cAMP Response Element-binding Protein Mediates Thyrotropin-releasing Hormone Signaling on Thyrotropin Subunit Genes*

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Transcription of pituitary α-glycoprotein hormone subunit (α-GSU) and thyrotropin β subunit (TSH-β) genes is stimulated by thyrotropin-releasing hormone (TRH). Since cAMP response element-binding protein (CREB)-binding protein (CBP) integrates a number of cell signaling pathways, we investigated whether CBP is important for TRH stimulation of the TSH subunit genes. Cotransfection of E1A in GH4 cells completely blocked TRH stimulation of the TSH subunit genes, suggesting that CBP is a key factor for TRH signaling in the pituitary. CBP and Pit-1 acted synergistically in TRH stimulation of the TSH-β promoter, and amino acids 1-450 of CBP were sufficient for the TRH effect. In contrast, on the human α-GSU promoter, CREB and P-Lim mediated TRH signaling. Intriguingly, CREB was phosphorylated upon TRH stimulation, leading to CBP recruitment to the α-GSU promoter. CBP also interacted with P-Lim in a TRH-dependent manner, suggesting that P-Lim is an important factor for non-cAMP response element-mediated TRH stimulation of this promoter. Distinct domains of CBP were required for TRH signaling by CREB and P-Lim on the α-GSU promoter, amino acids 450-700 and 1-450, respectively. Thus, the amino terminus of CBP plays a critical role in TRH signaling in the anterior pituitary via both Pit-1-dependent and -independent pathways, yielding differential regulation of pituitary gene products.

Thyrotropin (TSH) is a member of a pituitary and placental glycoprotein hormone family, which also includes follicle-stimulating hormone, luteinizing hormone, and chorionic gonadotropin. These hormones consist of two non-identical and non-covalently linked subunits termed α and β. The α subunit (α-glycoprotein hormone subunit (α-GSU)) is common to all members of this family and is expressed in molar excess in the pituitary to facilitate formation of the intact hormone (1) The β subunit is unique and confers specific biological activity to each dimeric hormone (2, 3). The TSH subunit genes are coordinately regulated at a transcriptional level by thyroid hormone (4), dopamine (5), and thyrotropin-releasing hormone (TRH) (6). Both thyroid hormone and dopamine reduce TSH subunit gene transcription in the anterior pituitary, whereas TRH increases transcription of these genes. However, the transcriptional response of the α-GSU and TSH-β subunit genes is not identical for these stimuli. For example, thyroid hormone treatment inhibits transcription of the α-GSU gene more slowly and to a lesser extent than that of the TSH-β gene. In contrast, transcription of the α-GSU gene is stimulated to a somewhat greater extent compared with the TSH-β gene by TRH (5). In the anterior pituitary, TRH bound to its receptor is known to activate phospholipase C, leading to calcium mobilization and protein kinase C activation (6–9). It has been suggested that the pituitary-specific transcription factor G HF-1/Pit-1 (hereafter referred to as Pit-1) is required for TRH regulation of the TSH-β and prolactin genes in humans and rats (10). Pit-1 is a member of a group of homeobox proteins containing both a homeodomain and a POU-specific domain, both of which are important for DNA binding (11). In addition to its signaling function in the anterior pituitary, Pit-1 is also necessary for cell-specific expression of prolactin, growth hormone, and TSH (12).

Interestingly, the TSH-β gene does not contain a consensus phorbol ester response element (AP-1 site), which is known to respond to activation of the protein kinase C pathway. Thus, the mechanism by which TRH mediates induction of this gene is unknown. We and others have shown that Pit-1 DNA-binding sites are required for TRH induction of the prolactin and TSH-β genes (13, 14).

In contrast, the human α-GSU gene, which is also stimulated by TRH, has no Pit-1 DNA-binding sites, but contains two cAMP response elements (CREs) (14–17). These data suggest that the signaling pathways responsible for TRH stimulation of the TSH subunit genes may diverge at least at the level of the transcription factors controlling gene transcription.

