Differential Expression of Parathyroid Hormone–Related Protein in Adrenocortical Tumors: Autocrine/Paracrine Effects on the Growth and Signaling Pathways in H295R Cells

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Abstract

Adrenocortical tumors (ACT) are rare and heterogeneous, but their pathogenesis is unclear. The oncoprotein parathyroid hormone–related protein (PTHrP), found in many common tumors, can regulate their growth in an autocrine/paracrine fashion through the PTH-R1 receptor. Little is known about the role of PTHrP in ACT. We monitored the synthesis of PTHrP and PTH-R1 in a series of 25 ACT: 12 adrenocortical carcinomas (ACC) and 13 adrenocortical adenomas (ACA), and investigated the effects of PTHrP(1-34) on H295R cells derived from an ACC. PTH-R1 mRNA and proteins were detected by real-time PCR and Western blotting in all the ACT samples and in H295R cells. Their concentrations did not differ significantly from one ACT to another. PTHrP mRNA was assayed by quantitative real-time PCR. It was detected in 90% of ACC, and in 10% of ACA. There was a positive correlation with the prognostic factors, McFarlane stage and Weiss score. Tissue-specific PTHrP protein processing was shown by Western blotting. Immunohistochemical staining revealed numerous, dense foci of PTHrP-containing cells in ACC, but few positive cells in ACA or normal tissue. PTHrP stimulated the growth of H295R cells, whereas a specific anti-PTHrP antibody and a PTHrP-R1 antagonist both enhanced their apoptosis. PTHrP activated both adenylate cyclase/protein kinase A and the intracellular calcium/protein kinase C pathways via PTHrP-R1. The active synthesis of PTHrP is linked to poor prognosis in ACC, in which it may act as an autocrine/paracrine factor in tumor growth and malignancy. (Cancer Epidemiol Biomarkers Prev 2008;17(9):2275–85)

Introduction

The parathyroid hormone–related protein (PTHrP) is present in many common malignancies, such as breast and prostate cancer, and in normal and malignant endocrine tissues, including pancreatic islet cells and endocrine tumors such as thyroid and ovarian tumors (1-3). Several investigators have shown that PTHrP acts as an autocrine or paracrine growth factor in malignancy, independently of its hypercalcemic effects (4, 5). PTHrP is an oncoprotein that seems to be implicated in tumor proliferation and differentiation. It is produced by tumors commonly associated with hypercalcemia, as well as nonneoplastic tissue and several endocrine glands and tumors (6). The human PTHrP gene is composed of nine exons. The products of exons 5 and 6 are present in all PTHrP transcripts, and encode the pre–pro-region and the majority of mature peptides (7). The NH2 terminus of PTHrP acts through a receptor that is common to PTH and PTHrP. The activated receptor initiates both the adenylate cyclase/protein kinase A (PKA) and the cytosolic calcium/inositol phosphate/protein kinase C pathways (8, 9). PTH and PTHrP belong to the vasoactive intestinal peptide-secretin-glucagon family of peptides, some of which, like vasoactive intestinal peptide and pituitary adenylate cyclase–activating peptide, modulate the secretory activity of the adrenal cortex in a paracrine manner. PTHrP immunoreactivity has been detected in the adult human adrenal cortex and in the adrenal cortex and medulla of human fetuses at ages 8 to 40 weeks (10, 11) PTH-binding sites have been located in the rat adrenal cortex by autoradiography, and PTH/PTHrP receptor mRNA has been found in the rat adrenal gland (12, 13). PTHrP was also detected in a case of adrenal cortical carcinoma associated with hypercalcemia (13, 14), in which the serum calcium concentration returned to normal after removal of the tumor. A secretagogue of PTHrP has been found in human normal adrenocortical cells (15), and in chicken adrenocortical cells in primary culture (16). PTHrP, like PTH, enhances the secretion of steroid hormones by human adrenocortical cells via a signaling
mechanism involving the activation of both the adenylate cyclase/PKA and PLC/protein kinase C cascades (15). The function and clinical importance of PTHrP are poorly understood, and little is known about its role in adrenocortical carcinoma (ACC) and adrenocortical adenoma (ACA).

Although PTHrP has been detected in the normal adrenal cortex, in adrenocortical tumors (ACT) and in ACC (6), no information is available on the relationship between PTHrP synthesis and the tumor phenotype. ACC is a rare tumor with a poor prognosis. The incidence of ACC has been estimated at 0.5 to 2 per million per year in adults. Symptoms may result from steroid oversecretion and/or tumor growth and metastases. The estimated 5-year survival rate is less than 30%, demonstrating the poor prognosis for this rare cancer (17, 18). However, the pathophysiology of ACTs is not well documented because very few genetic alterations have been identified in these tumors (19, 20). Nevertheless, recent progress has shed some light on the biology of ACTs, and discrete genetic markers are clearly associated with the malignant phenotype (21-23).

