Transcription of the Gene for Parathyroid Hormone-related Peptide from the Human Is Activated through a cAMP-dependent Pathway by Prostaglandin E$_1$ in HTLV-I-infected T Cells*

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Human T-cell leukemia virus type I (HTLV-I) is the etiologic agent of adult T-cell leukemia (ATL), and hypercalcemia frequently associated with ATL is mediated by parathyroid hormone-related peptide (PTHRP). The present study was undertaken to clarify the role of cAMP second messenger system in the regulation of human PTHRP gene expression in ATL cells, using an HTLV-I-infected T-cell line, MT-2. Forskolin and dibutyryl cAMP (Bt$_2$cAMP) caused a marked and transient increase in the steady-state level of PTHRP mRNA. The effects of these agents were dose-dependent, and the maximal effects were observed at 3 h. Nuclear runoff transcription assay showed that forskolin and Bt$_2$cAMP caused an increase in the transcription rate of the human PTHRP gene. In contrast, the stability of PTHRP mRNA was only modestly increased by these agents. Forskolin and Bt$_2$cAMP also increased the secretion of PTHRP by MT-2 cells, as determined by both a newly established immunoradiometric assay using two antibodies against human PTHRP-(1-34) and PTHRP-(50-83) and a radioimmunoassay using an antibody against human PTHRP-(109-141).

Prostaglandin E$_1$ (PGE$_1$) caused a marked stimulation of intracellular cAMP production in MT-2 cells, whereas PGE$_2$ and PGF$_2$,$\alpha$ had only modest effects. The ability of these PGs to stimulate cAMP production correlated well with their ability to increase PTHRP mRNA level and the secretion of PTHRP. Indomethacin did not affect the basal level of cAMP production or PTHRP mRNA, suggesting that endogenous PG was not involved in the basal production of cAMP or PTHRP. When PGE$_1$ was given to MT-2 cells together with interleukin 2, which is another stimulator of PTHRP gene expression, PTHRP secretion was synergistically stimulated. These results suggest that the transcription of the human PTHRP gene is enhanced through a cAMP-dependent pathway by PGE$_1$ and that PGE$_1$, as well as interleukin 2, plays an important role in the overexpression of the human PTHRP gene in HTLV-I-infected T cells leading to the development of hypercalcemia in ATL patients.

Parathyroid hormone-related peptide (PTHRP) has been identified in a number of carcinomas associated with humoral hypercalcemia (1). Molecular cloning of its cDNA has demonstrated that PTHRP is a novel tumor-derived peptide of 141 amino acids that has sequence similarity to parathyroid hormone at the amino terminus with complete divergence thereafter (2, 3). The isolation and characterization of the human PTHRP gene, which exists on chromosome 12 as a single copy, has revealed its complex organization consisting of two promoter regions and alternatively spliced 3′-exons (4, 5). However, the molecular mechanism by which PTHRP gene is activated in certain malignant cells remains to be clarified.

Hypercalcemia is a frequent and lethal complication of adult T-cell leukemia (ATL) (6), which is etiologically associated with the infection of a retrovirus, human T-cell leukemia virus type I (HTLV-I) (7). We have demonstrated by in vivo (8, 9) and in vitro (10, 11) studies that hypercalcemia associated with ATL is mediated, like solid tumors, through the production and secretion of PTHRP by the leukemic cells. Thus ATL cells provide a suitable model system to study the molecular mechanism of PTHRP gene expression. The present study was undertaken to clarify the role of cAMP, one of the major intracellular second messengers, in the activation of the human PTHRP gene, using an HTLV-I-infected T-cell line, MT-2. The results indicate that the transcription of the human PTHRP gene is activated by prostaglandin E$_1$ through the stimulation of intracellular cAMP production in ATL cells.

EXPERIMENTAL PROCEDURES

Materials—[α-32P]dCTP and [α-32P]UTP were purchased from Amersham Corp. Forskolin, isobutylmethylxanthine, prostaglandin E$_1$ (PGE$_1$), PGE$_2$, PGF$_2$,$\alpha$, indomethacin, actinomycin D, and dichlororibofuranosylbenzimidazole (DRB) were provided by Yamasa Shoyu Co. (Choshi, Japan). Recombinant human interleukin 2 (IL-2) was a generous gift from Dr. Y. Etoh (Ajinomoto Co. Inc., Kawasaki, Japan). Stock solutions of forskolin, actinomycin D, and PGs were prepared in 100% ethanol and DRB in 100% dimethyl sulfoxide. Control cells received an equal volume of vehicle (less than 0.1%).