Several investigators have shown that CREB-binding protein (CBP) integrates a number of diverse cell signaling pathways involving protein kinase A, protein kinase C, and nuclear hormone receptors (18). We and others have recently shown that CBP can constitutively bind to Pit-1 and synergistically activate transcription of promoters containing Pit-1 DNA-binding sites (19, 20). We have demonstrated previously that CBP and Pit-1 enhance protein kinase A induction of the prolactin gene in a synergistic manner (20). In this report, we explore the hypothesis that CBP may play a pivotal role in TRH induction of both of the TSH subunit genes and whether distinct TRH
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signaling mechanisms are responsible for induction of these genes.

EXPERIMENTAL PROCEDURES

**Plasmids**—The TSH-β-luciferase reporter construct contains bp −1192 to +37 of the human TSH-β promoter linked to the luciferase gene in the plasmid pA3Luc. The α-glycoprotein subunit gene (referred to as −846α/Luc) contains bp −846 to +44 of the human α-glycoprotein gene linked to luciferase in pA3Luc. 5′-Deletions of the −846α/Luc construct (−546, −250, −156, and −99) were prepared by polymerase chain reaction (PCR) using −846α/Luc as a template and were subjected to DNA sequencing. The truncated α/Luc CRE mutants were constructed using the −846α/Luc CRE mutant as a template. The TSH-β Mt (with all three Pit-1 DNA-binding sites mutated)/Luc and −846α/Luc CRE mutant constructs were prepared by PCR-directed mutagenesis as described previously (10).

All Pit-1, CBP, and CREB constructs are in the SV40 expression construct pSG5. Pit-1a and W261C are modifications of the original WT Pit-1 cDNA and therefore contain the same translation initiation site. CBP deletion mutants were made using restriction enzyme digestion and removal of mouse WT CBP domains as indicated: aa 1–1334, BamHI; Δ142–705, ApoI; and aa 1–450, EcoRI. The aa 1–700 construct of CBP was prepared by PCR (Expand™2000kbp Taq Long PCR system, Roche Molecular Biochemicals). The RSV-E1A12S and RSV-E1AΔCR1 plasmids were a kind gift of Dr. Tony Kouzarides (Cambridge University, Cambridge, United Kingdom). Mouse P-Lim was generated by reverse transcriptase-PCR and subcloned into pSG5. The reading frame and orientation of each construct were confirmed by DNA dideoxy sequencing (Amersham Pharmacia Biotech).

**Transfections and Luciferase Assay**—In a 6-well format, 2 μg of reporter, 8 μg of WT CBP in pSG5, 1.5 μg of mouse TRH receptor cDNA in pCDM8, and/or 1 μg of either Pit-1 or P-Lim construct were transfected per well by a calcium phosphate method (Specialty Media, Inc., Lavallette, NJ) for CV-1 cells or by the LipofectAMINE method (Lipo- fectAMINE PLUS®; Life Technologies, Inc.) for GH3 cells. Sixteen hours after transfection, cultures were treated with serum-free Dulbecco’s modified Eagle’s medium for 8 h. For TRH stimulation, 50 nM TRH was added to the medium. All transfections were balanced for the same amount of expression vector using empty vector as needed. All experiments were repeated at least three times. For analysis of the expression of transfected CBP constructs and Pit-1 constructs, their expression vectors were transfected as described above, and Western blotting of total cell lysate was performed using an anti-CBP antibody that recognizes the extreme N terminus (aa 2–22) of CBP or an anti-Pit-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). **CREB Phosphorylation Assay**—After incubation with serum-free Dulbecco’s modified Eagle’s medium overnight, 50 nM TRH or 10 μM forskolin was added to GH3 cells. Whole cell extracts from GH3 cells were prepared, and Western blotting was done using two antibodies, one recognizing the CREB protein and a second specific for the phosphorylated CREB species at serine 133 (New England Biolabs Inc., Beverly, MA), using previously described methods (20).

**cAMP Assay**—Nearly confluent GH3 cells in 10-cm plates were incubated with serum-free Dulbecco’s modified Eagle’s medium overnight, then 50 nM TRH or 10 μM forskolin was added to the cells for up to 60 min. Cells were harvested and precipitated with 6% trichloroacetic acid. To measure cAMP levels, a 125I-labeled cAMP radioimmunoassay kit (PerkinElmer Life Sciences) was employed in three different experiments.

