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RESEARCH ARTICLE

Coherent Expression Chromosome Cluster Analysis Reveals Differential Regulatory Functions of Amino-Terminal and Distal Parathyroid Hormone-Related Protein Domains in Prostate Carcinoma

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Parathyroid hormone-related protein (PTHrP) has a number of cancer-related actions. While best known for causing hypercalcemia of malignancy, it also has effects on cancer cell growth, apoptosis, and angiogenesis. Studying the actions of PTHrP in human cancer is complicated because there are three isoforms and many derived peptides. Several peptides are biologically active at known or presumed cell surface receptors; in addition, the PTHrP-derived molecules can exert effects at the cell nucleus. To address this complexity, we studied gene expression in a DU 145 prostate cancer cell line that was stably transfected with control vector, PTHrP 1-173 and PTHrP 33-173. With this model, regulatory effects of the amino-terminal portion of PTHrP would result only from transduction with the full-length molecule, while effects pertaining to distal sequences would be evident with either construct. Analysis of the expression profiles by microarrays demonstrated nonoverlapping groups of differentially expressed genes. Amino-terminal PTHrP affected groups of genes involved in apoptosis, prostaglandin and sex steroid metabolism, cell-matrix interactions, and cell differentiation, while PTHrP 33-173 caused substantial increases in MHC class I antigen expression. This work demonstrates the distinct biological actions of the amino-terminus compared to distal mid-molecule or carboxy-terminal sequences of PTHrP in prostate carcinoma cells and provides targets for further study of the malignant process.

INTRODUCTION

Parathyroid hormone-related protein (PTHrP) is best known as the factor responsible for humoral hypercalcemia of malignancy, but it exerts other important effects in cancers and cancer cells. Among them are regulations of cell growth, apoptosis, cytokine production, and angiogenesis [1, 2, 3, 4]. Given these properties, it is not surprising that PTHrP contributes to cancer progression and metastasis. PTHrP has been found to stimulate the growth of tumors in several models of human cancer [5, 6, 7] and PTHrP expression is associated with an increased incidence of metastases and early mortality in cancer patients with hypercalcemia [8].

The complex biology of PTHrP involves diverse mechanisms for its effects. PTHrP is a secretory protein that is processed and acts through both paracrine and autocrine pathways by binding at cell surface receptors. The amino-terminus of PTHrP, PTHrP 1-32, acts through the type I PTH/PTHrP receptor (PTH1R) [9] that also binds parathyroid hormone. Ligation of PTH1R is involved in the hypercalcemic sequelae of cancer, and can also mediate various growth-related effects. In addition, posttranslational processing releases non-amino forms of PTHrP that exert biological actions. For example, PTHrP

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38-94 promotes placental calcium transfer [10], PTHrP 67-86 inhibits growth of breast carcinoma cells [11] and sensitizes lung alveolar epithelial cells to apoptosis [12], peptides included in PTHrP 107-141 inhibit osteoclast function [13], and PTHrP 140-173 regulates metabolism of orthophosphate by articular chondrocytes and protects lung cancer cells from apoptosis [14]. Cell surface receptors that mediate the effects of non-amino-terminal peptides have been postulated but have yet to be identified. PTHrP possesses a nuclear localizing sequence and can enter the nucleus to exert effects through an intracellular or intracrine mechanism [15]. The intracrine actions of PTHrP are frequently growth, or apoptosis related [2, 16]. Thus, the effects of PTHrP in an individual tissue can be regulated by expression of PTH1R and receptors yet to be discovered, by the cellular processing apparatus, and by nuclear localization and intracrine pathways.

The goal of this study was to use microarray technology to identify genes regulated by PTHrP in a prostate carcinoma cell line. We compared gene expression profiles between cells transfected with control vector, PTHrP 1-173 and PTHrP 33-173. This strategy provided a means to distinguish between the effects of amino-terminal PTHrP and the rest of the molecule. For example, a gene that was upregulated only by PTHrP 1-32 would be upregulated by PTHrP 1-173 and not by PTHrP 33-173, while one that was under the control of a region within PTHrP 33-173 would be upregulated by both constructs.

MATERIALS AND METHODS

Cell culture

The DU 145 human prostate cancer cell line [17] was obtained from American Type Culture Collection (Manassas, Va) and was grown in monolayer in RPMI 1640 media (Mediatech Inc, Herndon, Va) supplemented with 5% fetal bovine serum (Gemini Bio-Products, Woodland, Calif) at 37°C in a humidified incubator with 95% air, 5% CO_2. This cell line was selected because it has low constitutive PTHrP expression.

