Parathyroid Hormone-related Protein Regulates Tumor-relevant Genes in Breast Cancer Cells*†§

Received for publication, September 26, 2005, and in revised form, March 17, 2006. Published, JBC Papers in Press, March 21, 2006, DOI 10.1074/jbc.M510527200

Angela Dittmer, Martina Vetter, Dario Schunke, Paul N. Span, Fred Sweep, Christoph Thomssen, and Jürgen Dittmer

The effect of endogenous parathyroid hormone-related protein (PTHrP) on gene expression in breast cancer cells was studied. We suppressed PTHrP expression in MDA-MB-231 cells by RNA interference and analyzed changes in gene expression by microarray analysis. More than 200 genes showed altered expression in response to a PTHrP-specific small interfering (si) RNA (siPTHrP). Cell cycle-regulating gene CDC2 and genes (CDC25B and Tome-1) that control CDC2 activity showed increased expression in the presence of siPTHrP. CDC2 activity was also found to be higher in siPTHrP-treated cells. Studies with PTHrP peptides 1–34 and 67–86, forskolin, and a PTH1 receptor (PTH1R)-specific siRNA showed that PTHrP regulates CDC2 and CDC25B, at least in part, via PTH1R in a cAMP-independent manner. Other siPTHrP-responsive genes included integrin α6 (ITGA6), KISS-1, and PAI-1. When combined, siRNAs against ITGA6, PAI-1, and KISS-1 could mimic the negative effect of siPTHrP on migration, whereas siKISS-1 and siPTHrP similarly reduced the proliferative activity of the cells. Comparative expression analyses with 50 primary breast carcinomas revealed that the RNA level of ITGA6 correlates with that of PTHrP, and higher CDC2 and CDC25B values are found at low PTHrP expression. Our data suggest that PTHrP has a profound effect on gene expression in breast cancer cells and, as a consequence, contributes to the regulation of important cellular activities, such as migration and proliferation.

PTHRP is a secretory protein that was discovered as a causative agent of hypercalcemia of malignancy (1–3). Its partial homology to parathyroid hormone allows PTHrP to activate the parathyroid hormone 1 receptor (PTH1R) (4). Activation of PTH1R in bone and kidney leads to bone absorption and renal calcium retention, respectively, inducing a rise in the blood calcium level (5). Besides the parathyroid hormone-like domain, which lies within the N-terminal part of the PTHrP protein, PTHrP contains two other functional domains, the mid-region and C-terminal domain. Post-translational cleavage of the PTHrP protein allows these domains to function independently (6–8). By activation of PTH1R, the N-terminal parathyroid hormone-like domain stimulates protein kinase A, protein kinase C, and/or the calcium-dependent pathways (5, 9, 10). Containing a bipartite nuclear localization sequence at residues 88–91 and 102–106 (11) and an importin-1-binding site at residues 66–94 (12, 13), the mid-region domain can enter the nucleus (14). In the nucleus, it can interfere with gene expression by a not yet defined mechanism (15). Nuclear traveling of the mid-region domain can be controlled by CDK1(CDC2)/CDK2-dependent phosphorylation (16). The C-terminal domain (osteostatin) is able to physically interact with β-arrestin (17), which can regulate internalization and desensitization of ligand-stimulated G-protein-coupled receptors (18). Osteostatin bears a number of phosphorylation sites that are important for the mitogenic activity of PTHrP in vascular smooth muscle cells (19).

PTHRP is expressed in almost all tissues (20) serving specific functions as an autocrine or paracrine factor (21–23). It plays an essential role in mammary gland and bone development (24–26). PTHrP is also expressed by many tumors (27). Tumors may benefit from this protein as suggested by the observation that its expression often correlates with unfavorable prognosis (28, 29). However, PTHrP expression is not always linked to decreased survival (30). By inducing local osteolysis, PTHrP has been shown to facilitate growth of metastatic breast cancer in the bone (31–33). This involves bone-borne transforming growth factor-β that, when activated by PTHrP, induces a vicious cycle by stimulating PTHrP expression in breast cancer cells (34–36). There are a number of reports suggesting that PTHrP also affects the proliferative and invasive activities of breast cancer cells and regulates their sensitivity to apoptotic stimuli (37–39). These results have been obtained by treating cells with PTHrP peptides or by overexpressing PTHrP. However, little is known about how suppression of endogenous PTHrP expression affects gene expression and behavior of tumor cells. We chose an RNA interference approach to down-regulate PTHrP expression in invasive MDA-MB-231 breast cancer cells. We found that a PTHrP-specific siRNA affected the expression of more than 200 genes, among which are some that play a role in tumor progression. Our data suggest that these genes mediate the effect of PTHrP on cell migration and growth.

MATERIALS AND METHODS

Cell Lines, siRNA, PTHrP Peptides, and Forskolin—MDA-MB-231 cells were maintained in RPMI medium (Invitrogen) supplemented with 10% fetal calf serum (Biochrom). No antibiotics were added. Small interfering RNA (siRNAs) were purchased from MWG Biotech or Dharmacon (Table 1). Cells were transfected by electroporation as described (40). Briefly, cells were trypsinized, washed once with RPMI medium (without serum), and resuspended in RPMI medium at a density of approximately 8 million cells per ml. For each transfection, 250 μl of the
**PTHrP Regulates Tumor-relevant Genes**