**Avidin-Biotin Complex DNA Binding Assay**—Whole cell extracts from GH3 cells were mixed with 3 μg of biotinylated oligonucleotides containing a P-Lim consensus DNA-binding site in binding buffer (phosphate-buffered saline, 1% Igepal CA-630 (Sigma), 0.5% sodium deoxycholate, 0.1% SDS, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 2% (v/v) Complete™ protease inhibitor mixture (Roche Molecular Biochemicals), and 0.1 mg/ml phenylmethylsulfonyl fluoride) at 4 °C for 1 h. Following this incubation period, 20 μl of streptavidinagarose (Life Technologies, Inc.) was added and incubated at 4 °C for an additional hour. Streptavidin-agarose resin was precipitated by centrifugation, washed intensively with binding buffer, and resolved on an 8% SDS-polyacrylamide gel. Western blotting performed to detect specific bound proteins. Rabbit anti-LIM-3 polyclonal antibody (Chemicon International, Inc., Temecula, CA) and the anti-CBP antibody (which recognizes the extreme N terminus (aa 2–22) of CBP) were employed for detecting P-Lim and CBP, respectively.

**RESULTS**

**E1A Blocks TRH Stimulation of Thyrotropin Subunit Gene Promoters in GH3 Cells**—The adenovirus oncoprotein E1A12S is known to bind CBP and to specifically inhibit CBP activity (21, 22). Since we hypothesized that CBP would play an important role in TRH stimulation of the TSH subunit genes, we cotransfected E1A12S and an E1A mutant (E1AΔCR1, where CR1 is conserved region 1) (22) that lacks the CBP-binding domain. -Fold basal activity is expressed as -fold induction over that of RSV (Vector) in the absence of TRH stimulation.

![Figure 1](image)

**FIG. 1.** E1A blocks TRH stimulation of the TSH subunit gene promoters in GH3 cells. Two micrograms of reporter plasmids and 1 μg of RSV-E1A, RSV-E1AΔCR1, or RSV vector plasmid were transfected into GH3 cells in a 6-well format. The mouse mammary tumor virus (MMTV) promoter was used as a negative control. E1AΔCR1 lacks the CBP-binding domain. -Fold basal activity is expressed as -fold induction over that of RSV (Vector) in the absence of TRH stimulation.
TSH-β Promoter—Since a physiological relevance for CBP in TRH signaling in the pituitary was suggested, we investigated a mechanism by which TRH stimulates the human TSH-β promoter. The human TSH-β promoter contains three well defined Pit-1 DNA-binding sites (14) (Fig. 2a), and these were collectively mutated (TSH-β Mt). These reporters were transfected to GH3 cells. -Fold basal activity is expressed as -fold induction over that of TSH-β Mt in the absence of TRH stimulation. c, CV-1 cells were transfected with an SV40 expression vector (pSG5) containing either CBP and/or WT Pit-1 and a mouse TRH receptor cDNA in the presence of the human TSH-β subunit gene promoter (bp -1192 to +37; Wt) and a Pit-1 DNA-binding mutant (MT; TSH-β Mt). These reporters were transfected into CV-1 cells as described for b in the presence of TRH stimulation. -Fold basal activity is expressed as -fold induction over that of pSG5 (Vector) alone in the absence of TRH stimulation. d, shown is a comparison of the wild-type TSH-β promoter (bp -1192 to +37) and a Pit-1 DNA-binding site mutant (TSH-β Mt). These reporters were transfected into CV-1 cells as described for b in the presence of TRH stimulation. -Fold basal activity is expressed as -fold induction over that of vector alone in the absence of TRH stimulation. e, shown is a Western blot of CV-1 cells transfected with pSG5 (Vector) or the indicated Pit-1 expression vectors probed with an anti-Pit-1 antibody.

