Hormonal regulation of the thyrotropin β -subunit gene by phosphorylation of the pituitary-specific transcription factor Pit-1

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ABSTRACT The pituitary-specific transcription factor Pit-1 is a cell-specific activator of prolactin and growth hormone gene transcription in the anterior pituitary. Pit-1 has also been shown to mediate both thyrotropin-releasing hormone (TRH) and cAMP stimulation of the prolactin and thyrotropin β -subunit (TSH β) genes. The molecular mechanism by which Pit-1 mediates these stimulatory effects remains unclear. At least three Pit-1-binding elements within the TSH β gene mediate responsiveness to TRH and cAMP. The present studies were designed to test the hypothesis that phosphorylation is an important modulator of Pit-1 interaction with the TSH β gene. TSH β elements bind less well to nonphosphorylated Pit-1 than to phosphorylated Pit-1 and are weak activators of gene expression, unlike high-affinity Pit-1 binding sites in the prolactin and growth hormone genes. Phosphorylation by protein kinase A or C enhances Pit-1 binding to TSH β elements 3- to 8-fold. Conversely, phosphorylation generally reduces binding of Pit-1 to elements within the prolactin and growth hormone genes. A variation within the consensus sequence for Pit-1 binding in TSH β gene elements [A(A/T)(A/T)AATNCAT in the TSH β gene versus A(A/T)(A/T)TATNCAT in the prolactin and growth hormone genes] could explain these differences. These elements may limit basal activation of the TSH β gene by binding less well to nonphosphorylated Pit-1 while conferring hormonal stimulation through enhanced binding of phosphorylated Pit-1.

The pituitary-specific transcription factor GHF-1/Pit-1 (hereafter referred to as Pit-1) is important for cell-specific expression of the pituitary hormones prolactin (Prl) and growth hormone (GH) (1–4). Pit-1 is a member of a group of the homeobox proteins containing both a homeodomain and a POU domain that are important for DNA binding (4). Despite the early observations that expression of this gene was primarily limited to GH-secreting (somatotrophs) and Prl-secreting (lactotrophs) cells of the anterior pituitary (3, 4), recent data indicate that Pit-1 is also expressed in the majority of thyrotropin (TSH)-secreting cells (thyrotrophs) (5). Moreover, a central role for Pit-1 in the development of each of these cell types was demonstrated in dwarf mouse strains containing mutations within the Pit-1 gene (6). It is unlikely, however, that Pit-1 subserves the same cell-specific role for TSH secretion in the thyrotroph as it does for GH and Prl secretion in the somatotroph and lactotroph, since TSH expression precedes Pit-1 expression in the anterior pituitary (5). Alternatively, it has been suggested that Pit-1 is necessary for thyroliberin (TRH) (presumably mediated by protein kinase C) and cAMP (mediated by protein kinase A) regulation of the TSH β -subunit (TSH β) gene in humans (7, 8) and rats (9) and the Prl gene in humans (10) and rats (11, 12). The present study was performed to elucidate the molecular mechanism(s) responsible for hormonal regulation by Pit-1.

We provide evidence that both TRH and cAMP stimulation of TSH β gene expression is mediated by phosphorylation and increased binding of Pit-1 to the same cis-acting elements.

METHODS

Plasmid Construction and DNA Transfection. Specific oligonucleotides and the polymerase chain reaction were used according to previously described methods (7) to introduce specific mutations within a DNA fragment consisting of base pairs (bp) –128 to +37 of the human TSH β (hTSH β) gene. These mutant DNA fragments were inserted between the *Kpn*I (5' end) and *Hind*III (3' end) sites of a chloramphenicol acetyltransferase (CAT) vector (13). Construction of luciferase (LUC) vectors containing various regions of the hTSH β gene have been described (14). The wild-type and mutant constructs (20 μ g) were transiently cotransfected along with a human GH expression plasmid (pTKGH, 5 μ g) into a clonal rat pituitary cell line (GH₃) by a calcium phosphate precipitation technique. Sixteen hours after application of the precipitate, the cell cultures were "shocked" for 2 min with 20% (vol/vol) glycerol in 150 mM NaCl/20 mM Hepes, pH 7.5; in some experiments, TRH (10 nM), forskolin (10 μ M), or both agents were applied to cell cultures. Basal as well as TRH-, forskolin-, or TRH/forskolin-stimulated expression was measured 24 hr after treatment, and CAT activity was corrected for transfection efficiency by measuring secretion of human GH into the culture medium (7, 8). Since TRH and forskolin induction of the wild-type construct varied somewhat between experiments (TRH, 3- to 5-fold induction; forskolin, 10- to 15-fold induction), induction of the mutant constructs was always expressed relative to the induction observed with the wild-type construct. In some experiments, LUC constructs (20 μ g), pTKGH (1 μ g), and a cytomegalovirus expression vector (2 μ g) containing either no insert ("empty vector," pCMV) or a rat Pit-1 cDNA insert (pCMVPit-1) (7) were cotransfected into the Pit-1-deficient embryonal cell line 293 (8). Cell cultures were harvested after 24 hr and LUC activity was determined and corrected for transfection efficiency as described above.

