Renin Gene Promoter Activity in GC Cells Is Regulated by cAMP and Thyroid Hormone through Pit-1-dependent Mechanisms*

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Transcriptional activity of human renin gene (hREN) 5'-flanking DNA sequences in pituitary cells is highly dependent on binding of the pituitary-specific transcription factor Pit-1. Pit-1 has been implicated in cAMP regulation of a number of pituitary genes and has also been shown to interact with thyroid hormone (T3) receptors in mediating T3 responsiveness of the rat growth hormone gene. In the present study we examine the effects of forskolin and T3 on the expression of luciferase hybrid genes containing hREN 5'-flanking DNAs (hREN.luc) transiently transfected into the pituitary cell line GC. Basal activities of all hREN.luc constructs transfected into cells grown in media containing serum stripped of hormones were low. Addition of forskolin stimulated expression up to 48-fold, depending on the hREN sequences present. The hREN sequence -148 to +18 was sufficient for both maximal expression and maximal stimulation by forskolin. Mutagenesis of the Pit-1 site between -82 and -58 reduced forskolin induction 4-5-fold. In addition to the Pit-1 site, the sequence between -148 and -96 was also required for maximal activity and forskolin induction. T3 on its own had no effect on hREN promoter activity in GC cells, but suppressed the effects of forskolin. Gel mobility shift and Western blot analyses indicated that forskolin treatment had no effect on Pit-1 DNA binding or Pit-1 levels. However, T3 reduced Pit-1 levels which was reflected in lower DNA binding under the conditions employed. Taken together, these findings emphasize the importance of cAMP-dependent mechanisms in directing renin gene expression.

Renin release from secretory granules of the renal juxtaglomerular cells is regulated by a number of extracellular stimuli whose effects are mediated by changes in intracellular cyclic AMP (1). Cyclic AMP has also been implicated in the transcriptional control of renin gene expression in a number of tissues (2, 3). We recently demonstrated that human renin gene (hREN) 5'-flanking DNA sequences -148/+18 direct maximal expression of a linked luciferase reporter gene transfected into the pituitary tumor cell line GC (4). In these cells, the main determinant of renin promoter activity is a binding site for the pituitary-specific factor Pit-1 (5, 6). Pit-1 has been implicated in mediating cAMP regulation of the rat growth hormone gene (rGH) (7, 8). Therefore, we postulated that Pit-1 might also be involved in cAMP regulation of hREN expression.

In addition to its role in mediating the effects of CAMP to RNA polymerase II, Pit-1 also appears to interact with thyroid hormone receptors (T3R), which may synergize the effect of increases in intracellular cAMP (7). Although expression of the ren-2 gene in mouse salivary gland is stimulated by T3 (9), little is known about the effects of T3 on renal renin gene expression. Studies of humans (10-12) and animals (13, 14) have shown positive correlations between thyroid status and plasma renin activity, suggesting that T3 may also stimulate renal renin gene expression.

In the present study, we investigated the effects of cAMP and T3 on the transcriptional activity of renin 5'-flanking DNA sequences. Transfection of GC cells (which contain high concentrations of Pit-1 and T3R) with renin-luciferase hybrid genes showed that the activity of the human renin gene promoter is positively regulated by cAMP and that this effect is dependent on the Pit-1 binding site. While, on its own, T3 had little effect, when added in combination with forskolin, it suppressed the effects of forskolin 2-3-fold. Gel mobility shift analysis of Pit-1 binding and Western blot analysis of Pit-1 levels indicated that the effect of T3 is indirect, being mediated through the effects of T3 on Pit-1 levels.

MATERIALS AND METHODS

Constructions, Cell Culture, and Transfections—Constructions used in this study are described in detail elsewhere (4). GC cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. Cells were transfected following previously established protocols either by lipofection (8) or by electroporation (8). To study the effects of hormones, cells were maintained in media containing charcoal-resin stripped serum for 24 h pre-transfection and were harvested for assay 48 h post-transfection. After transfection cells received either no treatment, thyroid hormone (3 x 10^-5 M), forskolin (10^-6 M), or a combination. Cells were harvested and lysed, and luciferase activity measured as described previously (4).