Cell Culture—MT-2 cells (provided by Dr. I. Miyoshi, Kochi, Japan) were maintained in RPMI 1640 supplemented with 10% fetal calf serum (12). For RNA experiments, cells were plated at a density of 6 x 10$^6$ cells/60-mm dish in RPMI 1640 with 0.1% bovine serum

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§The abbreviations used are: PTHRP, parathyroid hormone-related peptide; ATL, adult T-cell leukemia; HTLV-I, human T-cell leukemia virus type I; Bt$_2$cAMP, dibutyryl cyclic AMP; IRMA, immunoradiometric assay; RIA, radioimmunoassay; PG, prostaglandin; IL-2, interleukin 2; DRB, dichlororibofuranosylbenzimidazole; BSA, bovine serum albumin; bp, base pair(s); kb, kilobase(s); CRE, cAMP-responsive element.
Regulation of PTH-related Peptide Gene by PGE\(_1\) via cAMP

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RESULTS

Induction of PTHRP mRNA by cAMP—We first examined the effect of forskolin, an agent that is known to increase intracellular cAMP level, on PTHRP mRNA expression in MT-2 cells. As shown in Fig. 1, a marked and transient increase in the steady-state level of PTHRP mRNA was observed when the cells were treated with 20 \(\mu\)M forskolin under serum-free conditions. A shorter time-course experiment revealed that a clear-cut effect was observed as early as 1 h and that a maximal response occurred 3 h after the treatment (Fig. 1A). c-fos mRNA was also induced by forskolin with a maximal effect being observed at 3 h after the treatment. Based on these results, subsequent experiments were carried out at 3 h after the treatment.

As shown in Fig. 3, the effects of both forskolin and Bt\(_2\)cAMP on PTHRP mRNA were dose-dependent. The modest effects of forskolin and Bt\(_2\)cAMP on PTHRP mRNA were observed at 0.2 \(\mu\)M and 0.1 mM, and the maximal effects at 20 \(\mu\)M and 1 mM, respectively. In addition, the effects of forskolin (20 \(\mu\)M) in increasing PTHRP mRNA. Neither forskolin nor Bt\(_2\)cAMP influenced the actin mRNA level. Based on these results, subsequent experiments were carried out at 3 h after the treatment.
forksin and Bt2cAMP on PTHRP mRNA were completely abolished when RNA synthesis had been inhibited by prior treatment with actinomycin D for 10 min, suggesting a possible involvement of a transcriptional mechanism.

Mechanism of PTHRP mRNA Induction by cAMP—In order to obtain direct evidence of a transcriptional mechanism, the actual transcription rate of the PTHRP gene was measured by nuclear runoff experiments using nuclei isolated from forskolin-, Bt2cAMP-, and vehicle-treated MT-2 cells. [3P]-Labeled nascent transcripts (2 × 10^5 cpm) were hybridized to the target DNA sequences including a human PTHRP genomic fragment. As shown in Fig. 4, both forskolin and Bt2cAMP increased PTHRP gene transcription in MT-2 cells, whereas actin gene transcription was not affected by these agents.

The effects of forskolin and Bt2cAMP on the stability of PTHRP mRNA were also examined, since PTHRP mRNA contains in the 3'-untranslated regions several motifs of AUUA, which is known to confer the instability on certain agents. As shown in Fig. 5, both forskolin and Bt2cAMP increased PTHRP mRNA turnover rapidly after RNA synthesis was inhibited with the RNA polymerase II inhibitor DRB. When MT-2 cells had been pretreated with 1 mM Bt2cAMP for 40 min before the addition of DRB, PTHRP mRNA appeared to be slightly stabilized. Thus, in Bt2cAMP-treated cells, PTHRP mRNA level was modestly elevated at 0 h, remained elevated for at least 1/2 h, and then decreased quickly thereafter. Similar results were obtained with forskolin (data not shown). Although these results suggest a modest contribution of a post-transcriptional mechanism as well, taken together with the results of nuclear runoff experiments, it seems likely that the induction of PTHRP mRNA by cAMP occurs mainly at a transcriptional level.