This study investigates the synthesis of PTHrP in benign and malignant ACTs, and evaluates its physiologic role in the ACC cell line H295R (24, 25). We used quantitative real-time PCR to assay PTHrP gene expression, and measured the PTHrP protein and its processing in tumor tissues by immunohistochemistry and Western blotting. We studied the synthesis of PTHrP and the PTHrP receptor and cell signaling pathways using H295R cells. Lastly, we investigated the function of PTHrP in cell growth and apoptosis in vitro may well involve the cyclic AMP (cAMP)/PKA and PLC/protein kinase C pathways via activation of the PTHrP receptor.

Patients and Methods

Patients. All the patients included in the study underwent surgery for a sporadic ACT. None of the 25 patients had any adrenocortical tumor–predisposing syndromes (Beckwith-Wiedemann, Carney complex, McCune-Albright, multiple endocrine neoplasia type 1, or Li-Fraumeni syndrome). The clinical data, hormonal status, and tumor stage (McFarlane classification) shown in Table 1 were assessed as previously described (19-21, 26). The study was approved by an institutional review board (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, Hôpital Cochin, Paris), and patients gave their informed consent. After surgery, all patients were examined twice a year for 2 years, and annually thereafter. Each evaluation for ACC included hormone evaluation, chest X-ray, and computerized tomography scans of the abdomen and thorax. The patients were followed until their death, their last examination, or the end of the follow-up period. The minimum and the maximum follow-up periods were 3 and 119 months.

RNA Extraction and Reverse Transcription of Total RNA. Total RNA was extracted by homogenizing 50 to 100 mg of frozen tissue or 5 x 10⁶ H295R cells with RNA-ble reagent (Laboratoires Eurobio), followed by two separate elution steps in a final volume of 30 µL by the RNAeasy MiniKit (Qiagen) according to the manufacturer’s instructions. RNA was measured using a NanoDrop ND-1000 spectrophotometer (Labtech). Reverse transcription was done in triplicate on each sample using 2 µg of total RNA, according to the Superscript III priming (Invitrogen) manufacturer’s protocol. Negative controls were prepared under the same conditions, but without reverse transcriptase. Completed reverse transcription reactions were brought to a final volume of 20 µL by adding RNase-free/DNase-free double-distilled water.

Conventional Real-time PCR. The cDNA strand was synthesized from 2 µg of total RNA using the Superscript II reverse transcriptase enzyme protocol (Invitrogen). PCR was used to measure the synthesis of the cDNAs encoding PTHrP (285 bp), S14 (140 bp), and the PTH/PTHrP receptor (486 bp; refs. 1, 7, 9).

Quantitative PCR Analysis. Quantitative PCR amplification of PTHrP and β2-microglobulin cDNAs was done with previously published primers (27). For the PTHrP common region: forward primer (5'-GTCTCAGCCGGGGCCCTCAA-3') and reverse primer (5'-GGAAGAATCGTGCCTGGTAAA-3') corresponding to exons 5/6, and having a 93-bp amplicon. For β2-microglobulin (114 bp): forward primer (5'-GATGAGTATGCCCTGGCTGTG-3') and reverse primer (5'-CAATC-CAATGCAGCAGATCT-3') were used for normalization as described by Wellmann et al. (28). The plasmids of cDNA calibrators were generous gifts from Dr. T.J. Rosol (Department of Veterinary Biosciences, College of Veterinary Medicine, and Division of Human Cancer Genetics, Department of Molecular Virology, Immunology and Medical Genetics, Ohio State University, Columbus, OH). Assays were done in duplicate in two separate PCR assays. The SYBR Green reaction mixture (10 µL; Qiagen) contained 2 µL of sample (diluted 1:10) or calibrator cDNA, 1.2 µL of 25 mM MgCl₂, 0.5 µL of each primer (10 pmol/µL) for PTHrP or β2-microglobulin, 1 µL of DNA Master, and 4.2 µL of water. The mixture was run in a LightCycler apparatus (Roche Diagnostics) using QuantiTect SYBR Green PCR mixture (Qiagen), according to the manufacturer’s protocol. The Hot Start Taq DNA polymerase was activated at 95°C for 10 min, and 40 cycles were then run as follows: 95°C for 15 s, 58°C for 7 s (PTHrP) or 57°C for 7 s (β2-microglobulin), and 72°C for 15 s for extension synthesis. Total RNA from breast cancer cell lines was used as negative and positive control for PTHrP mRNA amplification (9). A melting curve analysis was routinely done (55-95°C) to verify the specificity of the PCR products (27, 28). Calibration curves were log-linear over the quantification range with correlation coefficients (r) of ≥-1 to (r) ≥-0.99, and slopes from -3.567 (β2-microglobulin) to -3.331 [PTHrP (1-139), common region], respectively. These indicated comparable PCR amplification efficiencies. The resulting cDNAs produced single bands on agarose gels of the expected size for β2-microglobulin (114 bp) and for PTHrP (1-139; common region = 93 bp; data not shown). Data were normalized using the
ratio of the PTHrP cDNA to that of β2-microglobulin cDNA to correct for differences in the amounts of RNA in samples.