Cell Extracts, Western Blot, and Gel Mobility Shift Assays—Whole cell extracts were prepared from cell monolayers grown in 10 cm dishes. Cells were rinsed in phosphate-buffered saline, harvested by scraping in 1 ml of phosphate-buffered saline, and centrifuged for 45 s in a microcentrifuge. Cells from one near-confluent 10-cm dish were resuspended in 100 µl of lysis buffer (25 mM HEPES pH 7.9, 200 mM KCl, 0.1% Nonidet P-40, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 0.1 mM Na3VO4, 5 mM EDTA, 5 mM dithiothreitol, 1% glycerol). After incubation on ice for 30 min with occasional vortexing, the suspension was centrifuged for 10 min in a microcentrifuge at 4 °C. The supernatant was divided into 10-µl aliquots, which were frozen in liquid nitrogen and stored at -76 °C. Proteins were separated by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated in a blocking solution containing 3% (w/v) non-fat dry milk and 0.1% (w/v) Tween 20 in Tris-buffered saline and then probed with antibodies against Pit-1 (5, 6) or with antibodies against T3R (7). The antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and chemiluminescent substrates.

The abbreviations used are: T3R, thyroid hormone receptor; T3, thyroid hormone; RSV, rous sarcoma virus; rGH, rat growth hormone; PRL, prolactin; TSH, thyroid-stimulating hormone; CREB, cAMP response element-binding protein.

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tein concentrations were determined using a Bradford assay (15). Nuclear proteins were fractionated on a 12% acrylamide gel (Novex) and transferred to nitrocellulose membrane using a Novex electrophoretic chamber following the manufacturer’s protocol. The blots were then incubated with either an affinity purified anti-Pit-1 antibody (gift of Carter Bannert) at a final dilution of 1:10,000 or an anti-rat growth hormone (rGH) antibody (Sigma) diluted 1:1,000. After washing off the unbound antibody, membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham Corp.), and immunoreactive bands were visualized using a chemiluminescence kit (Amersham Corp.) following the manufacturer’s recommendations. Gel mobility shift assays were carried out as described previously (4, 16).

RESULTS

hREN Sequences Required for cAMP Responsiveness in GC Cells—To examine whether the promoter activity of hREN 5'-flanking DNA sequences is responsive to changes in intracellular cAMP and to determine the sequence elements involved, we transfected a series of hREN-luciferase hybrid genes into GC cells. In the presence of resin-charcoal stripped serum, the basal activity of all constructs was low and differed less than 3-fold. Addition of forskolin stimulated expression up to 48-fold, depending on the 5'-flanking sequences present (Fig. 1). Deletion of sequence -555 to -148 had no significant effect on cAMP responsiveness. Indeed, deletion of a CREB binding site (CGTCA) (17), centered at position -215, led to a small increase in forskolin-stimulated activity. However, deletion of sequence from -148 to -98 resulted in 2-3-fold reductions in induction, whereas mutation of the Pit-1 site in the context of the -148 construct consistently resulted in 4-5-fold reductions in forskolin induction. These observations suggest that the Pit-1 site is required for maximal cAMP stimulation of the renin promoter, although sequences between -148 and -98 are also involved. This pattern of cAMP-stimulated activities is very similar to that observed when the same constructions were transfected into cells grown in medium containing 10% fetal bovine serum (4). Taken together, these findings suggest that cAMP provides the major transcriptional drive to the hREN promoter in GC cells.

Regulation of hREN Promoter Activity in GC Cells by T3 and cAMP—Pit-1 is known to interact with thyroid hormone receptors to stimulate transcription of growth hormone gene expression (7). To investigate the effect of T3 on renin gene expression, we transfected GC cells with human renin luciferase hybrid genes containing the human renin 5'-flanking DNA sequences -148+18 (-148hREN), which gave the highest activity in GC cells. To examine possible interactions between T3 and cAMP, we also transfected the construct containing a 4-base pair mutation in the Pit-1 site (-148A1hREN), which reduced by 4-5-fold both total activity (4) and forskolin responsiveness (Fig. 1). As positive controls we selected luciferase constructs containing the human choricion somatomammotropin (hCS) sequences -127/+14 (-127hCS) (18) and the Rous sarcoma virus long terminal repeat (RSV-LTR) (19). The hCS and hREN sequences are similar in that they contain one Pit-1 binding site and yield similar luciferase activities when transfected into GC cells (see below). The hCS sequence contains at least one thyroid hormone receptor binding site (−62/−48), which confers responsiveness to T3 (18). Activities were standardized to that of the maximally stimulated RSVluc construct transfected in parallel dishes in each experiment. The RSV-LTR is a strong promoter/enhancer, which is relatively insensitive to the effects of changes in intracellular cAMP and T3.