### TABLE 1
Primer sequences for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5' → 3')</th>
<th>Reverse primer (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC2</td>
<td>TGGAACCCAGCAAGACCTCAGC</td>
<td>GAATTTCCTTGCCCTGAGATCAT</td>
</tr>
<tr>
<td>CST4</td>
<td>GAACAGCGGTAGCTCAGAAGAA</td>
<td>AGCTAGCCTGGCTGAGATGTC</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>AATCATGACCTTTTGCCGTCG</td>
<td>TGATGAGGACCTTGGCAAGAA</td>
</tr>
<tr>
<td>HSPA1A</td>
<td>GAAGAGGCGAGAAGTTTGGCAAGACCA</td>
<td>TGATGAGGACCTTGGCAAGAA</td>
</tr>
<tr>
<td>ITGA6</td>
<td>GGCACCTAATCATCCCTGCA</td>
<td>AGCTAAGTGTCGACGACGTC</td>
</tr>
<tr>
<td>KISS-1</td>
<td>TTCTGCCCACCTGACCCCCG</td>
<td>AGGGTTCAGCTGCGGCC</td>
</tr>
<tr>
<td>PAI-1</td>
<td>GGCAGCGTGAAGGCAAGGAGA</td>
<td>GACCAACCCCTGAACGGTCG</td>
</tr>
<tr>
<td>PLAU</td>
<td>ATTCTCGACACCAAGGACTCG</td>
<td>CAGCTAAATGTCTGAAATGTC</td>
</tr>
<tr>
<td>PTHrP (exon 1C)</td>
<td>ACAATTACGCCGCCGCTGCC</td>
<td>TGACCCACACATCGAGGAGG</td>
</tr>
<tr>
<td>PTHrP (exon 2)</td>
<td>AGGAGCGGTGTTACCCCTG</td>
<td>TGACCCACACATCGAGGAGG</td>
</tr>
<tr>
<td>PTHrP (exon 4)</td>
<td>ACCCTGGAGGTGTCCCTACCAC</td>
<td>TGACCCACACATCGAGGAGG</td>
</tr>
<tr>
<td>PTH1R</td>
<td>TGCTCTCCACTCTGCCAGG</td>
<td>AGGTTGTACCCTCCCGTCCG</td>
</tr>
</tbody>
</table>

### TABLE 2
siRNA sequences

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sense strand (5' → 3')</th>
<th>Anti-sense strand (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>siLuc</td>
<td>CUACGCGUCGUACAGUCUCACATGT</td>
<td>UCGAAAGUUCAGCCAGUAGATG</td>
</tr>
<tr>
<td>siELF</td>
<td>CACAAAUUUUCGUCCAGUCCACATGT</td>
<td>UGACAAGGACCUAAGAAGATG</td>
</tr>
<tr>
<td>siPTHrP</td>
<td>UCUGUUGUCUGACGGUCACATGT</td>
<td>GUGUGGAGGAUCGUAGAAGATG</td>
</tr>
<tr>
<td>siITGA6</td>
<td>UCACCUGCCGUACCGAAGAAGATG</td>
<td>UGGUGGAGGAUCGUAGAAGATG</td>
</tr>
<tr>
<td>siPAI1</td>
<td>UGGAGCAGAUAGCTGACAGATG</td>
<td>UGUGAGGAGGAUCGUAGAAGATG</td>
</tr>
<tr>
<td>siKISS1</td>
<td>UCACACAGUGGGACGUCACATGT</td>
<td>UGGUGGAGGAUCGUAGAAGATG</td>
</tr>
<tr>
<td>siPrec</td>
<td>UGGAGGAGGAUCGUAGAAGATG</td>
<td>UGGUGGAGGAUCGUAGAAGATG</td>
</tr>
</tbody>
</table>

**cell suspension was mixed with 10–20 μl of an siRNA (250 pmol) stock solution in water or with 10–20 μl of water and electroporated by using a Bio-Rad GenePulserX-Cell (250 V, 800 microfarads). After incubation on ice for 30 min, cells were mixed with 8 ml of growth medium. For RNA or protein isolation, 3 ml of this cell suspension was transferred to a 60-mm cell culture dish (Nunc). Cells were grown for 3 days before harvesting. To analyze the effect of PTHrP peptides 1–34 and 67–86 (Bachem stock solution, 400 μM in water) on siPTHR-responsive genes, cells were incubated with 1 μM of the peptide for 18 h. Forskolin was used at final concentration of 2 or 20 μM. Cells were incubated with forskolin for 6 h. Quantitative RT-PCR—Preparation of RNA, cDNA synthesis, and PCR was carried out as described (35) with some modifications. Briefly, total RNA was isolated either by using RNeasy (Qiagen) or Nucleospin RNA II (Macherey & Nagel) according to the manufacturer’s protocol. For cDNA synthesis, 1 μg of total RNA was mixed with 1 μl of 10 mM dNTPs (Eppendorf), 1 μl of RNasin (Promega), and 1 μl of random hexamers or oligo(dT) (Amersham Biosciences) in a total volume of 13 μl and incubated at 65 °C for 5 min and quickly cooled on ice. After addition of 4 μl of 5× strand buffer and 2 μl of 0.1 M DTT, the primers were allowed to anneal to the RNA at 25 °C for 2 min. cDNA synthesis was achieved by addition of 1 μl of Superscript II (200 units/μl) Invitrogen) and sequential incubation at 25 °C for 10 min and at 42 °C for 50 min. The reaction was stopped by keeping the mixture at 70 °C for 15 min. Quantitative PCRs were performed by using ABSolute QPCR SYBR Green Fluorescin mix (ABgene). To 10 μl of SYBR Green mix, 2.5 μl of each primer (5 pmol), 2 μl of cDNA (1:20 diluted), and 3 μl of water were added, and the mixture was run in a Bio-Rad iCycler. After activation of the polymerase at 95 °C for 15 min, 40 cycles were run as follows: denaturing at 95 °C for 15 s, annealing at 60 °C for 1 min, and synthesis at 72 °C for 1 min. Each sample was analyzed in duplicate. The results were analyzed by using the iCycler iQ Optical System software version 3.1 (Bio-Rad). The relative RNA level of each gene of interest was calculated by the comparative C_{t} (2^{−ΔΔC_{t}}) method. GAPDH was used as a reference gene for normalization. The calibrator sample was either the cDNA obtained from cells that were treated with siLuc or with no siRNA. For gene expression comparison in tumor samples, normalized C_{t} values were used.**

Except for the CDC25B primers, all primers were purchased from MWG Biotec and are listed in Table 2. The CDC25B primers (QT00028350) were obtained from Qiagen and were used according to the manufacturer’s protocol.