Cell Culture. The human ACC cell line H295R was derived from a female patient aged 48 years who presented with weight loss, acne, facial hirsutism, a large ACC tumor (2,002 g), high steroid secretions (glucocorticoids, androgens, and mineralocorticoids), and metastasis. H295R is a pluripotential cell line that produces corticoids, androgens, and mineralocorticoids, and metastasizes. H295R cells were cultured for 48 h, then incubated with PTHrP(1-34), (Asn 10, Leu11, D-Trp12)-PTHrP(7-34) antagonist, or anti-PTHrP antibody. The medium was changed and fresh drugs added every 48 h. To determine the effects of antiproteasome, H295R cells were cultured for 48 h, then incubated for 20 min, 6 h or 24 h in fresh medium containing the proteasome inhibitor, MG-132 (25 μmol/L of cAMP to ensure activation of PKA. Bovine PKA (Promega) was used as a control for PKA activity, and protein kinase inhibitor (Sigma) was used as a specific PKA inhibitor. The PepTag A1 peptide substrate was subjected to electrophoresis for 20 min in 1% agarose gels, and the separated bands were photographed using a phosphoimager. The intensities of the bands were analyzed with Gene tools analysis system ver. 0.1 (1).

PKA Assay. The PepTag nonradioactive Protein Kinase Assay Kit (Promega) was used to measure the activity of PKA, as recommended by the manufacturer. The reaction buffer (supplied with the PepTag kit) contained 1 μmol/L of cAMP and 50 μmol/L of Fura-2/AM for 4 h with excess antigen (PTHrP 1-34 peptide; 100 μg/mL) at 4°C. The signals were digitized with the GeneTool analysis system ver. 0.1 (1).

Western Blotting. Frozen tissue samples were ground in liquid nitrogen, and the proteins extracted (1). The protein concentration was measured using the Bio-Rad assay, and equal amounts were loaded onto 15% SDS gels for electrophoresis. The separated proteins were electrophoretically transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences; ref. 1). These membranes were then incubated overnight at 4°C with the appropriate primary antibody: rabbit polyclonal (Biovalley) and mouse monoclonal (IDS) anti-PTHrP antibody (1-34) against PTHrP(1-34) (1/100), or mouse monoclonal anti-PTH/PTHr-P receptor antibody (1/100; Santa Cruz Biotechnology). An anti-β-actin antibody (1/500) was used for standardization. The antigen-antibody complexes were visualized using appropriate secondary antibodies (Santa Cruz Biotechnology), and the chemiluminescence detection system ECL kit (Amersham). Negative controls were obtained by incubating the anti-PTHrP antibody for 4 h with excess antigen (PTHrP 1-34 peptide; 100 μg/mL) at 4°C. The signals were digitized with the Gene Tool analysis system ver. 0.1 (1).