Avidin-Biotin Complex DNA-Binding (ABCD) Assay. The ability of ³⁵S-labeled Pit-1 to bind to regions of the hTSH β and rat GH genes was evaluated in this assay. DNA fragments were synthesized to contain the same biotinylated DNA extensions by methods previously described (14). hTSH β biotinylated DNA fragments contained 22 bp of one of the three Pit-1 binding sites flanked on either side by 10-bp biotinylated DNA extensions. The assay was also performed

Abbreviations: ABCD assay, avidin-biotin complex DNA-binding assay; CAT, chloramphenicol acetyltransferase; GH, growth hormone; LUC, luciferase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; Prl, prolactin; TRH, thyroliberin (thyrotropin-releasing hormone); TSH, thyrotropin (thyroid-stimulating hormone); hTSH β , human TSH β subunit.

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with a high-affinity Pit-1 binding site from the rat GH gene containing 30 bp of the binding site (rGH-1, bp -89 to -60; ref. 2) and the identical biotinylated DNA extensions. Prior to the binding assay in some experiments, radiolabeled Pit-1 was phosphorylated *in vitro* (15) with either purified cAMP-dependent protein kinase (PKA) or protein kinase C (PKC) (Promega). In general, 30,000–40,000 cpm of Pit-1 (trichloroacetic acid-precipitable counts) from an *in vitro* transcription-translation reaction and 1 pmol of biotinylated DNA fragment were used in each binding reaction (8).

RESULTS AND DISCUSSION

Pit-1 Is a Weak Transactivator of the TSH β Gene. Previous work (7, 8) has shown that TRH and cAMP response elements are located between bp -128 and -61 of the hTSH β gene and that this region contains three Pit-1 binding sites. To test the effect of Pit-1 on TSH β gene expression, hTSH β -LUC constructs were transfected into the Pit-1-deficient cell line 293. Cotransfection of a Pit-1 expression vector (pCMV-Pit-1) increased hTSH β expression 2- to 4-fold, and pRSV-LUC expression was reduced somewhat (Fig. 1). Cotransfection of different amounts of pCMVPit-1 did not result in a higher degree of stimulation (data not shown). These results are in sharp contrast, to the observation that transfection of a Pit-1 expression vector into a Pit-1-deficient cell line increased basal expression from the GH or Prl promoter $\approx$ 100-fold (4).

TRH and Forskolin Induction of the Wild-Type hTSH β Gene. We next tested the effect of TRH (10 nM), forskolin (10 μ M), or both agents on the expression of CAT reporter plasmids containing either the hTSH β promoter (bp -128 to +37) or a viral promoter (pTKCAT and pRSVCAT) in transfected GH $_3$ cells. TRH and forskolin induction of the hTSH β -CAT construct was 3.9- and 12.0-fold, respectively (Fig. 2). Induction with the combination of agents was similar to forskolin induction alone, suggesting that these agents may act through a shared pathway. On the other hand, TRH and forskolin induction of pTKCAT and pRSVCAT was less than or equal to 1.9-fold. This level of induction was considered nonspecific in GH $_3$ cells.

Mutation of Pit-1-Binding DNA Elements Reduces Basal Expression and TRH and Forskolin Induction. To begin to understand the importance of each of these sites for basal and hormonally stimulated expression, we mutated these sites, either individually or collectively, to sites that should not bind Pit-1 (M mutations; Fig. 3A and ref. 16) or to sites that

FIG. 1. Effect of Pit-1 on expression of the hTSH β gene in the 293 cell line. LUC plasmids containing either bp -1192 to +43 or bp -125 to +37 of the hTSH β gene or the Rous sarcoma virus promoter (RSV) were transfected into the Pit-1-deficient cell line 293 with either an "empty" CMV expression plasmid (pCMV) or one containing a rat Pit-1 cDNA (pCMVPit-1). Data are expressed on a logarithmic scale as the light units per ng of human GH per ml of medium (mean $\pm$ SEM from three independent experiments).