**Microarrays—For microarray analyses, Affymetrix probe array type HG-U133A was used. The analyses were carried out as described (40). For preparation of biotin-labeled cRNA and hybridization, the manufacturer’s protocol was strictly followed. Briefly, 1–8 μg of total RNA was first reverse-transcribed by using a T7-oligo(dT) promoter primer in the first-strand cDNA synthesis reaction. Following RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA was purified and transcribed into cRNA in the presence of T7 RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mixture. The biotinylated cRNAs were then cleaned up and fragmented. The fragmented, biotinylated cRNA (50 ng/μl) was hybridized to an HG-U133A GeneChip expression array in hybridization buffer (100 mM MES, 1 M NaCl, 20 mM EDTA, 0.01% Tween 20) containing 50 pm of a control oligonucleotide, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml bovine serum albumin, 10% dimethyl sulfoxide for 16 h at 45 °C. After stringent washings, hybridized biotinylated cRNAs were detected by streptavidin-phycoerythrin. Microarray analyses were performed with RNAs from three independent sets of transient transfection experiments. Only those genes that showed a detection p value of less than 0.05 (one-sided Wilcoxon’s rank test) and whose expression was similarly changed in all three comparative analyses (p < 0.05, t test) were considered to be siPTHrP-responsive genes.**

**Protein Extraction, Western Blot Analyses, uPA/PAI-ELISA, and Antibodies—Transfected cells grown for 3 days in a 60-mm plate were scraped off the plate and harvested by centrifugation. For extraction of cytosolic proteins, cells were resuspended in 150 μl of 250 mM Tris·HCl (pH 7.5) and lysed by three cycles of freezing and thawing. The lysate was cleared by centrifugation in a microcentrifuge at full speed for 10 min at 4 °C. Nuclear protein extraction was done as described (35). Briefly, after harvest, cells were resuspended in 400 μl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1...**
mm DTT) and kept on ice for 15 min. After addition of 25 μl of Nonidet P-40, the cell suspension was vortexed for 10 s and centrifuged for 30 s in a microcentrifuge at full speed. The pellet was resuspended in 100 μl of buffer C (20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) and rocked for 15 min at 4 °C. After centrifugation for 10 min, the supernatant was collected and stored at −80 °C. Extraction of plasma membrane proteins was carried out as described (41). Briefly, cells were resuspended in buffer A as described above and homogenized by five passes through a 20-gauge needle. The supernatant was stepwise centrifuged at 3000 (600 x g) and 6500 rpm (3500 x g) and then full speed in a microcentrifuge for 10 min each. The pellets after the first two centrifugations were discarded. The pellet of the last centrifugation contains the plasma membrane proteins and was dissolved in buffer D (5 mM HEPES (pH 7.9), 0.5 mM K-EDTA (pH 7.2), 1 mM DTT).

Western blot analysis was performed as described (35) with some modifications. Briefly, proteins were separated in a 10% gel by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Millipore). After blocking with 3% bovine serum albumin (fraction V; AppliChem) in washing buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA) for 20 min at room temperature, the membrane was incubated with the primary antibody in washing buffer containing 5% bovine serum albumin and 0.05% Tween 20 for 1 h at room temperature. In the case of the anti-CTNNB1 antibody, incubation was done overnight at 4 °C. The membrane was washed three times in washing buffer plus Tween 20 for 5 min each and incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (Cell Signaling or Amersham Biosciences) for 1 h at room temperature followed by three washes in washing buffer plus Tween 20 for 20 min each. Peroxidase activity was visualized by chemiluminescence using ECL Plus (Amersham Biosciences) followed by exposure to Hyperfilm ECL (Amersham Biosciences). Rabbit polyclonal antibodies recognizing CDC2, phospho-Tyr-15-CDC2, ERK1/2, phospho-Thr-202/Tyr-204-ERK1/2, and anti-rabbit peroxidase conjugates were purchased from Cell Signaling Technology (dilution 1:1000 each). Murine anti-CTNNB1 (1:2000) was from BD Transduction Laboratories. Anti-GAPDH (1:10,000; Ambion) was used to check for equal loading of cytosolic and nuclear proteins (42, 43). For detection of uPA and PAI-1, an enzyme-linked immunosorbent assay (ELISA) was used (Femtelle uPA/PAI-1 Measurement kit III; Roche Applied Science). Cells were washed with phosphate-buffered saline, and adherent cells were lysed in 200 μl of chloromphenicol acetyltransferase ELISA lysis buffer (Roche Applied Science) supplemented with 0.25% SDS. After 10 min of incubation, the lysate was transferred to a microtube and centrifuged for 10 min at full speed. The protein contents of the samples were determined by a BCA protein assay (Pierce) by following the instructions of the manufacturer. The protein content was used as a representative measure of the cell number. To determine the rate of DNA synthesis, siRNA-transfected cells (initial cell number, 5 ⋅ 10³/well) were grown in a 24-well plate for 3 days after which the medium was replaced by 250 μl of fresh medium/supplemented serum supplemented with 10 μM 5-bromo-2'-deoxyuridine (BrdUrd). After incubation for 2.5 h, cells were lysed and treated with nuclease, and BrdUrd was detected by anti-BrdUrd peroxidase conjugate essentially as described in the manufacturer’s protocol (BrdUrd labeling and detection kit III; Roche Applied Science).

Migration and Invasion Assays—For wound healing assays, cells transfected with siRNA were grown on 60-mm plates for 3 days before a gap was introduced into the monolayer cells by dragging a yellow tip across the cell surface. The phase contrast images of the wounds were recorded after 0, 24, and 48 h, and the wound closure was evaluated by measuring the width of the remaining wound. Cell migration was also studied by seeding transfected cells (2 × 10⁵) on a tissue culture insert (pore size, 8 μm; ThinCerts, Greiner Bio-One) inserted into a well of a 6-well plate and allowing them to migrate through the pores. After 48 h, the number of cells on the underside of the insert was determined by microscopic counting (15 fields/insert). For analyzing invasion, Matrigel invasion chambers (BD Biosciences) were used instead. The percentage of invasive cells was determined by comparing the number of cells that were able to pass Matrigel with those that migrated through the pores in the absence of Matrigel.