Table 1. Clinicopathologic features of 25 patients with sporadic ACTs

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (y)/sex</th>
<th>Clinical presentation</th>
<th>Functional status</th>
<th>Tumor weight (g)</th>
<th>Tumor size (cm)</th>
<th>McFarlane staging</th>
<th>Weiss score</th>
<th>Follow-up periods (mo)</th>
<th>Metastasis localization</th>
<th>Death</th>
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<tbody>
<tr>
<td>1 (ACC)</td>
<td>37/F</td>
<td>Tumor syndrome</td>
<td>GC + A + MC</td>
<td>1900</td>
<td>24</td>
<td>4</td>
<td>9</td>
<td>14</td>
<td>Liver/lung</td>
<td>Yes</td>
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<tr>
<td>2 (ACC)</td>
<td>42/F</td>
<td>Virilization</td>
<td>GC + A</td>
<td>68</td>
<td>5,5</td>
<td>2</td>
<td>4</td>
<td>12</td>
<td>No</td>
<td>No</td>
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<tr>
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<td>63/M</td>
<td>Cushing syndrome</td>
<td>GC + A + MC</td>
<td>135</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>1</td>
<td>Liver</td>
<td>Yes</td>
</tr>
<tr>
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<td>44/F</td>
<td>Virilization</td>
<td>GC + A + MC</td>
<td>1738</td>
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<td>2</td>
<td>5</td>
<td>119</td>
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<td>No</td>
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<tr>
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<td>15/F</td>
<td>Virilization</td>
<td>GC + A</td>
<td>22</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>47</td>
<td>No</td>
<td>No</td>
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<tr>
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<td>39/M</td>
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<td>GC + A</td>
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<td>11</td>
<td>2</td>
<td>3</td>
<td>26</td>
<td>Liver</td>
<td>Yes</td>
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<tr>
<td>7 (ACC)</td>
<td>59/F</td>
<td>Incidentaloma</td>
<td>GC + A + MC</td>
<td>16</td>
<td>2</td>
<td>6</td>
<td>13</td>
<td>Liver/lung</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>8 (ACC)</td>
<td>26/F</td>
<td>Virilization</td>
<td>A</td>
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<td>13</td>
<td>2</td>
<td>7</td>
<td>45</td>
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<td>No</td>
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<tr>
<td>9 (ACC)</td>
<td>53/F</td>
<td>Tumor Syndrome</td>
<td>NS</td>
<td>256</td>
<td>10</td>
<td>2</td>
<td>6</td>
<td>35</td>
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<tr>
<td>10 (ACC)</td>
<td>73/M</td>
<td>Tumor Syndrome</td>
<td>GC</td>
<td>620</td>
<td>12</td>
<td>4</td>
<td>8</td>
<td>1 Liver/lung</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>11 (ACC)</td>
<td>53/F</td>
<td>Tumor Syndrome</td>
<td>GC + A + MC</td>
<td>104</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>39</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>12 (ACC)</td>
<td>67/M</td>
<td>Incidentaloma</td>
<td>NS</td>
<td>330</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>26</td>
<td>Liver</td>
<td>Yes</td>
</tr>
<tr>
<td>13 (ACA)</td>
<td>68/F</td>
<td>Incidentaloma</td>
<td>NS</td>
<td>22</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>72</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>14 (ACA)</td>
<td>68/M</td>
<td>Incidentaloma</td>
<td>NS</td>
<td>30</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>73</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>15 (ACA)</td>
<td>71/M</td>
<td>Incidentaloma</td>
<td>NS</td>
<td>29</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>31</td>
<td>No</td>
<td>No</td>
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<tr>
<td>16 (ACA)</td>
<td>61/F</td>
<td>Incidentaloma</td>
<td>NS</td>
<td>45.3</td>
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<td>2</td>
<td>0</td>
<td>72</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>17 (ACA)</td>
<td>40/F</td>
<td>Incidentaloma</td>
<td>NS</td>
<td>23</td>
<td>3.3</td>
<td>1</td>
<td>0</td>
<td>54</td>
<td>No</td>
<td>No</td>
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<tr>
<td>18 (ACA)</td>
<td>44/F</td>
<td>Cushing syndrome</td>
<td>A</td>
<td>11.1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>69</td>
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<td>No</td>
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<tr>
<td>19 (ACA)</td>
<td>55/F</td>
<td>Cushing syndrome</td>
<td>GC</td>
<td>10</td>
<td>3.5</td>
<td>1</td>
<td>0</td>
<td>69</td>
<td>No</td>
<td>No</td>
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<tr>
<td>20 (ACA)</td>
<td>52/M</td>
<td>Incidentaloma</td>
<td>GC</td>
<td>40</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>No</td>
<td>No</td>
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<tr>
<td>21 (ACA)</td>
<td>49/M</td>
<td>HT</td>
<td>MC</td>
<td>10</td>
<td>1.5</td>
<td>1</td>
<td>0</td>
<td>15</td>
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<td>No</td>
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<tr>
<td>22 (ACA)</td>
<td>27/F</td>
<td>HT</td>
<td>MC</td>
<td>8</td>
<td>1.8</td>
<td>1</td>
<td>0</td>
<td>24</td>
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<tr>
<td>23 (ACA)</td>
<td>50/F</td>
<td>HT</td>
<td>MC</td>
<td>11</td>
<td>2</td>
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<td>0</td>
<td>16</td>
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<tr>
<td>24 (ACA)</td>
<td>51/F</td>
<td>HT</td>
<td>MC</td>
<td>5</td>
<td>1.5</td>
<td>1</td>
<td>0</td>
<td>12</td>
<td>No</td>
<td>No</td>
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<tr>
<td>25 (ACA)</td>
<td>65/F</td>
<td>HT</td>
<td>MC</td>
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<td>3</td>
<td>1</td>
<td>0</td>
<td>36</td>
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NOTE: Eight patients (66%) had localized tumors (McFarlane stage I and II). Four patients (33%) had disseminated tumors at diagnosis (McFarlane stages III-IV); six patients (20%) had distant metastases. Eleven patients (44%) had a Weiss score of 2, with obviously malignant tumors (stage II-IV). The patients with ACA included 13 patients ages 27 to 71 y; they were mainly (90%) females. Five patients (40%) were steroid-negative, and four patients (60%) presented with an adrenal steroid excess. Seven patients (90%) were stage I (McFarlane), with a Weiss score of 0 to 1. Abbreviations: F, female; M, male; GC, glucocorticoids; A, androgens; MC, mineralocorticoids; NS, nonsecreting; HT, hypertension.
Our research indicates that Adrenocortical Carcinoma (ACC) and Adrenocortical Adenoma (ACA) are distinct entities. Examples of ACC include the H295R cell line (lane 1) and ACA samples from lanes 14-26. Quantitative PCR analysis of PTHrP gene expression (exons 5/6, 93 bp amplicon) and β2-microglobulin gene activity (done in duplicate) for PTHrP cDNA concentration to β2-microglobulin (β2-M) mRNA ratio differences in RNA samples. The PTHrP/β2-microglobulin ratio was higher (10-fold) in carcinoma samples than in adenoma samples (P < 0.006).

We used immunohistochemistry to identify the cells containing PTHrP in six ACC samples and six ACA samples. Immunostaining techniques included PTHrP and PTH-R1 receptor antibodies with hematoxylin, and mounted in Vecta mount (Vector Clinisciences). Statistical analyses were done using the StatView 5.0 program (SAS Institute). Data were analyzed using ANOVA and regression analysis and Fisher’s projected least significant difference to compare means. Data are expressed as means ± SD. Significance was set at P < 0.05.