Increase in PTHRP Secretion by cAMP—In order to determine whether cAMP increased not only the PTHRP mRNA level but also the secretion of PTHRP by MT-2 cells, PTHRP concentrations in the conditioned medium were measured by a newly established IRMA using two antibodies against synthetic human PTHRP-(1-34) and PTHRP-(50-83). Compared with the vehicle-treated control cells, forskolin and Bt2cAMP increased the secretion of PTHRP approximately 2- and 3-fold, respectively, with maximal effects being seen at 24 h (Fig. 6). In agreement with the results of RNA analysis, the effect of Bt2cAMP (1 mM) on PTHRP secretion appeared a little greater than that of forskolin (20 μM). Similar results were obtained when RIA using an antibody against the carboxyl terminus of human PTHRP was employed to measure PTHRP concentrations in the conditioned medium (Table I).

Stimulation of Intracellular cAMP Production by PGE1—In order to identify a factor(s) that could stimulate intracellular cAMP production and thus enhance PTHRP gene expression in MT-2 cells, the effects of various PGs on cAMP production were examined, since PGs, especially of the E series, are known to modulate various T-cell functions through a cAMP second messenger system (26, 27). As shown in Fig. 7, PGE1 between 10^-9 and 10^-7 M caused a marked and dose-dependent increase in cAMP production in MT-2 cells, whereas PGF2α and PGF2α at similar concentrations had only slight effects. When PGF2α was added in the presence of forskolin (20 μM) or Bt2cAMP (1 mM), the effect of forskolin or Bt2cAMP was completely abolished when RNA synthesis had been inhibited by prior treatment with actinomycin D for 10 min, suggesting a possible involvement of a transcriptional mechanism.

Mechanism of PTHRP mRNA Induction by cAMP—In order to obtain direct evidence of a transcriptional mechanism, the actual transcription rate of the PTHRP gene was measured by nuclear runoff experiments using nuclei isolated from forskolin-, Bt2cAMP-, and vehicle-treated MT-2 cells. [3P]-Labeled nascent transcripts (2 × 10^5 cpm) were hybridized to the target DNA sequences including a human PTHRP genomic fragment. As shown in Fig. 4, both forskolin and Bt2cAMP increased PTHRP gene transcription in MT-2 cells, whereas actin gene transcription was not affected by these agents.

FIG. 4. The effects of forskolin and Bt2cAMP on PTHRP gene transcription in MT-2 cells. Nuclei were isolated from MT-2 cells treated with ethanol vehicle, forskolin (20 μM), or Bt2cAMP (DBcAMP) (1 mM) for 40 min, and runoff transcripts (2 × 10^5 cpm) were hybridized to the target DNAs indicated. pGEM vector itself was used as a negative control.

FIG. 5. The effect of Bt2cAMP on the stability of PTHRP mRNA. After MT-2 cells were cultured in the absence or presence of Bt2cAMP (DBcAMP) (1 mM) for 40 min, RNA synthesis was blocked by adding DRB (20 μg/ml) at 0 h, and total cellular RNA was harvested at the times indicated thereafter and analyzed by Northern blotting.
Regulation of PTH-related Peptide Gene by PGE₁ via cAMP

FIG. 7. The effects of various prostaglandins on the production of cAMP by MT-2 cells. MT-2 cells were plated at a density of 10⁶ cells/tube and treated with the indicated concentrations of PGE₁, PGE₂, PGF₂α, or indomethacin (Indo) for 15 min. The intracellular cAMP concentrations were measured by cAMP RIA kit as described under “Experimental Procedures.” Data were expressed as mean ± S.E. (n = 4).

modest effects. Indomethacin did not affect the basal level of cAMP production, suggesting that there was very little if any endogenous production of prostaglandins in MT-2 cells.

