Communication

Transcriptional Regulation of the Parathyroid Hormone-related Peptide Gene by Glucocorticoids and Vitamin D in a Human C-cell Line*

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A parathyroid hormone-related peptide (PTHRP) has been identified in human tumors associated with the syndrome of humoral hypercalcemia of malignancy. The PTHRPs and parathyroid hormone (PTH) genes appear to have arisen by duplication and to represent members of a gene family. PTHRPs mRNAs have been demonstrated in a number of normal tissues, but little is known concerning the regulation of PTHRPs gene expression in any site. We studied PTHRPs gene expression in TT cells, a human C-cell line which also produces calcitonin and calcitonin-related peptide. We found that both the synthetic glucocorticoid, dexamethasone, and the active vitamin D metabolite, 1,25-dihydroxyvitamin D₃, decreased steady-state PTHRPs mRNA levels in TT cells in a time- and dose-dependent fashion. The dexamethasone effect was completely blocked by the glucocorticoid antagonist RU-486. 24,25-dihydroxyvitamin D₃ was found to be inactive. Neither dexamethasone nor 1,25-dihydroxyvitamin D₃ appeared to influence PTHRPs mRNA stability in TT cells, and both agents were shown by nuclear transcription run-off assay to decrease PTHRPs gene transcription. These findings indicate that the PTHRPs gene is under the transcriptional control of glucocorticoids and vitamin D in a cell line with prototypical neuroendocrine features.

A novel parathyroid hormone-related peptide (PTHrp) has been isolated (1-3) and its cDNA cloned (4-6) from human tumors associated with the syndrome of humoral hypercalcemia of malignancy. The deduced PTHRPs structure is approximately twice the size of parathyroid hormone (PTH) and has sequence similarity to human PTH confined to its proximal amino terminus, following which the sequences of the two peptides diverge completely (4-6). Synthetic amino-terminal PTHRPs fragments have been shown to bind to classical PTH receptors (7-9), but the actual secretory form of the PTHRPs remains unknown and unique PTHRPs receptors have yet to be demonstrated.

The PTH and PTHRPs genes appear to have arisen by duplication from a common ancestral chromosome and to therefore represent members of a gene family (5, 10). Following this duplication event, the human PTHRPs gene has evolved to develop an organization which is considerably more complex than that of the PTH gene and which involves the apparent use of multiple promoters (10-12). In addition, whereas the PTH gene seems to be expressed exclusively in parathyroid cells (13), PTHRPs transcripts have been identified in a variety of normal tissues, including sources as diverse as skin, lactating mammary tissue, and the central nervous system (5, 14, 15). The biological role(s) of the PTHRPs in these tissues has not been defined, and nothing is known concerning the regulation of PTHRPs gene expression in any site.

We studied the regulation of PTHRPs gene expression in TT cells, a continuous human C-cell line derived from a medullary thyroid carcinoma (16). TT cells have neuroendocrine characteristics and produce both calcitonin and calcitonin gene-related peptide (CGRP) (16-18). Glucocorticoids have been reported to increase (17) and 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) to decrease (18) steady-state calcitonin mRNA levels in these cells. We have recently found that TT cells also express the PTHRPs gene (15, 19), and we report here that both dexamethasone and 1,25-(OH)₂D₃ decrease steady-state PTHRPs mRNAs in these cells. Both steroids appear to act at a transcriptional level.

EXPERIMENTAL PROCEDURES

Cell Culture—TT cells (courtesy of P. J. Gkonos and B. A. Roos, University of Washington, Seattle) were maintained in RPMI-1640 supplemented with 10% fetal calf serum (17, 18). Cells were plated at a density of 3 x 10⁶ cells/100-mm dish and allowed to attach for 24 h before experiments were begun. In experiments extending beyond 2 days, medium (including experimental additives) was changed every 48 h. Stock solutions of dexamethasone, 1,25-(OH)₂D₃, 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃) (each at 0.1 mM), and RU-486 (courtesy of D. Martini, Roussel Uclaf, Romainville, France) (2 mM) were prepared in 100% ethanol, and dichloro-ribofuranosylbenzimidazole (25 mg/ml) was prepared in 100% dimethyl sulfoxide. Control cells received an equal volume of vehicle (0.1%).