Measurement of Proliferation—To measure cell growth, 5 × 10⁵ or 10⁴ siRNA-transfected cells were seeded to each well of a 24-well plate and incubated for 3 days. Cell layers were washed with phosphate-buffered saline, and adherent cells were lysed in 200 μl of chloromphenicol acetyltransferase ELISA lysis buffer (Roche Applied Science) supplemented with 0.25% SDS. After 10 min of incubation, the lysate was transferred to a microtube and centrifuged for 10 min at full speed. The protein contents of the samples were determined by a BCA protein assay (Pierce) by following the instructions of the manufacturer. The protein content was used as a representative measure of the cell number. To determine the rate of DNA synthesis, siRNA-transfected cells (initial cell number, 5 ⋅ 10³/well) were grown in a 24-well plate for 3 days after which the medium was replaced by 250 μl of fresh medium/supplemented serum supplemented with 10 μM 5-bromo-2'-deoxyuridine (BrdUrd). After incubation for 2.5 h, cells were lysed and treated with nuclease, and BrdUrd was detected by anti-BrdUrd peroxidase conjugate essentially as described in the manufacturer’s protocol (BrdUrd labeling and detection kit III; Roche Applied Science).

Breast Cancer Biopsies—Fifty tumor samples from patients with unilateral operable breast cancer who underwent resection of their primary tumor between 1987 and 1997 were selected by availability of frozen tissue in the tumor bank at the Department of Chemical Endocrinology, Radboud University Nijmegen Medical Centre (The Netherlands). Breast cancer biopsies were pulverized under liquid nitrogen, and total RNA was isolated for cDNA synthesis and quantitative RT-PCR. To compare the expression of PTHrP with that of various siPTHrP-responsive genes, Spearman rank correlations were calculated by using SPSS 11.5 software.

RESULTS

PTHRP-specific siRNA Down-regulates PTHrP in MDA-MB-231 Cells—In order to identify genes that are regulated by PTHrP in invasive breast cancer cells, we designed a PTHrP-specific siRNA (siPTHrP) to specifically down-regulate PTHrP expression. To confirm that PTHrP expression was suppressed, we performed quantitative RT-PCR. We used PTHrP exon 4-specific primers that are capable of recognizing all PTHrP transcripts. The study was carried out with the MDA-MB-231 breast cancer cell line, which is known to produce and secrete PTHrP (34, 44, 45) and which is a well established model system for PTHrP research (34, 44). When MDA-MB-231 cells were treated with siPTHrP, the PTHrP-RNA level decreased by 4–5-fold...


**PTHRP Regulates Tumor-relevant Genes**

(1). In contrast, control siRNAs (siLuc, siElf1) had no effect on PTHrP expression.

In the Presence of siPTHRP the Expression of More than 200 Genes Is Changed—To study the effect of siPTHRP on gene expression in MDA-MB-231 cells, microarrays analyses by using Affymetrix HG-U133A gene chips were carried out. The RNAs derived from cells treated with siPTHRP or siLuc in three independent transfection experiments were analyzed. The data are deposited in Gene Expression Omnibus as series GSE4292. Of the 44,928 genes and ESTs present on the chip, more than 200 genes were found to have altered expression in the presence of siPTHRP versus siLuc ($p < 0.05$; t test) (supplemental Table 3).

Although statistically significant, many siPTHRP-induced changes in expression were in the range of 3-fold or less, and in some cases, variations between individual measurements were large. Among the genes that responded to siPTHRP were four annexin genes and four metallothionein genes. In addition, many genes involved in ubiquitination and proteasome-dependent degradation were affected by siPTHRP. Furthermore, the expression of four cell cycle-regulating genes (CDC2, CDC25B, GRCC8, and CKS2) were induced by siPTHRP. Interestingly, three of these genes (CDC2, CDC25B, and GRCC8) are important for G1/M transition. CDC2 (CDK1) forms complexes with cyclin B to initiate entry to mitosis (46). More importantly, CDC2 is overexpressed in many breast cancer cells (47). CDC25B (cell division cycle 25B) activates the CDC2-cyclin B complex by dephosphorylating CDC2 (48, 49). GRCC8 (Tome-1, trigger of mitotic entry 1) indirectly activates CDC2 by inducing the degradation of wee-1 (50), an inhibitor of the CDC2-cyclin B complex. GRCC8 is an F-box protein. F-box proteins direct the SCF (Skp1,Cdc53/cullin1, F-box protein) ubiquitin-ligase complex to specific targets to initiate their proteolysis via a proteasome-dependent mechanism (51). GRCC8 targets the SCF complex to wee-1. Another siPTHRP-responsive F-box protein-encoding gene was found to be FBXO7 (F-box protein only 7 or FBX7). FBXO7 specifically recognizes and induces the degradation of HURP (hepatoma up-regulated protein), a mitotic protein that is overexpressed in a variety of cancer types, including breast cancer (52). Interestingly, FBXO7 requires phosphorylation by CDC2 to be able to interact with HURP (52). The siPTHRP-responsive CKS2 gene is thought to be important for targeting CDK-cyclin complexes to certain substrates (53). Thus, siPTHRP not only interferes with CDC2 expression but also modulates the expression of CDC2-regulatory genes and genes that are CDC2 substrates. In the light of these results it is intriguing that PTHrP itself is a CDC2 substrate (16).

Also of interest were siPTHRP-responsive genes that play a role in tumor progression. Among these are integrin α6 (ITGA6), catenin β1 (CTNNB1), KISS-1 metastasis suppressor gene (KISS-1), plasminogen activator inhibitor 1 (SERPINE1, PAI-1), and uPA (PLAU). siPTHRP down-regulated the expression of ITGA6, KISS-1, and PAI-1 by 4.5-, 6.5-, and 3.8-fold and up-regulated that of CTNNB1 and uPA by 3.7- and 2.9-fold, respectively.

To verify the microarray data, we performed quantitative RT-PCR. A number of selected genes (CDC2, CDC25B, ITGA6, CTNNB1, KISS-1, uPA, PAI-1, HSPA1A, and CST4) were chosen for this analysis. In all cases, the RT-PCR data confirmed those obtained by microarray analyses, except that fold changes were generally lower in RT-PCR-based RNA measurements (Fig. 2).