FIG. 2. TRH and forskolin induction of hTSH β and viral promoters in GH $_3$ cells. GH $_3$ cells were transfected with CAT constructs containing either bp -128 to +37 of the hTSH β promoter (WT), the herpes simplex virus thymidine kinase (TK) promoter, or Rous sarcoma virus (RSV) promoter. CAT activity is expressed as percent chloramphenicol acetylation per hr per ng of human GH per ml of medium (mean $\pm$ SEM from three independent experiments). Fold induction from basal activity by TRH, forskolin (FSK), or both agents is shown above the appropriate bar on the graph.

match the Pit-1 binding-site consensus sequence derived from studies of the rat Prl and GH genes (P mutations; Fig. 3B and ref. 2).

Point mutations that should abolish Pit-1 binding at site 1 (M1), site 2 (M2), site 3 (M3), or all three sites (M1-3) each reduced basal expression by about 60% (Fig. 4A). This reduction in basal expression by a factor of $\approx$ 3 with mutation of Pit-1 binding sites is consistent with a 3-fold stimulation of the hTSH β promoter by Pit-1 in a Pit-1-deficient cell line (see Fig. 1). TRH or forskolin (Fig. 4B) induction was reduced $\approx$ 70% by either the M1, M3, or M1-3 mutation. The M2 mutation, however, resulted in only a 20–30% reduction. Since these point mutations abolished both TRH and forskolin induction to a similar extent, we conclude that both stimulatory responses are mediated through the same cis-acting elements in the wild-type hTSH β construct.

When we mutated these sites to a consensus Pit-1 binding site defined in the Prl and GH genes (2), basal expression was

FIG. 3. Mutations of the human TSH β gene used in this study. Mutations of a hTSH β gene fragment (bp -128 to +37) were introduced into a CAT expression vector. DNA sequences homologous to the consensus Pit-1 binding sites are indicated by the bars below the DNA sequence (2). The most-conserved sequences among Pit-1-binding DNA elements are indicated by the black portion of the bars. Mutations introduced in the hTSH β gene to alter Pit-1-binding DNA sequences are indicated by nucleotide changes above the boxed wild-type DNA sequences. Mutations to abolish Pit-1 binding (M mutations) (A) and mutations to a consensus Pit-1 binding site (P mutations) (B) are illustrated.

FIG. 4. Transfection studies in rat pituitary cells with M mutations. GH₃ cells were transfected with the indicated mutant plasmid or the wild-type (bp -128 to +37) hTSH β plasmid (WT). (A) Basal activity of the constructs is expressed as in Fig. 2. (B) Fold induction by TRH or forskolin is expressed as a percentage of wild-type induction (mean $\pm$ SEM from three independent experiments).

either unchanged or slightly greater with individual site mutations (Fig. 5A; P1, P2, or P3) and was significantly increased when all three sites were mutated (Fig. 5A, P1-3). TRH or forskolin stimulation, on the other hand, was reduced $\approx$ 40% by all mutations except P3. Thus, in most cases, substitution of consensus Pit-1 binding sites for wild-type hTSH β sites reduced TRH or forskolin stimulation of the hTSH β gene by $\approx$ 40% even though basal expression was either unchanged or increased.

Phosphorylation of Pit-1 Increases Its Binding to TRH and Forskolin Response Elements in the hTSH β Gene. Since stimulatory TRH and forskolin response elements in the hTSH β gene bind Pit-1 (7, 8), we next tested whether phosphorylation of Pit-1 would affect its binding to wild-type and mutated Pit-1-binding DNA elements from the hTSH β gene. Previous investigators have suggested that Pit-1 is not a target for direct phosphorylation (17). However, recent evidence clearly demonstrates that Pit-1 is phosphorylated in rat pituitary cells (GC cells) in response to either cAMP or phorbol ester treatment and that phosphorylation alters binding of Pit-1 to response elements in the rat Prl, GH, and Pit-1 genes (15). At all but one site (Prl 3P site), Pit-1 DNA-binding affinity was either unchanged or decreased by phosphorylation (15). Therefore, since Pit-1 is phosphorylated in rat pituitary cells, we tested whether *in vitro* phosphorylation of Pit-1 by PKA or PKC would affect its binding to cAMP and TRH response elements in the hTSH β gene (Fig. 6). These sites bound 10% (hTSH β -3), 20% (hTSH β -2), and 50% (hTSH β -1) of nonphosphorylated Pit-1 protein as compared to the rat GH-1 site (bp -89 to -60) in an ABCD assay. Phosphorylation by either kinase resulted in a 3-fold increase in binding to site 1 and an 8-fold increase in binding to site 3. Pit-1 binding to site 2, on the other hand, was only increased

FIG. 5. Transfection studies in rat pituitary cells with P mutations. Data are expressed as in Fig. 4.