RNA Preparation, Northern Blot Analysis, and RNase Protection Analysis—Total cellular RNA was prepared using a modification of the guanidinium thiocyanate-cesium chloride technique (15, 20) and quantitated by A₂₆₀. For Northern analysis, RNA was decatured and electrophoresed on a 1.0% agarose-formaldehyde gel, transferred to a nylon membrane, and prehybridized and hybridized as described (15, 20). The calcitonin probe was a 0.6-kilobase (kb) BglI calcitonin-specific fragment corresponding to exon 4 of the rat calcitonin-CGRP gene (21) (courtesy of S. S. Murray and M. G. Rosenfeld, University of California, San Diego), and the γ-actin probe was a 1.0-kb PstI XbaI cDNA fragment corresponding to the coding region of human γ-actin (22) (courtesy of C. Stolle, Yale University). The probes were labeled by the random primer technique (23). RNase protection analysis was performed as described (24, 25) using a 306-base pair PacI-SacI PTHRPs coding-region fragment subcloned into pGEM-

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3-blue (Promega Biotec) (5, 14). The antisense RNA probe (2 \times 10^5 cpm) was hybridized to 40–80 \mu g of total RNA, and protected fragments were fractionated on a 5% polyacrylamide 7 M urea gel (24, 25). Autoradiograms were quantitated using a computer-assisted scanning densitometer (Hoefer model GS 300).)

**Nuclear Run-off Transcription Assay**—Nuclei were isolated and the transcription reaction carried out essentially as described (26, 27). Run-off transcripts (4 \times 10^5 cpm) were hybridized for 3 days at 42°C to target DNA (5 \mu g each) applied to nylon membranes using a slot-blot manifold. The target DNA included (a) pGEM-3-blue vector to determine background hybridization, (b) the human \gamma-actin fragment (22) and a 0.8-kb BamHI coding region fragment of rat cyclophilin (28) (courtesy of F. Van Itallie, Yale University) as internal controls, (c) a 0.8-kb EcoRI cDNA fragment corresponding to the common region and calcitonin sequences of the human calcitonin/CGRP gene (courtesy of S. S. Murray and M. G. Rosenfeld) (29), and (d) a 0.9-kb NarI fragment corresponding to exons 1, 2, and 3 of the human PTHRP gene (10). Prehybridization, hybridization, and washing conditions were as described previously (15, 20).

**RESULTS**

We have previously reported that poly(A)^+ RNA prepared from TT cells contains the major 1.5- and 2.1-kb PTHRP mRNA species (15, 19) that have been identified in RNAs from a variety of human tumors and normal tissues and/or cells (5, 15, 20). Because of the low abundance of these transcripts in TT cells, an RNase protection assay was employed to study the effects of dexamethasone and 1,25-(OH)\_2D\_3 on steady-state PTHRP mRNA levels in these cells. Both steroids have been previously shown to influence steady-state calcitonin mRNA in TT cells (17, 18), and these responses were used in the present experiments as positive controls. Calcitonin mRNA is abundant in TT cells and was analyzed by Northern blotting of total RNA.

We first examined the time course of the effects of 100 nM dexamethasone (Fig. 1) and 100 nM 1,25-(OH)\_2D\_3 (Fig. 2) on PTHRP and calcitonin mRNA levels in TT cells. As shown in Fig. 1, dexamethasone decreased steady-state PTHRP mRNA levels, with a clear-cut reduction being seen as early as 6 h and a maximal and sustained response by 4–8 days. As anticipated, dexamethasone increased calcitonin mRNA levels, with a major effect first seen at day 4 and beyond (17). Based on densitometric scanning, the maximal responses (4–8 days) were an 80% reduction in PTHRP mRNA and a 2.5-fold increase in calcitonin mRNA.