Increase in PTHRP mRNA and Secretion by PGE₁—Since the results thus far obtained indicated that both the mRNA level and the secretion of PTHRP were increased by cAMP in MT-2 cells and that cAMP production in these cells was markedly stimulated by PGE₁, we next examined the effects of PGE₁ as well as various other PGs on PTHRP mRNA expression and its secretion. As shown in Fig. 8, PGE₁ caused a marked and transient increase in the steady-state level of PTHRP mRNA, whereas PGE₂ and PGF₂α had only modest effects. The effect of PGE₁ on PTHRP mRNA followed a similar time course to those of forskolin and Bt2cAMP, with a maximal response being observed at 3 h. PGE₁ also stimulated the secretion of PTHRP by MT-2 cells, as determined by IRMA, while PGE₂ and PGF₂α caused only a modest increase (Fig. 9). Again, indomethacin did not change the basal level of PTHRP mRNA (Fig. 8) or the basal secretion of PTHRP into the culture medium (Fig. 9). Similar results were obtained when PTHRP concentrations were measured by RIA (Table II).

Synergism between PGE₁ and IL-2 in the Stimulation of PTHRP Secretion—T-cell growth factor or IL-2 appears to also be an important stimulator of PTHRP gene expression in HTLV-I-infected T cells based on our findings that IL-2 increases PTHRP mRNA level as well as its secretion in MT-2 cells. Since IL-2 does not increase intracellular cAMP level in MT-2 cells (data not shown) and presumably utilizes another signal transduction system for its biological effects, we examined the combined effect of IL-2 and PGE₁ on PTHRP secretion. As shown in Fig. 10, PGE₁ and IL-2 acted synergistically to stimulate the secretion of PTHRP by MT-2 cells.

DISCUSSION

In the present study, we have demonstrated, using HTLV-I-infected T cells, that both the production and the secretion of PTHRP are increased by cAMP, that these effects are due mainly to the transcriptional activation of the human PTHRP gene by cAMP, and, finally, that PGE₁ is one of the agonists that stimulate the intracellular cAMP production and thus enhance PTHRP gene transcription.

Human PTHRP gene has a complex organization consisting of six exons (4, 5). The gene has two promoter regions and encodes three peptides with different carboxyl termini by alternative RNA splicing (4, 5). The present results demonstrated for the first time that agents that are known to

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TABLE II

Effects of various prostaglandins on secretion of PTHRP by MT-2 cells

| Treatment | Cell number | PTHRP secretion |
|-----------|-------------|-----------------|
|           | ×10⁶ cells  | fmol eq PTHRP-(109-141)/10⁶ cells/24 h |
| Control   | 1.79 ± 0.02 | 117 ± 4         |
| PGE₁      | 1.67 ± 0.06 | 206 ± 14        |
| PGE₂      | 1.76 ± 0.03 | 129 ± 2         |
| PGF₂α     | 1.80 ± 0.06 | 120 ± 2         |

* Significantly different from the control group.

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increase cAMP, one of the major intracellular second messengers, enhance PTHRP gene transcription in HTLV-I-infected T cells, suggesting the presence of a functional cAMP-responsive element (CRE) in the 5'-flanking region of the human PTHRP gene. CRE has been identified in the regulatory regions of a number of genes, including enkephalin (30), somatostatin (31), vasoactive intestinal polypeptide (32), chonic gonadotropin (33), and c-fos (23) with the consensus core sequence being a palindromic octanucleotide 5'-TGACGTCA-3' (34). In addition, nuclear transcription factors, which bind to CRE and confer cAMP responsiveness, have been identified (35, 36) and their structures determined by cDNA cloning (37, 38). These include CRE-binding protein (37, 38), CRE-BP1 (39), activating transcription factor (40), and Tax-responsive element binding protein (41). It has also been shown that phosphorylation of Ser 135 of the CRE-binding protein by cAMP-dependent protein kinase, protein kinase A, is critical for the transcription of cAMP-responsive genes (42, 49).