Amino acids 1–450 of CBP Are Sufficient for the Synergistic Response with Pit-1 in TRH Stimulation of the TSH-β Promoter—We next determined which domains of CBP are responsible for the synergistic effect with Pit-1 in TRH stimulation of the TSH-β promoter.
TSH-β promoter. Various CBP deletion constructs (Fig. 3a) were cotransfected with WT Pit-1 in CV-1 cells, and TRH stimulation of the TSH-β promoter was measured. As shown in Fig. 3b, the aa 1–1334 construct, which lacks the carboxyl terminus of CBP, was sufficient to mediate the TRH effect. Moreover, the aa 1–700 construct of CBP, which contains both the cysteine/histidine-rich domain 1 (designated the C/H1 domain) and the CREB-binding domain, was also able to mediate a full TRH response. Finally, the aa 1–450 construct, which lacks the CREB-binding domain but contains the C/H1 domain, was fully sufficient to mediate the TRH response, indicating that a transactivation domain for TRH stimulation of the TSH-β promoter is located in this amino-terminal region of CBP. In contrast, the Δ142–705 construct, which lacks the C/H1 and CREB-binding domains, was defective (10% of the wild type) in mediating TRH stimulation (Fig. 3b). Taken together, these data indicate that aa 1–450 are fully sufficient for TRH stimulation of the TSH-β promoter and that the C/H1 domain contained within this construct is necessary for the effect. To prove that these CBP deletion constructs were expressed in CV-1 cells, Western blot analysis of cell lysate from transfected CV-1 cells was performed. In Fig. 3c, an anti-N terminus antibody was used to detect the transfected CBP proteins, and proteins of the correct size were detected in this analysis. Thus, the sufficiency of the aa 1–450 construct in TRH stimulation was not due to enhanced expression of this CBP construct relative to WT CBP in transfected CV-1 cells.

**CREs in the Human α-GSU Promoter Are Involved in TRH Stimulation**—We next studied the common α-GSU promoter. Since the human α-GSU promoter contains two consensus CREs (8-base palindrome, 5’TGACGTCA-3’) in the proximal promoter, and CBP is known to bind phosphorylated CREB (29), we mutated the CREs in the α-GSU promoter (Fig. 4a) and determined the effect of this change on TRH stimulation. GH3 cells were transfected with both wild-type (−846W) and CRE mutant (−846M) α-GSU promoter constructs. As shown in Fig. 4b, the −846W reporter construct was strongly stimulated by TRH treatment of cells, and the −846M reporter construct displayed significantly reduced TRH stimulation. Next, we tested a CRE reporter construct containing four copies of a consensus CRE upstream of a minimal promoter in GH3 cells. As shown in Fig. 4c, this CRE reporter was markedly stimulated by TRH in the rat pituitary cell line. The minimal reporter construct, lacking CREs, was not responsive to TRH stimulation. These data indicate that a cAMP response element mediates, in part, TRH stimulation of the human α-GSU gene.

**TRH Elevates cAMP Levels and Phosphorylates CREB in Pituitary Cells**—Based on evidence showing that CBP is an important factor for TRH stimulation in the pituitary (Fig. 1), we hypothesized that CREB is phosphorylated by the TRH signaling pathway (8). Previously, TRH was thought to act almost exclusively through the protein kinase C pathway in the pituitary, such that phosphorylation of CREB at site 133 (30) would seem unlikely. Given our results, however, we tested whether the TRH signaling pathway phosphorylates CREB in the same way that the protein kinase A signaling pathway phosphorylates this protein in GH3 cells. We utilized a phospho-specific antibody directed against serine 133 and a control antibody to CREB to prove this hypothesis. As shown in Fig. 4d, forskolin (a direct activator of adenyl cyclase) phosphorylated CREB at Ser133, as expected. Unexpectedly, TRH treatment of GH3 cells also phosphorylated CREB at this site. CREB phosphorylation by either forskolin or TRH treatment

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**Figure 3. Determination of CBP domains required for TRH induction of the human TSH-β subunit gene.**

*Panel a:* Schematic diagram of the CBP deletion constructs. C/H1 and C/H3 are the cysteine/histidine-rich domains 1 and 3, respectively. CREB BD, CREB-binding domain; HAT, histone acetyltransferase domain.