As shown in Fig. 2, 1,25-(OH)\_2D\_3 treatment decreased both PTHRP and calcitonin mRNA levels in TT cells. The reduction in PTHRP mRNA was observed as early as 6 h and was maintained throughout the 8-day experiment (Fig. 2). There were modest changes in the level of calcitonin mRNA at the early time points, but a pronounced effect was not apparent until day 4, in agreement with previous findings (18). By densitometric scanning, the maximal decreases were about 40–50% for each mRNA. Neither dexamethasone nor 1,25-(OH)\_2D\_3 influenced \gamma-actin mRNA, analyzed as an internal control (Figs. 1 and 2). Poly(A)^+ RNA was prepared from selected samples and analyzed by Northern analysis using a coding region PTHRP RNA probe, confirming that equivalent reductions in the 1.5- and 2.1-kb PTHRP mRNA species were observed in response to both dexamethasone and 1,25-(OH)\_2D\_3 (data not shown).

As shown in Fig. 3, the effects of both agents were dose-dependent. When included at a 10-fold molar excess, the glucocorticoid antagonist RU-486 (30) completely blocked the response to 100 nM dexamethasone, whereas 1 \mu M RU-486 by itself has no influence on the level of PTHRP mRNA (data not shown). At 100 nM, 24,25-OH\_2D\_3 had no effect on PTHRP mRNA (not shown).

We next explored the mechanism of the dexamethasone- and 1,25-(OH)\_2D\_3-induced decreases in steady-state PTHRP mRNA levels. The stability of PTHRP mRNA was examined in control cells and in cells exposed to 100 nM dexamethasone or 1,25-(OH)\_2D\_3 using the RNA polymerase II inhibitor dichloro-ribofuranosylbenzimidazole (DRB) (31). In this experiment, 40 \mu g of total RNA from control cells and 80 \mu g from steroid-treated cells were analyzed in attempt to generate comparable PTHRP signals at the time of DRB addition. As shown in Fig. 4, PTHRP mRNA turned over rapidly in TT cells, with an estimated half-life in control cells of about 3 h. Neither dexamethasone nor 1,25-(OH)\_2D\_3 appeared to influence the PTHRP mRNA disappearance rate (Fig. 4).

To address directly whether the effects of dexamethasone and 1,25-(OH)\_2D\_3 reflected control at a transcriptional level, we performed nuclear transcription run-off assays. Cells were exposed to medium containing vehicle, 100 nM dexamethasone, or 100 nM 1,25-(OH)\_2D\_3 for 24 h, and 32P-labeled nascent transcripts were prepared from isolated nuclei and hybridized...
plotted as the fraction (percent) of the mRNA remaining at each cells harvested in guanidinium solution at the times indicated. Total of calcitonin mRNA did not decline appreciably during the 6 h of this mRNA has a half-life of about 15 h in TT cells (17) (data not shown). Densitometric analyses of the films are shown to the right, DRB treatment, in agreement with previous results indicating that to a membrane bearing immobilized target DNA sequences.

FIG. 4. Lack of an effect of dexamethasone or 1,25-(OH)_{2}D_{3} on PTHRP mRNA stability. TT cells were pretreated with medium plus vehicle (control), dexamethasone (100 nM), or 1,25-(OH)_{2}D_{3} (100 nM) for 4 days, following which DRB was added (25 μg/ml) and the cells harvested in guanidinium solution at the times indicated. Total RNA (40 μg for control cells and 80 μg for steroid-treated cells) was analyzed by RNase protection assay (13-h film exposure). The level of calcitonin mRNA did not decline appreciably during the 6 h of DRB treatment, in agreement with previous results indicating that this mRNA has a half-life of about 15 h in TT cells (17) (data not shown). Densitometric analyses of the films are shown to the right, plotted as the fraction (percent) of the mRNA remaining at each time point.