The maximal activities of hREN and hCS constructs in GC cells were approximately 1–2% of the RSV. Whereas T3 exerted the greatest effect on hCS promoter activity (15-fold), its effect on the RSV and renin promoters was relatively weak (2-4-fold). Conversely, forskolin treatment stimulated the hREN promoter 7-20-fold but had a smaller effect on RSV (5-6-fold) and hCS (6-12-fold). When T3 was added together with forskolin, their effects on hCS were more than additive, while on RSV, T3 plus forskolin stimulated activity little more than forskolin alone. However, in the case of the hREN construct, when T3 was added together with forskolin, it halved the induction by forskolin alone. Mutation of the Pit-1 binding site in the hREN promoter reduced activity maximally stimulated by for-
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Fig. 2. Differential effects of thyroid hormone on the basal and cAMP-stimulated activities of hREN, hCS, and RSV-luciferase hybrid genes in GC cells. Cells were replated in medium containing charcoal/resin-stripped serum 24 h before transfection and transfected by electroporation as described previously (8). Media and hormones were replaced after 12-16 h and the cells harvested after 40 h. A, activity normalized to the activity of maximally stimulated RSV luciferase (T3 + forskolin (T3+F)-treated (mean ± S.E.) = 1.8 ± 0.4 × 10^6 light units; n = 5). B, fold induction treatment (T3, F, or T3+F) untreated (-). Values shown are mean ± S.E. for five separate experiments.

skolin 8-15-fold and reduced forskolin induction to one-half to one-third that of the native sequence (Fig. 2). Moreover, in the absence of the native Pit-1 binding site, there was no significant suppression of forskolin-stimulated activity by T3.

Effects of Hormones on Pit-1 and T3R Levels and DNA Binding—The positive effect of forskolin on hREN promoter activity in GC cells appeared to be mediated by the Pit-1 site, suggesting several possible mechanisms. These include: (i) modulation of Pit-1 concentration, (ii) modulation of Pit-1 DNA binding, and (iii) post-translational modifications that affect Pit-1 interactions with other transcription factors. Because the negative effect of T3 also appeared to require the Pit-1 site, the effects of T3 might also be mediated indirectly through some effect of T3 on Pit-1. To investigate these possibilities, Pit-1 DNA binding was examined in gel mobility shift assays, and the intracellular concentration of Pit-1 determined by immuno-detection on Western blots using a Pit-1 antibody. In these analyses, a constant amount of total protein was loaded in each track. However, total protein concentrations were similar under the different hormone treatments (not shown). The results of these experiments are shown in Fig. 3. Whole cell extracts were prepared from cells electroporated with non-expressing DNA which received the various hormone treatments. Protease and phosphatase inhibitors were included to minimize proteolytic degradation and dephosphorylation of proteins.

DNA binding was assessed in gel mobility shift assays using the hREN sequence -80/55 and hCS -95/65 as probes (Fig. 3A). Earlier competition experiments suggested that the hCS sequence binds Pit-1 with 5-10-fold higher affinity than the hREN sequence (4). Accordingly, under the assay conditions used, the hCS probe bound approximately 10 times more Pit-1 than the hREN probe. With both probes, treatment with forskolin had little or no effect on complex formation, suggesting that the transcriptional effects of forskolin are not mediated through changes in Pit-1 levels or through modifications that affect its ability to bind DNA. Treatment of cells with thyroid hormone reduced Pit-1 complex formation, although the effect was more evident with the hCS probe, where presumably the concentration of Pit-1-probe complex had reached a level closer to equilibrium.

To examine the effects of the various hormone treatments on intracellular Pit-1 levels, Western blot analysis of GC cell extracts was carried out with an anti-Pit-1 antibody (Fig. 3B). To serve as a control, an identically prepared gel blot was incubated in parallel with an anti-rGH antibody (Fig. 3B). Preliminary titration experiments established the optimal antibody dilutions and staining conditions. The anti-Pit-1 antibody bound two bands of approximately 31 and 33 kDa corresponding to the alternatively spliced forms of Pit-1 previously reported (20, 21). The anti-rGH antibody bound a single band of approximately 12 kDa corresponding to mature rGH (22). Quantification of band intensities showed that, while forskolin had no effect on Pit-1 levels, T3 decreased Pit-1 levels approximately 2-fold, both in the presence and absence of forskolin. Conversely, rGH showed an opposite pattern in which levels were increased by T3 and decreased by forskolin. When T3 and forskolin were added together, the positive effect of T3 was predominant. rGH is endogenously expressed by GC cells, and its transcription is known to be stimulated by T3. Therefore, these results suggest that hormone treatments on rGH expression show that the common pattern of Pit-1 and T3R binding does not represent a general phenomenon exhibited by all GC cell proteins.