### siPTHRP Affects uPA/PAI-1 Protein Expression and CDC2 Activity

Next we studied whether the siPTHRP-dependent changes in RNA levels are translated into alterations in protein expression. uPA and PAI-1 protein levels were determined by ELISA. Being a secretory protein, PAI-1 protein was not only detected in cytosolic extracts but also in the medium. Like PAI-1 RNA levels, PAI-1 protein levels decreased in response to siPTHRP by ~2-fold (Fig. 3A). In contrast to PAI-1, uPA protein was not present in the medium (data not shown), and only little uPA protein could be detected in the cytosolic extract (Fig. 3B). However, high amounts of uPA were found in the plasma membrane fraction, which is in accordance with the notion that uPA is complexed with the uPA receptor on the cell surface (54). The uPA protein level in siPTHRP-treated cells was more than 2-fold higher than in control cells confirming the data obtained by RT-PCR.

Protein expression of CDC2 was studied by Western blot analysis. CDC2 levels were found to be increased in the presence of siPTHRP (Fig. 3C), which again is in agreement with the RT-PCR results. We next explored the possibility that the activity of CDC2 may also be altered in siPTHRP-treated cells. We probed the Western blots with an anti-phospho-Tyr-15-CDC2 antibody that specifically recognizes the inactive form of CDC2. Although the total amount of CDC2 was higher in siPTHRP-transfected cells, Fig. 3C shows that the level of inactive CDC2 was similar in the siLuc and siPTHRP samples. This suggests that, in siPTHRP-transfected cells, more CDC2 protein is in its active state. To quantify the difference in CDC2 activity in the presence of siPTHRP versus siLuc, we analyzed the intensities of total CDC2 and phospho-CDC2 bands by AIDA image quantitation software. The analysis revealed that siPTHRP significantly increased CDC2 activity by ~2-fold ($p < 0.05$, Student’s t test; Fig. 3D). For comparison, we treated MDA-MB-231 cells with forskolin, known to inactivate the CDC2-cyclin B complex (55). Forskolin increased CDC2 protein phosphorylation by ~2-fold without having an effect on CDC2 protein expression (Fig. 3C). To mimic a 2-fold difference, 5 and 10 μg of total protein of the same MDA-MB-231 cells were run in the same gel. Western blot analysis of this gel revealed a similar difference in phospho-CDC2 signal intensities between these samples as between the mock and forskolin samples. This suggests that the data as obtained by quantitation by AIDA software (Fig. 3D) reflect the real changes in signal intensities as seen in Fig. 3C. Collectively, these data show that siPTHRP increases both CDC2 expression and CDC2 activity. The increased CDC2 activity in the presence of siPTHRP is in accordance with the observation that siPTHRP up-regulated the expression of CDC25B and GRCC8, which both activates CDC2 directly or indirectly, respectively.
We also analyzed the effect of siPTHrP on the CTNNB1 protein level. Although siPTHrP up-regulated the RNA level of CTNNB1, it failed to affect CTNNB1 protein expression (Fig. 3C). This is likely to be due to the fact that CTNNB1 protein expression is regulated by adenomatous polyposis coli protein on a post-transcriptional level (56). This may moderate changes in RNA levels prevent from being translated into changes in protein expression.

**FIGURE 3.** Altered protein expression in the presence of siPTHrP. A and B, ELISA of PAI-1 (A) and uPA (B). MDA-MB-231 cells were transfected with either siLuc, siPTHrP, or siPAI-1, incubated for 3 days, and harvested. After centrifugation, the supernatants (medium/serum) were collected, and the cells were lysed for extraction of cytosolic and membranous proteins. Membrane and cytosolic fractions were measured for uPA protein expression, whereas medium and the cytosolic extracts were determined for PAI-1 protein levels. The background PAI-1 level in the medium (30 ng/ml) was subtracted from the medium samples (A). Values are given in either nanograms per ml (medium) or nanograms per mg (cytosolic or membranous proteins). Values are averages of two to three independent experiments. Standard deviations are indicated. C, Western blot analysis of cytosolic and nuclear extracts of siluc- or siPTHrP-transfected or forskolin- or mock-treated MDA-MB-231 cells to determine protein expression of CDC2, CTNN1B, and CDC2 phosphorylation. exp. 1–3 indicate three independent transfection experiments. D, quantitation of the intensities of the CDC2 and phospho-CDC2 bands as displayed in C. The intensity of each band was determined by AIDA image quantitation software. Relative CDC2 activity was calculated by dividing the band intensity of CDC2 by that of the inactive phosphorylated form of CDC2.

**FIGURE 4.** PTHrP regulates genes in MDA-MB-231 cells via different pathways. Confluent MDA-MB-231 cells were either treated with PTHrP peptide 1–34, PTHrP peptide 67–86, or mock (water) for 18 h or forskolin (forsk) or mock (Me2S O ) for 6 h or cells were transfected with siluc or siprec and grown for 3 days. Cell were then lysed for RNA analysis. Fold induction values are calculated by normalizing against mock or siluc, respectively. Each bar represents the average value (+ S.D.) of two to three independent transfection experiments.