*Panel b:* Western blot analysis of CV-1 cells transfected with pSG5 (Vector) or the indicated CBP expression vectors using a common anti-CBP N terminus antibody.

*Panel c:* Cotransfection of CBP deletion constructs with the human TSH-β reporter. CV-1 cells were cotransfected with SV40 expression vectors (pSG5) containing Cre-1 binding sites and CBP constructs relative to WT CBP induction. Data are shown as mean ± S.E. of TRH-stimulated activity relative to percent WT CBP induction.

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**Figure 4. TRH-induced cAMP levels and CREB phosphorylation in GH3 cells.**

*Panel a:* Determination of CREs in the α-GSU promoter. GH3 cells were transfected with both wild-type (−846W) and CRE mutant (−846M) α-GSU promoter constructs. As shown in Fig. 4b, the −846W reporter construct was strongly stimulated by TRH treatment of cells, and the −846M reporter construct displayed significantly reduced TRH stimulation. Next, we tested a CRE reporter construct containing four copies of a consensus CRE upstream of a minimal promoter in GH3 cells. As shown in Fig. 4c, this CRE reporter was markedly stimulated by TRH in the rat pituitary cell line. The minimal reporter construct, lacking CREs, was not responsive to TRH stimulation. These data indicate that a cAMP response element mediates, in part, TRH stimulation of the human α-GSU gene.

*Panel b:* Cotransfection of CBP deletion constructs with the human TSH-β reporter. CV-1 cells were cotransfected with both wild-type (−846W) and CRE mutant (−846M) α-GSU promoter constructs. As shown in Fig. 4b, the −846W reporter construct was strongly stimulated by TRH treatment of cells, and the −846M reporter construct displayed significantly reduced TRH stimulation. Next, we tested a CRE reporter construct containing four copies of a consensus CRE upstream of a minimal promoter in GH3 cells. As shown in Fig. 4c, this CRE reporter was markedly stimulated by TRH in the rat pituitary cell line. The minimal reporter construct, lacking CREs, was not responsive to TRH stimulation. These data indicate that a cAMP response element mediates, in part, TRH stimulation of the human α-GSU gene.
was maximal at 5 min and then gradually decreased in TRH-treated cells, whereas forskolin-induced phosphorylation of CREB was relatively constant. We next investigated whether cAMP levels were elevated in GH3 cells after TRH stimulation. As shown in Fig. 4e, forskolin elevated cAMP levels as expected, and TRH also raised cAMP levels to maximum levels within 5 min, which declined thereafter. These data indicate that TRH rapidly phosphorylates CREB at Ser133 through elevation of intracellular cAMP levels, suggesting a potential mechanism for signaling cross-talk in pituitary cells.

A Second Region of the α-GSU Promoter Binds P-Lim and Mediates TRH Stimulation—It has been suggested that several non-CRE cis-elements on the α-GSU promoter play a pivotal role in its tissue-specific expression (12, 31–36). We employed truncated α-GSU promoters lacking consensus CREs (e.g., −846M) and transfected them into GH3 cells. As shown in Fig. 5a, there was a significant reduction in TRH stimulation when the region between bp −346 and −250 was deleted, suggesting that a non-CRE cis-acting element responsible for TRH stimulation is located there. Within this region is a P-Lim/Lhx-3 (hereafter referred to as P-Lim)-binding site known to activate the α-GSU promoter (12, 38); we hypothesized that P-Lim may be important for TRH stimulation and mutated this P-Lim-binding site (Fig. 5b) (12). To prove this hypothesis, we utilized the −346 α-GSU promoter construct containing a CRE-binding site mutation (−346M), a P-Lim-binding site mutation (−346M Lim mut), or both mutations in GH3 cells. As shown in Fig. 5c (left panel), the P-Lim mutation (−346W Lim mut) showed a 55% reduction in TRH stimulation compared with the wild-type construct (−346W). On the other hand, when both the CRE- and P-Lim-binding sites were mutated (−346M Lim mut), TRH stimulation was abolished (right panel).