Results

Clinical Results. Pathologic variables of the 25 ACTs are summarized in Table 1. The patients with ACC (n = 12) were ages 15 to 73 years. The patients with ACA (n = 13) were ages 27 to 71 years. Five ACA (40%) were nonsecreting, and eight ACA (60%) produced excess adrenal steroids.

Eight patients (66%) had localized tumors (McFarlane stage I and II). Four patients (34%) had disseminated tumors at diagnosis (McFarlane stages III-IV), whereas six patients (50%) had distant metastases. Eleven patients (96%) had a Weiss score of 2, with obviously malignant tumors (stages II-IV). The patients with ACA included 13 patients ages 27 to 71 years. This group was mostly (90%) female. Five patients (40%) were steroid-negative, and four patients (60%) presented an adrenal steroid excess. Seven patients (90%) were stage I (McFarlane) with a Weiss score of 0 to 1.

Quantitative Real-time PCR of PTHrP mRNA. The PTHrP/β2-microglobulin ratio was significantly higher in the ACC samples (0.008 ± 0.014) than in the ACA samples (0.001 ± 0.001, P < 0.006; Fig. 1A). The concentrations of PTHrP mRNA were positively correlated with the markers of malignancy: McFarlane stage (r² = 0.225, P < 0.0001) and Weiss score (r² = 0.175, P < 0.004). The concentrations of PTHrP mRNA in ACC were also correlated with metastases (P < 0.05).

Immunolocalization of PTHrP in Tissue Sections. We used immunohistochemistry to identify the cells containing PTHrP in six ACC samples and six ACA samples, normal adrenal tissue, and H295R human adrenocortical cancer cells. The ACC sections contained dense staining and numerous foci of positive cells (Fig. 2A). The ACA sections contained few positive cells, and these were located mainly in the endothelial layer.
within the interstitial tissue delimiting the nodules. Normal tissue sections also contained a few cells with PTHrP in their cytoplasm/nucleus (Fig. 2A). The positive cells were mainly in the interstitial zone of endothelial cells and in vacuolated cells in the zona fasciculata. H295R cells stained positively for PTHrP in the cytoplasm or nucleus (Fig. 2B).

**Immunoblotting.** Western blotting of tissue lysates confirmed the presence of PTHrP in all tumors and in H295R cells. The band patterns were characteristic, with bands at 25, 27, and 35 kDa and at 50 and 70 kDa, respectively (data not shown), suggesting that each tumor may specifically regulate its PTHrP protein products. The same pattern was obtained for H295R lysates, except for the 70 kDa band (Fig. 2Ca). The specificity of the immunoreactive bands of pro-PTHrP (25 kDa) and pre–pro-PTHrP (27 kDa), and the 35 and 50 kDa bands produced by the endopeptidase cleavage

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**Figure 2.** PTHrP and PTH-R1 synthesis. **A,** right, immunohistochemical staining of ACT sections; positive cells (arrows): normal (N), adrenocortical adenoma (ACA), adrenocortical carcinoma (ACC). PTHrP immunohistochemistry of normal tissue showing its cytoplasmic/nuclear location in some endothelial cells and in vacuolated cells of the zona fasciculata. ACA sections with positively stained cells within the interstitial tissue delimiting the nodules. ACC sections contain extensive, dense, positively stained cells with foci in the vacuolated cells. **Left,** control sections incubated with rabbit anti-IgG showing no staining. **B,** Immunostaining of PTHrP and PTHrP receptors in the H295R cell line. The negative control was incubated with rabbit IgG for PTHrP and with mouse IgG for the PTHrP receptor (bottom). **C,** Western blot of PTHrP in H295R cells using monoclonal antibody to PTHrP(1-34). **a,** Left lane, the blot shows multiple peptide bands: 25 and 27 kDa for pre–pro-PTHrP, 35 and 50 kDa bands indicating products of proteolysis, and a faint 17 kDa PTHrP(1-34) band. **b,** Right lane, the immunoreactive bands were enhanced after treatment with the antiprotease MG132. Actin was used to assure equal loading. **D,** Western blot of the same samples incubated with anti-PTHrP antibody that had been incubated with its blocking antigen, showing the specificity of the multiple bands. **E,** Real-time PCR of the PTH/PTHrP receptor (PTH-R1) showing one 481-bp band in all the tumor samples. The ratio of PTH-R1 mRNA to S14 mRNA was not significantly different between tumors. **F,** Western blot of the PTH/PTHrP receptor showed one 75 kDa band. Nonsecreting adrenocortical adenoma (NSACA); secreting adrenocortical adenoma (SACA).
of the PTHrP, was confirmed by incubating the blots with anti-PTHrP antibody that had been incubated with blocking peptide (PTHrP 1-34; data not shown). However, the 17 kDa band corresponding to PTHrP(1-34) appeared only in lysates of H295R cells (Fig. 2Ca). We checked that PTHrP was indeed degraded by proteasomes. Blots of equal amounts of H295R proteins treated with the proteasome inhibitor (MG132) showed more intense protein bands, and particularly that of PTHrP(1-34) (17 kDa), than did blots of untreated protein (Fig. 2C). The specificity of these immunoreactive bands was confirmed by incubating the blot with anti-PTHrP antibody that has been previously saturated with blocking peptide PTHrP(1-34) (Fig. 2Cb).