**PTHRP Regulates Tumor-relevant Genes**

PTHRP Acts through Different Pathways to Modulate Gene Expression in MDA-MB-231 Cells—In order to study the mechanism underlying the siPTHrP effect on gene expression, we treated MDA-MB-231 cells with a number of agents. To determine the role of the PTH/PTHrP receptor PTH1R for the PTHrP-dependent regulation in these cells, PTHrP peptide 1–34 and PTH1R-specific siRNA siprec were used. PTHrP-(1–34) activates PTH1R while siprec inhibits this receptor by down-regulating its expression (Fig. 4). Experiments with adenylate cyclase activator forskolin were carried out to explore whether PTHrP-activated PTH1R acts through the protein kinase A/cAMP pathway to modulate gene expression (22). Mid-region PTHrP peptide 67–86 was tested to examine the importance of a PTH1R-independent PTHrP function (57). Of the genes that were analyzed, CDC2, CDC25B, and KISS-1 reacted on PTHrP-(1–34) showing an ~2-fold decreased expression in the presence of this peptide (Fig. 4). Conversely, down-regulation of PTH1R by siprec increased the RNA levels of these genes. The effects of siprec on CDC2 and CDC25B were the same as that of siPTHrP (Fig. 2) suggesting that, at least in part, PTHrP used the PTH1R pathway to activate CDC2 and CDC25B expression. In contrast, siPTHrP and siprec had opposite effects on KISS-1. This suggests that the regulation of KISS-1 by PTHrP is more complex and that a second PTHrP-induced pathway may override the PTH1R-dependent effect of PTHrP on this gene. Neither nor 20 μM forskolin did affect CDC2 or CDC25B expression, and only at high concentration did forskolin have an effect on KISS-1 RNA levels (Fig. 4). This suggests that the PTH1R-dependent effects of PTHrP on CDC2 and CDC25B are not mediated by the protein kinase A/cAMP pathway. Interestingly, although neither PAI-1 nor uPA (PLAU) responded to PTHrP-(1–34) or siprec, they both reacted to forskolin. This may imply that, in general, cAMP is not important for the PTHrP effects on gene expression in MDA-MB-231 cells.
PTHrP-(67–86) interfered with the expression of KISS-1, PAI-1, CDC25B, and CTNNB1. Interestingly, except for CDC25B, these genes responded to PTHrP-(67–86) in the same way as to siPTHrP. Collectively, the data suggest that PTHrP affects gene expression in MDA-MB-231 cells in at least three different ways as follows: one that depends on the PTH/PTHrP-receptor, a second that is sensitive to the PTHrP peptide 67–86, and a third that did not depend on PTH1R nor was sensitive to PTHrP-(67–86).

**ITGA6, CDC2, CDC25B Expression Is Linked to PTHrP Expression in Primary Breast Carcinomas**—Next, we analyzed the expression of ITGA6, PAI-1, CDC2, CDC25B, and uPA in 50 primary breast carcinomas and compared it with the expression of PTHrP. To obtain a measure of the quality of such comparative RT-PCR measurements, we first compared the level of all (exon 4-specific) transcripts of PTHrP with the levels of exon 1C- and exon 2-specific PTHrP transcripts. Exon 1C- and exon 2-specific PTHrP transcripts are generated by promoter P1/2 or promoter P3 usage, respectively. Promoters 2 and 3 have been shown to be the major promoters that drive PTHrP transcription (27, 35, 58, 59). The levels of both promoter-specific transcripts correlated well with that of all PTHrP transcripts (Fig. 5). Expression of ITGA6 also positively correlated with PTHrP expression provided that the three samples with the highest PTHrP expression are not taken into consideration. These samples show low ITGA6 expression. Without these three samples, the correlation between ITGA6 and PTHrP expression was significant by Spearman’s rank analysis. This result support the data obtained by the siPTHrP experiments where ITGA6 and PTHrP expression was down-regulated along with that of PTHrP. Although the expression levels of CDC2 and CDC25B were relatively constant over a large range of PTHrP expression, higher levels of CDC2 and CDC25B were only observed when PTHrP levels were very low. This is in accordance with the notion that PTHrP is able to down-regulate CDC2 and CDC25B.

The expression of all other siPTHrP-responsive genes that were analyzed did not show any relationship to the expression of PTHrP (data not shown). In summary, the results suggest that in breast cancer the expressions of ITGA6, CDC2, and CDC25B are linked to that of PTHrP.

**siPTHrP Changes Growth and Migration of MDA-MB-231 Cells**—The siPTHrP-responsive genes ITGA6, PAI-1, and KISS-1 have been shown to interfere with cell cycle, migration, and invasion (60, 61). This prompted us to test whether siPTHrP modulates these cellular activities and whether ITGA6, PAI-1, and KISS-1 contribute to such possible effects. For this purpose, we designed siRNAs against ITGA6, PAI-1, and KISS-1 and compared the effects of each of these siRNAs to those of siPTHrP. All three siRNAs (siITGA6, siPAI1, and siKISS1) specifically and efficiently depleted the RNA expression of its corresponding gene by 20-, 20-, and 5-fold, respectively (Fig. 6).

To study cell migration, we performed wound closure assays. In this assay, the cell layer was injured by a pipette tip, and the resettlement of
this wounded area by migrating cells was examined after 24 and 48 h. A representative result of three independent experiments is shown in Fig. 7. Mock- or siLuc-treated cells were similarly able to migrate into the wounded area. In contrast, cells transfected with siPTHrP and siPAI1 cells were less motile, whereas siITGA6 and siKISS1 cells showed the highest migratory activity. Of all siRNAs, siPAI1 had the strongest negative and siITGA6 the strongest positive effect on cell motility. When cells were transfected with siITGA6, siPAI1, and siKISS1 combined, cell migration was similar to that found in the presence of siPTHrP. Hence, the combinatorial effect of the siPTHrP-induced lower levels of ITGA6, PAI-1, and KISS-1 on cell migration could explain the diminished migratory ability of the siPTHrP-transfected cells. To further substantiate the data obtained by the wound closure assay, we examined the ability of the cells to migrate through 8-μm pore membranes. As shown in Fig. 8A, treatment with siPTHrP reduced migration by 2-fold relative to the siLuc control. A stronger decrease in migratory activity was observed with siPAI1, whereas siITGA6 and siKISS1 increased migration by 13.5- or 3.5-fold, respectively. These results confirm the data obtained by the wound healing assay.

To examine the effect of siPTHrP on cellular invasion, we compared cell migration through 8-μm pore membranes in the presence versus the absence of an artificial extracellular matrix (Matrigel). As shown in Fig. 8B, transfection of MDA-MB-231 cells with siPTHrP did not change the invasive capabilities of the cells. siITGA6 had no effect on invasion either, whereas siPAI1 and siKISS1 decreased the ability of the cells to invade Matrigel. In the presence of siPTHrP, the negative effects of PAI-1 and KISS-1 deficiency on invasiveness may have been compensated by the action of one or more of the other 200 genes that were affected by siPTHrP. These data suggest that PTHrP has no influence on invasiveness of MDA-MB-231 cells.