Next, we examined whether P-Lim would interact with CBP on the P-Lim DNA-binding site. As shown in Fig. 5d, we performed an avidin-biotin complex DNA binding assay using a biotinylated WT or mutant P-Lim DNA-binding site and GH3 whole cell extracts, which were obtained before or after treatment with TRH. Western blot analysis showed that a biotinylated P-Lim DNA fragment specifically bound both P-Lim and CBP. P-Lim binding to this DNA fragment was constitutive, whereas CBP binding was increased in extracts prepared from TRH-treated GH3 cells. Since TRH treatment did not increase...
CBP content in the cells or the amount of P-Lim bound to DNA, these data suggest that CBP recruitment to the P-Lim site is augmented by TRH in GH3 cells.

**P-Lim and CBP Act Synergistically in TRH Stimulation of the Human α-GSU Promoter—**We next investigated whether P-Lim and CBP would act synergistically in TRH stimulation of the human α-GSU promoter. For this purpose, we employed the CV-1 cell line, which is devoid of TRH receptors and P-Lim and contains a low amount of endogenous CBP. To reconstitute TRH stimulation, a mouse TRH receptor expression vector was cotransfected into GH3 cells. Relative -fold activity is expressed as -fold induction over that of the −99W wild-type reporter (−99W) in the absence of TRH stimulation, b, shown are the sequence and mutation of a P-Lim-binding site (Lim mut) in the human α-GSU promoter. The P-Lim-binding site is a palindrome as indicated. c, a P-Lim-binding site is involved in TRH signaling. The −346 α-GSU promoter construct (−346W) or one containing a CRE-binding site mutation (−346M), a P-Lim-binding site mutation (−346W Lim mut), or both mutations (−346M Lim mut) was transfected in GH3 cells. Relative -fold activity is expressed as -fold induction over that of the −99W wild-type reporter (−99W) in the absence of TRH stimulation (not shown). d, shown are the results from the avidin-biotin complex DNA binding assay using a biotinylated P-Lim-binding site (P-Lim wt) or a mutant (P-Lim mut) fragment. The WT P-Lim fragment contains bp −341 to −314 of the α-GSU promoter. The mutant P-Lim fragment contains the exact mutation as indicated in b. B, biotin; SA, streptavidin-agrose. e, CV-1 cells were cotransfected with the α-GSU promoter, CBP, Pit-1, P-Lim, and the mouse TRH receptor. -Fold basal activity is expressed as -fold induction over that of vector alone in the absence of TRH.

**DISCUSSION**

In this report, we show that CBP mediates TRH signaling on both the human α-GSU and TSH-β promoters via different mechanisms. We have shown that CBP enhances expression of pituitary genes such as growth hormone and prolactin (20, 39). In this study, E1A clearly blocked TRH stimulation of the TSH subunit genes in a pituitary cell line, indicating that CBP may play an important role in TRH stimulation of the anterior pituitary (Fig. 1). We and others have also shown that Pit-1 plays an important role in TRH stimulation of the prolactin (19, 20). CBP binds to Pit-1 via two distinct domains, the C/H1 and C/H3 domains. On the rat proximal prolactin promoter, CBP synergistically enhances Pit-1-dependent TRH stimulation via the C/H1 domain and protein kinase A stimulation via the C/H3 domain (20). Our data on the human TSH-β promoter are consistent with the observation that the amino terminus of CBP is sufficient to mediate TRH stimulation via Pit-1 DNA response elements. Since Pit-1 binds to CBP via its C/H1 and
C/H3 domains, CBP may be a direct target of the TRH signaling pathway. This is further supported by the work of others studying the prolactin gene or isolated Pit-1 DNA response elements (13, 42). CBP bound to Pit-1 could also recruit other factors that contain histone acetyltransferase activity such as p/CAF (43) and p/CIP (44) to the transcription complex during TRH stimulation. To date, however, these coactivator proteins have been shown to bind to the C terminus of CBP, which our data clearly show is not required for TRH stimulation of the TSH subunit promoters. Moreover, Chawla et al. (45) suggested that CBP action is also controlled by nuclear calcium- and calmodulin-dependent kinase IV via the C terminus. This mechanism could be involved since TRH signaling is known to increase Ca\(^{2+}\) influx in the pituitary. However, calcium signaling also appears to act on the C terminus of CBP. Regardless of the mechanism, the amino terminus of CBP contains a coactivator function sufficient for TRH stimulation.