**PTHrP-R1 Receptor in ACTs.** The amplified cDNA from ACCs and adenomas had similar intensities, and a single 489-bp band was revealed by agarose gel analysis. PTHrP-R1 mRNA was also found in H295R cells (Fig. 2D). Western blots showed a 74 kDa band, corresponding to the PTH/PTHrP receptor, in the lysates of all the tumors and H295R cells. However, the concentrations of PTHrP-R1 in the nonsecreting adrenocortical adenomas and the secreting adrenocortical adenomas were not significantly different (Fig. 2E).

**Intracellular Calcium.** The basal intracellular calcium concentration ([Ca$^{2+}$]i) in confluent H295R cells was 110 ± 10 nmol/L (mean ± SD, n = 6). The NH$_2$-terminal PTHrP(1-34) (10 nmol/L) triggered a transient increase in intracellular calcium concentration ([Ca$^{2+}$]i) that decreased rapidly after 15 seconds, but remained above the basal level (plateau phase, 23 ± 5%; mean ± SD, n = 4; Fig. 3Aa). The PLC inhibitor U-73122 inhibited the transient peak when added 60 seconds before PTHrP, but had no effect on the plateau phase (Fig. 3Ab). U-73343 (0.5-5 μmol/L), an inactive analogue of U-73122, had no effect (data not shown). The calcium channel inhibitor, verapamil (1 μmol/L), inhibited part of the transient peak when added 60 seconds before PTHrP; it also abolished the plateau phase (Fig. 3Ac). Thus, PTHrP increased the [Ca$^{2+}$]i via two mechanisms, one involving a calcium influx from the extracellular milieu (verapamil), and the other, a mobilization of calcium from the endoplasmic reticulum (U-73122).

**Adenylyl Cyclase and PKA Activation.** The kinase activity in cells treated with 10 to 100 nmol/L of PTHrP for 15 minutes was higher than that in untreated cells (Fig. 3Ba). Exposure of protein lysates of both controls and PTHrP-treated cells to cAMP significantly ($P < 0.0001$) increased their kinase activity (Fig. 3Bb). Incubation with protein kinase inhibitor inhibited the enhancement of PKA activity (data not shown).

**Cell Growth and Viability.** PTHrP(1-34) stimulated the growth of H295R cells in serum-free and growth factor–free medium in a dose-dependent fashion (Fig. 4A). PTHrP(1-34) increased cell proliferation from day 2 to day 5. The PTHrP-induced proliferation was

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**Figure 3.** Effects of PTHrP(1-34) on intracellular calcium and PKA activity in H295R cells. **A.** a. Direct effects of 10 nmol/L of PTHrP on intracellular calcium. Cells were loaded for 30 min with 1 μmol/L of Fura-2/AM. The response profile is a peak followed by a plateau phase. b. Cells were incubated for 1 min with 1 μmol/L of U-73122, a direct inhibitor of PLC, before adding 10 nmol/L of PTHrP(1-34). c. Cells were incubated for 1 min with 1 μmol/L of verapamil, a calcium channel inhibitor, before adding 10 nmol/L of PTHrP(1-34). **B.** PKA activity: the PKA activity in H295R cells incubated for 15 min with PTHrP(1-34) (10 nmol/L; n = 3), and cells not incubated with PTHrP shown at time 0, without (a) and after exposure to cAMP (b). Cells exposed to PTHrP have a higher PKA activity than untreated cells (**, $P < 0.001$).
neutralized by the receptor antagonist PTHrP(7-34) (100 nmol/L) and anti-PTHrP antibody (0.5 μg/mL) when added with PTHrP(1-34) (100 nmol/L) for 48 hours (Fig. 4B).

Effects of PTHrP, Anti-PTHrP Antibody, and PTH-R1 Antagonist on Apoptosis. The H295R cells were incubated with a potent, specific PTH/PTHrP receptor antagonist, PTHrP(7-34) (Asn10, Leu11, D-Trp12), or an anti-PTHrP(1-34) antibody recognizing all the NH2-terminal–containing PTHrP peptides to further show that endogenous PTHrP is also required to maintain increased cell proliferation. Cells were analyzed for apoptosis by Annexin V incorporation. Incubation with PTHrP reduced the percentage of apoptotic cells (Fig. 5B) compared with control-depleted medium (Fig. 5A). Incubation with PTHrP(7-34) (1 μmol/L) or anti-PTHrP antibody (1-2.5 μg/mL) induced apoptosis on day 5 (Fig. 5C and D). Incubation of cells with PTHrP(1-34) (100 nmol/L) plus either the PTHrP(7-34) or anti-PTHrP antibody reduced the percentage of apoptotic cells (Fig. 5C and D).

