuk *et al.* 2002). In fact, it is likely that CREB acts in concert with other GH-regulated transcription factors, since mutation of the CRE only partially blocks GH-stimulated *c-fos* promoter activation. The residual response to GH may reflect the contribution of the other GH-regulated factors, and their potential interactions with CREB. Whether the phosphorylation of Pol II on Ser 5, which can also be mediated by ERKs (Trigon *et al.* 1998, Oelgeschlager 2002), reflects activation of ERKs by GH remains to be determined.

The relatively similar mechanisms for phosphorylation of CREB and C/EBP β by GH, as well as their cooperative occupancy on *c-fos* in response to GH, suggest that the regulation of their phosphorylation might be a coordinating factor for their cooperative regulation of *c-fos*. In fact, ERKs might themselves be associated with a complex on *c-fos*. Accumulating evidence indicates that MAPKs can become associated with the gene promoters that they regulate in the yeast genome (Edmunds & Mahadevan 2006, Pokholok *et al.* 2006). If ERK1/2, like p38 (Simone *et al.* 2004), are similarly present in complexes on a mammalian gene such as *c-fos*, activation by a signal such as GH could serve to phosphorylate transcription factors constitutively occupying the promoter DNA. Phosphorylation and activation of several factors such as CREB and C/EBP β could thereby be coordinated, facilitating their recruitment of other nuclear factors such as CBP or p300 to activate transcription. GH may also regulate

additional factors whose phosphorylation enables them to occupy *c-fos*, or other genes, which coordinately regulate GH-induced transcription.

GH induces the combined occupancy of P-CREB and P-C/EBP β on c-fos DNA

CREB has been considered to serve as a platform for formation of nucleoprotein complexes on target genes. Such complexes contain co-activators such as CBP (Chrivia *et al.* 1993, Kwok *et al.* 1994), enzymes such as PP1 and HDACs (Canettieri *et al.* 2003), and other transcription factors (Chen *et al.* 2003). The present study confirms that CREB and C/EBP β cooperatively enhance *c-fos* promoter activation and demonstrates that GH induces the combined occupancy of activated CREB and C/EBP β on the *c-fos* promoter. Endogenous P-CREB occupies the same *c-fos* DNA immunoprecipitated by anti-C/EBP β , and GH increases the appearance of P-CREB on the same DNA occupied by C/EBP β . This novel finding suggests that P-CREB and P-C/EBP β may be part of a regulated complex that occupies *c-fos* in response to GH. In previous studies, the combined occupancy of C/EBP β and the co-activator p300 on *c-fos* was induced by GH (Cui *et al.* 2005). Taken together, these findings raise the possibility that a regulatory complex occupying *c-fos* in response to GH contains P-CREB and P-C/EBP β , and a co-activator(s) such as p300 and/or the homologous factor CBP.

This study suggests that a complex on *c-fos* containing CREB and C/EBP β might be tethered at the CRE. C/EBP β might associate with CREB in a complex that occupies the CRE, since C/EBP β co-precipitates with CREB (Chen *et al.* 2003), and this study shows that mutation of the CRE prevented the enhancement of transcription with the simultaneous expression of CREB and C/EBP β . The CRE and the C/EBP site in the murine and human *c-fos* promoters are separated by ~200 bp. A highly positioned nucleosome has been identified on human *c-fos* between the C/EBP site and the CRE (Herrera *et al.* 1997). One can envision that the nucleosome brings the C/EBP site and the CRE, and the proteins associated with them, into proximity, facilitating interactions that allow formation of a complex containing C/EBP β and CREB on *c-fos*. Alternatively, CREB and/or C/EBP β might be present in multiple complexes that occupy different sites on *c-fos*. Analysis of CREB and C/EBP β on other promoters such as phosphoenolpyruvate carboxykinase (PEPCK; Wilson *et al.* 2002), as well as genome-wide analysis of CREB target genes (Impey *et al.* 2004, Zhang *et al.* 2005) and expression profiling of C/EBP β target genes (Friedman *et al.* 2004), support the importance of additional regulatory partners cooperating with CREB and C/EBP β in transcriptional activation. A complex containing CREB and C/EBP β is likely to recruit p300 and/or CBP. It will be of interest to identify other components of GH-induced complexes that contain CREB and C/EBP β .

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