Like the TSH-\(\beta\) gene, CBP is also able to activate TRH stimulation of the \(\alpha\)-GSU gene. Unlike the TSH-\(\beta\) gene, however, Pit-1 is not required for TRH stimulation. Thus, at the level of the TSH subunit genes, TRH signaling pathways diverge. Previous workers suggested that CREs could in some way be involved in TRH stimulation of the human \(\alpha\)-GSU promoter (36). We demonstrated that phosphorylation of CREB by TRH plays an important role in the ability of CBP to enhance TRH stimulation of this gene. Since the CRE-binding domain of CBP is also required for TRH stimulation of the \(\alpha\)-GSU gene, we suggest that phosphorylation of CREB by TRH recruits CBP to the \(\alpha\)-GSU promoter to increase transcription (Fig. 7). Traditionally, Ser\(^{133}\) of CREB was thought to be an exclusive target of the protein kinase A signaling pathway (30) and, in contrast, that the protein kinase C signaling pathway regulates phosphorylation of the Jun-Fos (AP-1) complex (46). However, others have suggested that calcium/calmodulin-dependent kinases, Ras/mitogen-activated protein kinase, and protein kinase C can phosphorylate CREB at Ser\(^{133}\) (47–49). Their data are consistent with our findings since we note that the TRH signaling pathway, which activates protein kinase C and Ras/mitogen-activated protein kinase pathways and increases intracellular levels of calcium, also phosphorylates CREB. The data showing an elevation in intracellular cAMP levels upon TRH stimulation in GH\(_3\) cells also support these findings.

However, we also found that CBP was able to enhance TRH stimulation on this promoter in the absence of CREs, albeit to a lesser extent. This finding is important since the CREs found

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**Fig. 6.** Mapping of CBP domains responsible for TRH stimulation of the \(\alpha\)-GSU promoter. CBP deletion constructs were cotransfected with the \(-346W\) (a), \(-346W\) Lim mut (b), or \(-346M\) (c) \(\alpha\)-GSU reporter construct. CV-1 cells were cotransfected with a SV40 expression vector (pSG5) containing CBP deletion constructs or P-Lim. A mouse TRH receptor cDNA was also cotransfected. Data are shown as means ± S.E. of TRH-stimulated activity relative to percent WT CBP induction.

**Fig. 7.** Model of TRH stimulation of human TSH subunit gene promoters. The three pairs of white ovals on the TSH-\(\beta\) promoter represent Pit-1 dimers bound to DNA, and the two white ovals and two black ovals on the \(\alpha\)-GSU promoter represent P-Lim (dimer) and phosphorylated CREB (dimer) bound to DNA, respectively. The phosphorylation state of Pit-1 and P-Lim bound to TSH subunit gene promoters after TRH stimulation is unknown and is not indicated in the model.
in the human α-GSU gene are not conserved in lower mammalian species such as the mouse (37). Since a significant reduction in TRH stimulation was found when the region between bp −346 and −250 of the α-GSU promoter was deleted, we focused on the region. Located between bp −346 and −313 is a pituitary glycoprotein hormone basal element (12) that is known to bind LIM homeodomain proteins. Mutation of the region in the context of the CRE mutant revealed loss of TRH signaling on the α-GSU promoter. We also demonstrated that P-Lim binds to CBP in TRH-dependent manner on this site and that these proteins synergistically activate the human α-GSU promoter during TRH stimulation. We hypothesize that the P-Lim-CBP complex could be a key mediator of TRH signaling of the α-GSU gene in lower mammals in which the α-GSU promoter contain no consensus CREs.

In conclusion, CBP plays a pivotal role in TRH stimulation of the human TSH subunit genes via different amino-terminal domains. As suggested in the model in Fig. 7, recruitment of CBP to the TSH subunit genes involves different DNA-binding transcription factors. The involvement of CBP in TRH stimulation might allow for the integration of other pathways impinging on these genes, such as their negative regulation by thyroid hormone.

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