To study the effects of the different siRNAs on cell growth, two different dilutions of siRNA- or mock-transfected MDA-MB-231 cells were seeded on 24-well plates and were allowed to grow for 3 days. Adherent cells were lyzed, and cell growth was determined by measurement of total protein amount. In the presence of siPTHrP, siITGA6, or siKISS1, the protein amount was significantly reduced by ~20% relative to that in the presence of siLuc or absence of any siRNA (Fig. 9A). In the presence of siPAI1, protein amount was 10% lower than under control conditions. We also measured the rate of DNA synthesis by using a BrdUrd-based assay. DNA synthesis was significantly decreased in the presence of siPTHrP, siITGA6, or siKISS1, and the protein amount was significantly reduced by 2.5–4-fold (Fig. 9B). This demonstrates that siPTHrP and siKISS1, and also to lesser extent siITGA6, have similar effects on cell proliferation.

Extracellular signal-responsive kinase 1/2 (ERK1/2) is highly activated in MDA-MB-231 cells (40) and has been shown to be important for the proliferation of these cells (62). In addition, siPTHrP-responsive genes ITGA6 and KISS-1 have both been found to interfere with ERK1/2 activity (63–65). This prompted us to analyze the effect of different siRNAs on ERK1/2 activation in MDA-MB-231 cells. To measure ERK1/2 activity, Western blot analyses were performed by using a phospho-ERK1/2-specific antibody. The data show that siPTHrP, siKISS1, and siPAI1 down-regulated ERK2 phosphorylation by 2.5–4-fold (Fig. 9C and D). In contrast, a moderate effect or no effect of these siRNAs was found on ERK1. Interestingly, siITGA6 increased phosphorylation of both ERK1 and ERK2. The data may suggest that siPTHrP and siKISS1 reduced cell proliferation in part by decreasing the phosphorylation status of ERK2.

**DISCUSSION**

To identify PTHrP target genes, we have suppressed PTHrP expression in MDA-MB-231 breast cancer cells by RNA interference and gene expression analyzed by microarray analysis. More than 200 genes were affected by the PTHrP-specific siRNA suggesting that PTHrP regulates gene expression in breast cancer cells.

A number of genes (ITGA6, KISS-1, PAI-1, uPA, CTNNB1, CDC2SB,
and CDC2) that responded to siPTHrP were selected for further studies. RT-PCR analyses confirmed the microarray data showing that siPTHrP down-regulates the RNA levels of ITGA6, PAI-1, and KISS-1 and up-regulates those of uPA, CTNNB1, CDC25B, and CDC2. For uPA, PAI-1, and CDC2, we also verified the microarray data on the protein level.

A number of agents (PTHrP peptides 1–34 and 67–86, forskolin, and siPrec) were used to investigate the mechanism underlying gene regulation by PTHrP in MDA-MB-231 cells. siPrec mimicked the positive effect of siPTHrP on CDC2 and CDC25B, whereas PTHrP-(1–34) negatively affected these genes. This suggests that PTHrP regulates CDC2 and CDC25B expression, at least in part, via the PTHrP receptor. Although KISS-1 also responded to PTHrP-(1–34) and siPrec, siPrec and siPTHrP had opposite effects on KISS-1. This may be explained by the possibility that PTHrP uses several (opposing) pathways to regulate KISS-1. None of these three genes responded to forskolin suggesting that the PTH1R-dependent PTHrP effect did not rely on a rise in the cAMP level. Alternatively, PTHrP-activated PTH1R may have induced the protein kinase C pathway or increased the intracellular calcium level. Protein kinase C is constitutively activated in MDA-MB-231 rendering these cells unresponsive to phorbol ester (66, 67). Hence, it is unlikely that protein kinase C activation played a role in PTHrP-dependent gene regulation in these cells. Instead, calcium may be involved, which seems to be a preferred target of PTH1R in the more progressed breast cancer cells (10). Interestingly, calcium has been shown to have a negative effect on CDC2 by activating calcium/calmodulin-dependent protein kinase II (68).

KISS-1, PAI-1, CDC25B, and CTNNB1 were responsive to PTHrP-(67–86). CTNNB1 has been demonstrated previously to be a target of PTHrP in UMR106 cells (69). In these cells, however, CTNNB1 showed responsiveness to the PTHrP peptide 1–34 and the cAMP pathway. Although PTHrP-(67–86) is a mid-region PTHrP peptide, it is unable to travel into the nucleus because of the lack of essential sequences for importin/H9252 binding (12). Nevertheless, it has biological activities. It is thought that PTHrP-(67–86) interacts with a unique G-protein-coupled receptor different from PTH1R and induces a rise in the intracellular calcium level (57, 70). Although it has been shown in the sqcc/Y1 cancer cells that both PTHrP-(1–34) and -(67–86) can up-regulate the calcium level (57, 70), it is unlikely that this happens in MDA-MB-231 cells as PTHrP-(1–34) and PTHrP-(67–86) affected different genes (except KISS-1 and CDC25B). This suggests that in MDA-MB-231 cells, different pathways are activated by PTHrP-(1–34) and PTHrP-(67–86).

Interestingly, the effects of PTHrP-(67–86) on KISS-1, PAI-1, and...
CTNNB1 equaled the effects of siPTHrP. In contrast, PTHrP-(67–86) and siPTHrP had opposite effects on CDC25B. Depending on the gene that is targeted by PTHrP, this suggests that PTHrP-(67–86) can mimic PTHrP action or inhibit PTHrP activity. In the latter case, PTHrP-(67–86) may interfere with a component of the particular PTHrP-induced pathway that is required to regulate certain genes, such as KISS-1, PAI-1, and CTNNB1.

None of the other genes that were analyzed showed a response to any of the two PTHrP peptides or to siPtec. The observation that ITGA6 expression was not modulated by either PTHrP-(1–34) or PTHrP-(67–86) is in agreement with previous results obtained with MCF-7 cells (71). In these cells, the integrity of the mid-region domain is required for PTHrP to regulate ITGA6, suggesting that PTHrP must enter the nucleus to modulate the expression of this gene.

Collectively, these data suggest that at least three different mechanisms are involved in the PTHrP-dependent regulation of the siPTHrP-responsive genes in MDA-MB-231 cells. These include a PTHR1-dependent, a PTHR1-(67–86)-sensitive, and possibly a mid-region-dependent pathway.