1.5- to 2.0-fold by phosphorylation. *In vitro* phosphorylation of transcription-translation reaction mixtures was confirmed by using [γ -³²P]ATP. These data correlate with data on function in GH₃ cells (Fig. 4B), where mutations at either site

FIG. 6. DNA binding of *in vitro* phosphorylated Pit-1 in the ABCD assay. Pit-1 was synthesized in an *in vitro* transcription-translation system using rabbit reticulocyte lysate (8). The ABCD assay was performed with the DNA fragments shown at the bottom of the figure; each fragment contained the same biotinylated DNA extension to ensure accurate comparisons among the fragments. Pit-1 binding is expressed relative to basal binding of Pit-1 to a site in rat GH DNA (rGH-1, bp -89 to -60; refs. 2 and 8) and is the mean $\pm$ SEM of three to six experiments. In this assay, phosphorylation with PKA inhibited Pit-1 binding to the rGH-1 site by a factor of 4 (data not shown). Kapiloff *et al.* (15) reported a decrease by a factor of 10-20 with phosphorylation in their assay.

1 (M1) or site 3 (M3) decreased TRH or forskolin induction by 70% but mutation at site 2 (M2) resulted in only a 20–30% decrease in TRH or forskolin induction. These data suggest that the stimulatory effect of TRH and forskolin on gene expression is due to direct phosphorylation of Pit-1, via either PKA or PKC, and increased binding of phosphorylated Pit-1 to predominately sites 1 and 3.

DNA Sequence Determinants for Pit-1 Binding in Response to Phosphorylation. We utilized the same mutations of site 1 (M1 and P1) in the ABCD assay and tested the effect of phosphorylation on Pit-1–DNA binding. Mutation of site 1 (M1) prevented an increase in binding of phosphorylated Pit-1. Since this mutation should abolish binding of Pit-1 to the core consensus sequence (Fig. 6), this result suggests that phosphorylated Pit-1 binds to site 1 at this DNA sequence. On the other hand, mutation P1 resulted in a nearly 10-fold increase in Pit-1 binding to site 1 and a 25–30% reduction in Pit-1 binding in response to phosphorylation. Inspection of the hTSH β Pit-1 binding sites (Fig. 6) suggests that the deoxyadenosine at position +4 (conserved in all three sites but different from the consensus sequence, arrowhead) may limit binding of nonphosphorylated Pit-1, since the P1 mutation in the ABCD assay displays a 10-fold increased binding to nonphosphorylated Pit-1. The deoxyguanosine at position +1 was also changed to a deoxyadenosine residue in the P1 mutation. This change, however, is unlikely to be the cause of increased Pit-1 binding, since a deoxyadenosine residue is also found at this same position in site 3 (Fig. 6, Wt3); and Wt3 also displays low binding to nonphosphorylated Pit-1. Moreover, each site, but especially sites 1 and 3, displays increased binding to phosphorylated Pit-1 and mutations within the core DNA sequence (M1 or P1) abolish this enhanced binding to site 1. In the case of either the M1 or the P1 mutation of site 1, phosphorylation of Pit-1 actually inhibited binding of Pit-1 to these DNA fragments.

From these data we conclude that the stimulatory effect of TRH or forskolin on hTSH β gene expression is the result of the phosphorylation of the Pit-1 protein and the subsequent increased binding of this modified protein to at least two of the Pit-1 binding sites in the hTSH β gene. The Pit-1 binding sites within the hTSH β gene appear to interact, since P mutations at all three sites are required to increase basal expression and since M mutations at either site 1 (M1) or site 3 (M3) reduce TRH and forskolin induction to the same extent as mutations at all three sites. These Pit-1 binding sites: (i) bind nonphosphorylated Pit-1 less well than the previously described cell-specific sites in the rat Prl and GH genes, (ii) display enhanced binding to phosphorylated Pit-1, and (iii) are responsible for hormonal responsiveness to TRH

and forskolin in the hTSH β gene. The deoxyadenosine residue at +4 (Fig. 6, arrow), which is conserved in each of the TSH β sites but is different from the core consensus Pit-1 binding site defined in the rat Prl and GH genes, appears to be responsible for at least part of the difference between these elements. Thus, the TSH β gene contains a unique subset of Pit-1 DNA-binding sites that appear to mediate, in part, both TRH and cAMP responsiveness in the thyrotroph.

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