Effects of PTHrP, Anti-PTHrP Antibody, and PTH-R1 Antagonist on Cell Cycle Distribution. Flow cytometry analyses corroborated the observation that PTHrP increased cell proliferation. There was no change in the cell cycle distribution after 48 hours of treatment (data not shown). The number of cells in S phase was increased on day 5 of treatment with PTHrP(1-34), whereas the number of cells in G1 phase was decreased (Fig. 5E), followed by an increase in the number of cells in S phase on day 7 (PTHrP, 65.30 ± 0.01; controls, 57.14 ± 0.57; P < 0.01; Fig. 5F). Thus, the proliferative profile of the cells incubated with PTHrP (65.30 ± 0.01) was similar to that of cells cultured in normal medium (75.05 ± 1.2; Fig. 5F). PTHrP-R1 antagonist and anti-PTHrP antibody also decreased the number of cells in G1 phase and increased the number of cells in S phase at day 5 (Fig. 5D). However, in contrast to PTHrP(1-34) treatment, cells incubated with PTHrP(7-34) or anti-PTHrP antibody for 7 days had entered apoptosis, as indicated by the sustained accumulation of cells in S phase (PTHrP 7-34, 32.40 ± 0.03, P < 0.001; antibody, 32.07 ± 0.7, P < 0.01 as compared with controls, 25.80 ± 0.49). Similarly, the percentage of cells in G2 phase was increased (PTHrP 7-34, 24.21 ± 2.30; controls, 17.04 ± 1.04; P < 0.001), whereas the percentage of cells in G1 phase was markedly decreased (PTHrP 7-34, 43.41 ± 2.06; antibody, 50.05 ± 0.5; controls, 57.14 ± 0.57; P < 0.001). Adding PTHrP(1-34) to either PTHrP(7-34) or antibody-treated cells caused them to enter the G1 phase on days 5 and 7 (Fig. 5E and F). A similar cell cycle profile was obtained when H295R cells were triggered to apoptosis by 5 ng/mL of transforming growth factor (data not shown).

Discussion

This study examines the relationship between the concentration of PTHrP mRNA and clinical-pathologic variables of human ACC and adrenal adenoma by quantitative real-time PCR. All the tumors contained the PTHrP 93-bp amplicon (common exon VI), but there were significantly more in the ACCs and the H295R cells than in the adenomas. The highest PTHrP mRNA concentration was correlated with the usual criteria of aggressive malignancy and elevated steroid secretion. And differences in the immunolocalization of PTHrP enabled us to distinguish between ACC, ACA, and normal tissue. ACC tumors contained many cells with a dense, focal PTHrP staining, whereas the PTHrP in ACT adenoma was mainly in endothelial cells within the interstitial tissue delimiting the nodules. Normal tissue contained few PTHrP-positive cells, and these were endothelial cells and vacuolated cells in the zona fasciculata. The intense PTHrP staining observed in invasive and metastatic tumors, both pituitary and thyroid tumors, also varies with the type of tumor (5). Western blots show PTHrP processing by these tumors...
and H295R cells. The observed multiple immuno-reactive bands correspond to pro- and pre–pro-PTHrP (25-27 kDa), and to higher molecular weights in all the tissue extracts and H295R cells. However, only the H295R cell line possesses the 17 kDa band, corresponding to the PTHrP(1-34) peptide. The amino acid sequence of pro-PTHrP, a precursor of PTHrP, can serve as a substrate for the prohormone convertase furine (29). The PTHrP(1-34), detected in H295R cells, but not in adrenal tumors, is probably due to ubiquitination and to the proteasome-dependent degradation of the peptide (29, 30). PTHrP(1-34) (17 kDa) and other immunoreactive proteins accumulated in H295R cells incubated with a proteasome inhibitor. The different patterns of intensities within the tumors may be due to different processing of PTHrP by the tumors, as reported for pancreatic adenocarcinoma (31). PTHrP contains many basic amino acid motifs that allow extensive posttranslational processing before secretion; these products have short half-lives, as do many regulatory proteins, including oncoproteins.

The PTH/PTHrP receptor mRNA and protein were found in all the tumors and in the H295R cells, suggesting that PTHrP acts as an autocrine/paracrine factor, influencing proliferation and differentiation. The signaling pathway activated by PTHrP depends on the cell type. PTHrP has no effect on PKA activity in Walker 256 tumor cells or in human mammary invasive cells, but it activates the phospholipase C pathway (8, 32). Low (10 pmol/L-10 nmol/L) concentrations of PTHrP(1-34) increased the concentration of intracellular calcium in H295R, whereas higher concentrations (100 nmol/L-1 µmol/L) activated the cAMP/PKA signaling pathway. This suggests that the concentration of PTHrP is an important discriminating factor for using a particular route in these H295R human adrenocortical cancer cells. The aldosterone and cortisol responses to PTHrP are totally or partially blocked by inhibitors of adenyl cyclase or phospholipase C, when they are added alone or together to dispersed human adrenocortical cells in primary culture (15). Thus, there may be a positive association between PTHrP and steroid secretion in human adrenocortical cancers and in H295R cells, revealing another crucial role of the peptide in ACTs.