FIG. 5. Influence of dexamethasone and 1,25-(OH)_{2}D_{3} on PTHRP and calcitonin gene transcription. 

\[ \text{ACTIN} \]
\[ \text{CYCLOPHILIN} \]
\[ \text{pGEM} \]
\[ \text{CALCITONIN} \]
\[ \text{PTHRP} \]

\text{Control}
\text{Dexamethasone}
\text{1,25-(OH)_{2}D_{3}}

\text{DISCUSSION}

The human PTHRP gene is a complex transcriptional unit spanning more than 15 kb of genomic DNA (10–12). The gene appears to use two promoters, neither bearing a structural resemblance to the PTH gene promoter in any species or containing perfect consensus hormone response elements (10–12, 32, 34). In addition, the gene contains alternatively spliced 3' exons which give rise to three PTHRP mRNA classes (4–6, 10–12, 33). The 3'-untranslated region of each of these three mRNA classes is AU-rich and shares multiple copies of an AU motif (4–6, 33) that has been found to be associated with rapid turnover of many cytokine and some protooncogene mRNAs (55, 36). Our findings indicate that PTHRP mRNAs also turnover rapidly, as had been suggested earlier by studies in vivo (14). The identification of these AU sequences in mRNAs which are constitutively expressed is unusual (35, 36), and the biological implications of the rapid disappearance of PTHRP mRNAs are unknown.

We found that both dexamethasone and 1,25-(OH)_{2}D_{3} repressed PTHRP gene transcription in TT cells. We also found that dexamethasone stimulated and 1,25-(OH)_{2}D_{3} inhibited the transcription of the calcitonin gene in these cells, as had been anticipated by earlier studies (17, 18) but not previously demonstrated using transcriptional techniques. Glucocorticoids have been shown to increase calcitonin gene transcription in a rat C-cell line (37). Oppositional effects of glucocorticoids and vitamin D on gene transcription, as typified by the calcitonin and prolactin genes (17, 18, 38, 39), appear to be the rule and inhibition (or stimulation) by both agents, as we reported here for the PTHRP gene, to be the exception. Because glucocorticoid and vitamin D receptors are widely distributed (40, 41) and the PTHRP gene appears to be widely expressed (5, 14, 15), it may be that these agents will be found to regulate PTHRP expression in a number of different tissues. The finding of transcriptional regulation of the PTHRP gene by 1,25-(OH)_{2}D_{3} is of particular interest, given the well defined effects of 1,25-(OH)_{2}D_{3} on PTH gene expression (42, 43) and the evolutionary relationship of the PTH and PTHRP genes (5, 10–12). It is also of interest to note that 1,25-(OH)_{2}D_{3} has the same directional effect on the expression of the PTH, PTHRP, and calcitonin genes (18, 42, 43). We would anticipate finding so-called negative glucocorticoid and vitamin D response elements in the 5' flanking region of the PTHRP gene, but such elements have not yet been identified by sequencing and/or transfection techniques. Negative glucocorticoid response elements are less well defined than are the so-called positive response elements (44), and negative vitamin D response elements have yet to be defined.

We have previously identified PTHRP transcripts in several established cell lines (5, 15, 19, 20) and chose to initially focus on the TT line because it represents a well studied system with a neuroendocrine phenotype (16–18). In addition to calcitonin and CGRP, human and/or rat C-cell lines have been found to produce a number of neuropeptides, including somatostatin, cholecystokinin, and thyrotropin-releasing hormone (17, 18, 45). Using in situ hybridization techniques, we have recently demonstrated PTHRP mRNA in specific neurons in several regions of rat brain. Since glucocorticoid and vitamin D receptors appear to be expressed in some of these same neurons (46, 47), it is possible that glucocorticoids and 1,25-(OH)_{2}D_{3} may regulate PTHRP gene expression in the central nervous system.

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\[ \text{REFERENCES} \]

1. M. Mangin, unpublished observations.