A gene expression analysis with 50 breast carcinomas showed that ITGA6 expression correlated with that of PTHrP confirming the data obtained with the siPTHrP approach in MDA-MB-231 cells. In contrast to ITGA6 expression, CDC25B and CDC2 levels were higher at lower PTHrP expression suggesting a down-regulating effect of PTHrP on these genes as predicted from the siPTHrP data. These data imply that ITGA6, CDC2, and CDC25B are common targets of PTHrP in breast cancer.

siPTHrP affected migration and proliferation. ITGA6, PAI-1, and KISS-1 are known to play a role in regulation of these biological activities. The combined suppression of these genes by RNA interference mimicked the effect of siPTHrP on migration, and depletion of KISS-1 decreased cellular growth and DNA synthesis to a similar extent as siPTHrP. Interestingly, like siPTHrP, siKISS1 decreased the ERK2 phosphorylation status. A potential target of ERK2 is C/EBPβ (72), which is involved in breast cancer development (73). Interestingly, the expression of C/EBPβ was also affected by siPTHrP (supplemental Table 3). It is possible that a KISS-1-C/EBPβ pathway is involved in PTHrP-dependent regulation of proliferation.

We show here that KISS-1 is a target of PTHrP. Originally defined as a metastasis suppressor gene (74), KISS-1 expression has now been shown to correlate with unfavorable prognosis in breast cancer (75) suggesting that it can also act as a tumor promoter. We found that in MDA-MB-231 cells, siKISS1 has a positive effect on migration but a negative effect on invasion and proliferation. By using the same cell line, Martin et al. (75) reported an increase in both migration and invasion in response to siKISS1 overexpression. In contrast, exposure of NIH3T3 cells to the KISS-1 peptide reduced migration as well as proliferation (60). Therefore, it seems that KISS-1 acts differently in different cell types.

The importance of ITGA6 for tumor development has been well documented. ITGA6 interacts with the extracellular matrix proteins laminin and is involved in the regulation of cell adhesion, migration, and growth (76, 77). In addition, its expression correlates with unfavorable prognosis in breast cancer (78). However, we found that loss of ITGA6 in response to siITGA6 increased rather than decreased cellular mobility of MDA-MB-231 cells. It is important to note that, in previous experiments, that the function of ITGA6 was inhibited by antibodies and not by RNA interference. The gradual decline of ITGA6 expression in the course of the 3-day incubation with siITGA6 might have allowed the cells to compensate for the loss of ITGA6. Other integrins might now stimulate migration with even higher efficiency than ITGA6. Alternatively, the non-integrin 67-kDa laminin receptor (79) may have substituted for ITGA6 to regulate laminin-dependent migration. This laminin receptor is up-regulated in and correlates with the invasive and metastatic potential of cancer cells (79).

uPA and its inhibitor PAI-1, two important prognostic factors in cancer (80, 81), were also found to be targeted by siPTHrP. uPA is a serine-specific protease that converts inactive plasminogen into active plasmin, a protease involved in proteolysis of extracellular matrix. By regulating plasmin activation and vitronectin-dependent migration, uPA is a key player in controlling cell migration and invasion (82). PAI-1 can also affect migration independent of uPA. Depending on the cell type and the extracellular matrix protein to which it binds, PAI-1 can either promote or inhibit migration (83). Here we found that siPAI1-induced PAI-1 deficiency led to the inhibition of migration as well as invasion. In the case of MDA-MB-231 cells, this suggests that PAI-1 is required to maintain migration and invasion independent of uPA. The strong inhibitory effect of PAI-1 deficiency on migration on the one hand and the strong stimulatory effects of ITGA6 and KISS-1 deficiency on migration on the other hand may have resulted in a net inhibitory effect on migration as observed in the presence siPTHrP.

Also of particular interest is the response of CDC2 and some of its regulators to siPTHrP. CDC2/cyclin B is essential for G2/M transition and therefore for proliferation. Given the higher proliferative activity of cancer cells, it is not surprising that CDC2 expression is markedly up-regulated in breast cancer cells as compared with normal breast cells and is higher in more aggressive MDA-MB-231 cells versus less aggressive MCF-7 cells (84). Regulation of the cell cycle seems not to be the only function of CDC2. In complex with cyclin B2, CDC2 has also been shown to induce the displacement of caldesmon from actin filaments (85). This allows these filaments to be more dynamic as is required for migration (86). In addition, CDC2 regulates the degradation of the mitotic gene HURP (52) and the nuclear activity of PTHrP (16). Phosphorylation of PTHrP at Thr-85 leads to cytoplasmic retention of the PTHrP mid-region domain.

siPTHrP increased the expression of CDC2 and its regulators CDC25B and GRCC8. Both CDC2 regulators activate CDC2, either directly (CDC25B) or indirectly through initiating degradation of the CDC2 inhibitor wee1 by GRCC8. Accordingly, CDC2 activity (decrease in CDC2 phosphorylation) was found to increase in response to siPTHrP. This suggests that PTHrP negatively regulates CDC2 activity on three levels as follows: by decreasing the expression of CDC2, by reducing the expression of CDC2 activator CDC25B, and by inhibiting the expression of GRCC8, the inhibitor of CDC2’s inhibitor. In addition, by keeping the expression of CKS2 low, PTHrP may also interfere with CKS2-dependent targeting of CDC2 to certain substrates. As PTHrP itself is a CDC2 substrate, it is tempting to assume that PTHrP keeps CDC2 activity low to reduce the likelihood that its nuclear activity is inhibited by CDC2. As PTHrP-dependent regulation of ITGA6 expression is likely to be dependent on the nuclear activity of PTHrP (71), down-regulation of CDC2 activity would allow PTHrP to keep ITGA6 levels high.

In summary, our data suggest that endogenously expressed PTHrP regulates many genes in breast cancer cells. Some of these genes, such as ITGA6, PAI-1, KISS-1, and CDC2, are key regulators of important biological activities, such as proliferation, migration, and/or invasion, and are known to be important players in tumor progression. By interfering with the expression of these genes, PTHrP is likely to play a role in tumorigenesis.

Acknowledgments—We thank Pearl Campbell and Katayoun Sheikheleslami for performing the microarray analyses.