Taken together, the results of Western blot analysis and DNA binding indicate that forskolin does not act by changing either the concentration or DNA binding of Pit-1, whereas thyroid hormone reduces Pit-1 concentration, which results in proportionally lower DNA binding and lower transcriptional activation of the hREN promoter in GC cells.

DISCUSSION

Pit-1 is a pituitary-specific transcription factor that plays an important role in pituitary development and in the expression of several pituitary-specific genes, including GH and PRL (23, 24), TSH (25-27), growth hormone releasing factor (28), and PRL itself (29). We recently demonstrated that Pit-1 also activates the human renin gene promoter (4), although the role of Pit-1 in directing expression of pituitary renin is not known. Pit-1 may also regulate transcription in response to extracellular signals. Pit-1 appears to mediate cAMP regulation of GH (30, 31), PRL (32), and TSH (33, 34) genes. In each of these genes, full cAMP responsiveness depends on sequences outside the Pit-1 binding site. In the TSH promoter, an AF-1 site is required (34); in the rat and human PRL promoters, a sequence with some identity to the CREB consensus, but unable to bind CREB itself, is involved (32, 35); and in the hGH gene, two apparently functional CREB/ATF binding sites con-
ttribute to cAMP responsiveness (30).

In the present study, we showed that the activity of the hREN promoter was increased by forskolin in the GC cell line. Basal activities in media containing charcoal/resin stripped serum were low and differed less than 3-fold among constructs. Addition of forskolin stimulated the activity of certain constructs up to 48-fold, depending on the hREN 5'-flanking sequences present. Most notably, the relative forskolin-stimulated activities of the constructions tested were very similar to the pattern of serum-stimulated activities we reported previously (4). Taken together, these findings suggested that cyclic AMP, acting through Pit-1, is the major stimulus for transcription of the hREN in GC cells. In the absence of an intact Pit-1 binding site both serum-stimulated activity and the effect of forskolin were greatly diminished.

Deletion of a low affinity, non-palindromic CREB binding site (CGTCA) (17) contained between positions -220 and -215 had no deleterious effect on cAMP induction. However, deletion of sequence between -148 and -98 reduced forskolin induction more than 2-fold, suggesting that this region may bind some additional factor(s) that acts in concert with Pit-1 to stimulate cAMP responsiveness.

Inspection of the sequence -148 to -98 revealed no apparent homologies with known transcription factor binding sites. Burt et al. (36) had previously suggested that this region might contain cAMP-responsive elements based on similarities to a cAMP consensus derived from alignments of cAMP responsive genes (37). However, in functional assays an heterologous promoter construct containing the -148 to +18 hREN sequence linked to a thymidine kinase gene 5-flanking DNA sequence was induced only about 2-fold by forskolin after transfection into JEG-3 cells (36). This may reflect the absence of cell-specific factors able to bind the Pit-1 site.

Because several studies have demonstrated that Pit-1 interacts with thyroid hormone (7) and estrogen (38) receptors in the regulation of growth hormone and prolactin genes by these hormones, we postulated that similar interactions might occur in the hREN promoter. Expression of the ren-2 gene in mouse salivary gland is regulated by T3 (9), and studies of humans (10–12) and animals (13, 14) have shown positive correlations between thyroid status and plasma renin activity. In the present study, however, T3 suppressed the stimulatory effects of forskolin. This apparent discrepancy may reflect different mechanisms. Thyroid hormones may exert an indirect effect on renin secretion from renal JG cells by regulating β-adrenergic receptors (39, 40). Accordingly, hypothyroid patients with low renin hypertension exhibit decreased sensitivity to catecholamines (41), while propranolol reduces basal levels of AI and plasma renin activity, prevents the increase in PRC in hyperthyroid rats (42), and reverses the effects of T3 on plasma renin activity and serum aldosterone levels (43). Thus the effects of thyroid hormones on circulating renin may be independent of any effects on renin gene expression. Moreover, the effects observed in the present study may be specific to renin gene expression in pituitary cells.