Inspection of the nucleotide sequence in the 5'-flanking region of the human PTHRP gene revealed the presence of several CRE-like motifs in the upstream promoter region as well as in exon 2 approximately 30 bp 3' of the putative downstream transcription start site (44). It is tempting to speculate, therefore, that the activation of the human PTHRP gene by cAMP is mediated through the specific interaction of a CRE-binding protein with one of these CRE-like DNA elements. Alternatively, activator protein 2, which has been shown to confer both cAMP and 12-O-tetradecanoylphorbol-13-acetate responsiveness (45, 46), may be involved since several sequence motifs similar to the activator protein 2 binding site are found in the regulatory region of the human PTHRP gene and our preliminary experiments have shown that the PTHRP gene transcription is increased by 12-O-tetradecanoylphorbol-13-acetate in MT-2 cells. It is also possible that a novel CRE and CRE-binding protein(s) may be involved in the regulation of PTHRP gene by cAMP in HTLV-I-infected T cells. Studies using a transient transfection system are under way in our laboratory to identify a cis-acting DNA sequence(s) in the 5'-flanking region of the human PTHRP gene that is responsible for the regulation by cAMP.

HTLV-I is the etiologic agent of ATL (47, 48). A nuclear phosphoprotein of 40 kDa (p40 Tax) or Tax, which is encoded by the pX region of the provirus, plays a key role in the transactivation of the viral transcription through the interaction with the direct repeats of a 21-bp enhancer in the U3 region of the 5' long terminal repeat (7). The Tax-responsive element in this enhancer has been known to share a consensus sequence with CRE, and nuclear proteins that bind directly to the 21-bp enhancer have recently been identified by molecular cloning and found to be members of the CRE-binding protein/activating transcription factor family (41). Taken together with the present results, these observations raise the possibility that a transcription factor(s) of this family may play an important role not only in the viral replication in host cells through binding to the HTLV-I long terminal repeat but also in the development of hypercalcemia through the interaction with a CRE-like sequence(s) in the regulatory region of the human PTHRP gene.

HTLV-I Tax is also known to trans-activate various cellular genes, including IL-2 receptor, IL-2, c-fos, and granulocyte-macrophage colony-stimulating factor (7). Interestingly, we have found that the IL-2/IL-2 receptor system, implicated in the proliferation of ATL cells, also increases the production and secretion of PTHRP by MT-2 cells mainly through a posttranscriptional mechanism. Although the exact mechanism underlying the intracellular signal transduction of IL-2 remains to be clarified, the data thus far available suggest that it is distinct from the cAMP second messenger system (28, 29). In fact, the present findings that IL-2 and PGE1 act synergistically to increase the production and secretion of PTHRP in MT-2 cells indicate not only that both IL-2 and PGE1 are important stimulators of PTHRP gene expression in HTLV-I-infected T cells but also that there is a cooperative interaction between the genomic action and/or intracellular signaling pathway of IL-2 and that of PGE1 in these cells.

The 3'-untranslated regions of the human PTHRP gene have multiple AU motifs, which have been shown to confer the instability of mRNA (24, 25). The present findings indicate that PTHRP mRNA turns over rapidly in HTLV-I-infected T cells and that a posttranscriptional mechanism may also be involved, although as a minor component compared with the transcriptional control, in the activation of PTHRP gene expression by cAMP.

Cyclic AMP has been shown to modulate various T-cell functions, including inhibition of T-cell receptor-mediated T-cell activation (49), and inhibition of IL-2-stimulated T-cell proliferation and gene expression (50-52). It has been reported that cAMP is an intracellular second messenger that mediates the effects of prostaglandins, especially of the E series (26, 27) and interleukin-1 (53) in T cells. In these studies, the effects of cAMP on T-cell functions appeared generally inhibitory. It is to be noted that agents that increase intracellular cAMP cause an inhibition, although modest, of cell proliferation and yet a marked stimulation of PTHRP gene expression in MT-2 cells, whereas IL-2 stimulates both the cell proliferation and PTHRP gene expression.

In conclusion, the present study suggests that PGE1 and IL-2 are stimulators of PTHRP gene expression in HTLV-I-infected T cells and thus may play an important role in the development of hypercalcemia in ATL patients. Since the observation that PGE1 activates the transcription of the PTHRP gene through a cAMP-dependent pathway indicates that the 5'-flanking region of the human PTHRP gene contains a functional cAMP-responsive element, the identifica-
tion of a cis-acting DNA sequence(s) as well as a trans-acting nuclear factor(s) seems important to understand the molecular mechanism underlying the overexpression of PTHRP gene in ATL cells.

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