One of the main actions of PTHrP on tumors is to increase cell multiplication (8, 32). PTHrP(1-34) stimulates the proliferation of H295R cells in medium lacking both serum and growth factors in a dose-dependent fashion. The specific, competitive antagonist of the PTH/ PTHrP receptor blocks the PTHrP-induced increase in cell proliferation, showing that PTHrP promotes tumor cell survival by interacting with the PTH/PTHrP-R1. Specific anti–NH2-terminal PTHrP antibodies also neutralize endogenous PTHrP-induced cell proliferation. This corroborates data showing that reducing PTHrP concentrations with neutralizing antibodies in vivo or in vitro with PTHrP siRNA or shRNA in vitro reduces cell invasion, tumor size, and metastases (33-39). Treating H295R cells with (1-2.5 µg/mL) anti-PTHrP antibody enhances their apoptosis by day 5, revealing that the cells produce endogenous PTHrP. Adding PTHrP reverses the inhibitory effect of the antibody by reducing the percentage of apoptotic cells. This suggests that PTHrP has an antiproliferative effect on H295R cells, in agreement with its effect on cell proliferation.

Increasing cell growth and division with PTHrP(1-34) could well be associated with increases in the percentages of cells in the S and G2-M cell cycle phases. PTHrP did indeed activate the cell cycle in H295R cells and released the G1-S checkpoint, resulting in a redistribution of cells in the S and G2-M phases. Flow cytometry analyses showed that cells incubated with antibodies against PTHrP increased the percentage of cells in S phase, and decreased the percentage of cells in G1 phase, whereas the PTH/PTHrP-R1 antagonist decreased the percentage of cells in G1 phase and increased the percentage in the S-G2 phase. The anti-PTHrP antibody induced apoptosis after 5 days, probably after a premitotic block (40, 41), whereas PTHrP counteracted the effect of the anti-PTHrP antibody causing cells to enter the G1 phase after day 5. The blockade of cells in the S or G2 phase may be linked to cell cycle–regulating genes. Specific PTHrP siRNA affects the CDC2 and CDC25B cell cycle–regulating genes implicated in the progression from S-G2 phase to the mitotic phase (34). PTHrP was shown to protect cells from apoptosis by treating a human medulloblastoma with antisense PTHrP (42). PTHrP also regulates the synthesis of the antiapoptotic protein Bcl2 in HEK293 cells, and inhibits the apoptosis induced in chondrogenic cells and HEK 293 cells by TNFα (43, 44). These results indicate that PTHrP participates in the growth of H295R cells, and by extension, is involved in tumor growth.

In conclusion, the concentration of PTHrP mRNA may be used to discriminate between ACCs and adenomas. The high activity of the PTHrP gene may play a role in the development of ACCs and could be implicated in steroid hormone dysregulation and tumor metastasis. The experiments on H295R cells show that PTHrP is a key regulator of important biological activities, such as proliferation, cell cycle dysregulation, and apoptosis. PTHrP uses two signaling pathways, intracellular

**Figure 5.** Apoptosis induced by the anti-PTHrP antibody and antagonist of PTH/PTHrP receptor. Control cells cultured in depleted medium for 48 h were then incubated in normal medium (A), medium containing 100 nmol/L of PTHrP(1-34) (B), or medium containing 1 µmol/L of PTHrP(7-34), the antagonist of PTHrP receptor, or 2 µg/mL of anti-PTHrP antibody, with or without 100 nmol/L of PTHrP(1-34) (C and D). All cells were gated, and apoptosis was determined by Annexin V-FITC/IP staining. Numbers within quadrants represent percentages of live (bottom left, Annexin V-FITC/IP−), early apoptotic (bottom right, Annexin V-FITC+/IP−), and late apoptotic or early necrotic (top right, Annexin V-FITC+/IP+) cells. This is a representative experiment of three duplicate experiments. 

E. Distribution of cell cycle phases by flow cytometry multicycle analysis. Percentages of cells in G1, S, and G2 in untreated cells (Control), in cells treated with 100 nmol/L of PTHrP(1-34), with 1 µmol/L of PTHrP(7-34) antagonist or 2 µg/mL of anti-PTHrP antibody with or without 100 nmol/L of PTHrP(1-34) for 5 days or 7 days (F). The Fisher test was used for statistical analysis: a*, P < 0.01; a**, P < 0.001 compared with controls; b*, P < 0.01; b**, P < 0.001; b***, P < 0.0001 compared with PTHrP-treated cells.
cAMP/PKA and calcium/PLC in H295R cells that can be involved in the mitogenic effects of PTHrP because they are largely dependent on mitogen-activated protein kinase, whose activity can be modulated by both PKA and protein kinase C (45, 46). Further studies are needed to characterize the cascade of intracellular proteins activated by PTHrP in ACT cells leading to the activation of mitogen-activated protein kinase that in turn activates early genes involved in cell proliferation and differentiation (46, 47).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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