Differences in the interactions that occur between Pit-1 and other factors that bind the hREN and hCS promoters are exemplified in their responsiveness to cAMP as well as to T3. Although both promoters contain a single Pit-1 binding site the hCS promoter was 2–3-fold less responsive to cAMP than the hREN promoter, and only the hCS promoter responded directly to T3. The lower cAMP responsiveness of the hCS promoter is most likely due to the absence of sequence elements contained in the -148 to -98 region of the hREN promoter which was required for maximal cAMP induction. However, the lower affinity of the hREN Pit-1 site, which may reflect differences in
the mode of Pit-1 binding, may also affect responses to both cAMP and T₃. The DNA binding domain of Pit-1 and other POU family transcription factors may be divided into two subdomains: a POU-homeobox (POU₆D) similar to the DNA binding domain of homeobox family transcription factors, and a POU-factoric domain (POU₆P) unique to this family (44). Although the POU₆D binds DNA with low affinity, the POU₆P is required for high affinity binding (45, 46). The relatively low affinity binding of Pit-1 to the hREN sequence ~85–60, predominantly as a monomer, may be because this site contains only the motif shown by Verrijzer et al. (46) to bind the POU₆P (Fig. 4). In the absence of a recognition sequence for the POU₆D, dimer formation is either reduced or prevented. These differences in the mode of Pit-1 DNA binding may affect interactions with T₃Rs and with other components of the transcriptional machinery.

The low affinity of the hREN Pit-1 site may also account for the sensitivity of the hREN promoter to small changes in intracellular Pit-1 concentration. In the present study, Pit-1 levels appeared to be negatively regulated by T₃. This resulted in a T₃-dependent attenuation in the forskolin-induced activity of the native promoter, which was lost when the Pit-1 binding site was mutated. Despite the diminution in Pit-1 levels by T₃ and forskolin had a synergistic effect on hCS promoter activity. While this may require interactions among Pit-1, T₃R, and other factors specific to the hCS promoter, the higher affinity of the hCS Pit-1 site most likely reduces its sensitivity to small changes in Pit-1 levels.

Pit-1 has recently been shown to be phosphorylated by cAMP-dependent protein kinase A and also protein kinase C (47). The latter study demonstrated that phosphorylation of Pit-1 by either protein kinase reduced the binding of Pit-1 to certain sites depending on the flanking sequences. Conversely, phosphorylation of Pit-1 increases its affinity for Pit-1 binding sites in the TSH promoter and has been implicated in the mechanism of cAMP regulation of TSH expression (33). In the present study, we found no effect of forskolin treatment on the binding of Pit-1 to the hREN or hCS Pit-1 binding sites. While the core sequence in the hCS Pit-1 binding site used in the present study is identical to the high affinity rGH Pit-1 site analyzed by Kapiloff et al. (47), the flanking sequences differ at exactly the positions that were found to modulate the differential binding of phosphorylated and unphosphorylated forms of Pit-1 (19). No homologies were evident between the hREN sequence and rGH regulatory region. These observations suggest that Pit-1 binding to the hREN and hCS sequences used in this study is unaffected by phosphorylation. Phosphorylation of Pit-1 may, however, regulate transcription by modulating interactions with other transcription factors.

Expression of Pit-1 is limited to pituitary cells (6). Therefore, other transcription factors, possibly related to Pit-1, might directly or indirectly regulate gene expression in other tissues. Indeed, we have evidence to suggest that the Pit-1 site is important in directing placental renin gene expression (52) and preliminary studies indicate that a Pit-1-like factor is expressed in the renin-expressing renal cell line As4.1 (48). These factors might also confer CAMP responsiveness to the hREN promoter. Renin release from renal juxtaglomerular cells is stimulated by an increase in intracellular cAMP (49). Thus, renin secretion and synthesis might be coupled to the same intracellular signaling pathway, leading to de novo synthesis to replenish depleted stores when renin is released from storage granules. It is noteworthy that Pit-1 has been proposed to play a role in mediating calcium regulation of the PRL gene (50, 51). Therefore, it is tempting to speculate that a Pit-1-like factor might also confer calcium responsiveness to renal renin expression. This could provide another mechanism to co-ordinate the secretion and synthesis of renin by reducing renin synthesis in response to angiotensin II.

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