Stable transfection

PreproPTHrP 1-173 and preproPTHrP 33-173 constructs were directionally subcloned in the pCI-neo mammalian expression vector that utilizes the cytomegalovirus promoter for robust expression of the transgene (Promega Corp, Madison, Wis). The fidelities of the plasmids were confirmed by DNA sequencing and site-specific PTHrP immunoassays of cells transduced with the constructs [18]. The pCI-neo vector contains a neomycin resistance cassette that allows G418 selection of stable clones of DU 145 cells that express preproPTHrP 1-173 and preproPTHrP 33-173. The prepro-forms were used to facilitate PTHrP secretion [19].

Microarray analysis

The prostate cancer cells were seeded at a confluency of 75% in 100 mm tissue culture dishes at 37°C in a humidified incubator with an atmosphere of 5% CO_2. The next day, the media were aspirated from the dishes, the cells were washed with PBS, and serum-free medium was added to the cells for 18 h. The RNA was harvested by the RNeasy kit (Qiagen, Valencia, Cailf) protocol, as per manufacturer's recommendations. cDNAs were synthesized from the total RNA using a reverse transcriptase enzyme and biotin-labeled cRNAs were made using an RNA transcript labeling kit. The biotin-labeled RNA fragments were next hybridized to the probe array of a U133A Affymetrix GeneChip (Santa Clara, Cailf), which contains 19 571 probe sets corresponding to approximately 16 000 separate genes. The hybridized array was stained with a streptavidin phycoerythrin conjugate and scanned by a GeneChip Scanner. The amount of light emitted at 570 nm was proportional to the bound target at each location on the probe array.

Six experiments were performed: two with the cells transfected only with the pCI-neo control vector, two with the cells transfected by preproPTHrP 1-173, and two with cells transfected by preproPTHrP 33-173.

Bioinformatic analysis

Initial signal preprocessing was performed as recommended by Affymetrix. We used the Affymetrix software normalization tools to enable the comparison of results from different chips. We restricted the analyses to the data for those genes in which two members of the experimental group (vector, PTHrP 1-173, PTHrP 33-173) each had "P" (present) detection levels. Gene expression values for the cells transfected by PTHrP were normalized to the average expression levels in the profiles for the cells transfected with control vector. To provide a biological framework for the observed changes in gene expression, we analyzed the genes that were up- or downregulated by PTHrP in terms of their biological process, molecular function, cell compartment, and signaling pathway, utilizing the terms established by the Gene Ontology (GO) Consortium [20]. We considered a significant change in gene expression after PTHrP transfection to be an increase of more than twofold or a decrease of more than 40% compared to cells transfected with vector control. We also included genes that were upregulated by a factor of 1.75-fold if they were closely clustered in one chromosomal location and shared gene ontology terms with other upregulated genes in that locus. A lower threshold was accepted in these cases because the location and ontology data represented additional information supporting a differential effect of PTHrP on gene expression.

Criteria used to define coherent expression chromosome clusters

Gene Ontology uses the following classes to group genes: molecular function (MF), cell compartment (CC),

biological process (BP), and signaling pathway (PW). We consider the cluster of genes on any chromosome as a "coherent expression chromosome cluster (CECC)" when a cluster of genes in a chromosome express together in a common pattern that is consistent with the following rules:

(1) all genes of this region are over- or underexpressed due to exposure to a common factor (in our case PTHrP);

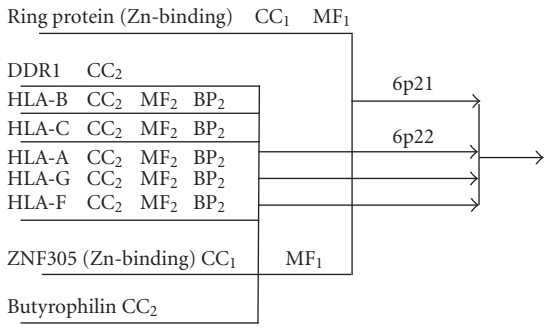
(2) the genes are located in the same chromosomal banding region (or in the neighboring localization in two adjacent chromosome band regions);

(3) the genes flanking the CECC have similar ontological definition(s) (MF or CC).

All of the genes that are regulated by PTHrP and lie between the two flanking genes having similar GO definitions are considered members of the CECC.

Examples of such analysis appear below.

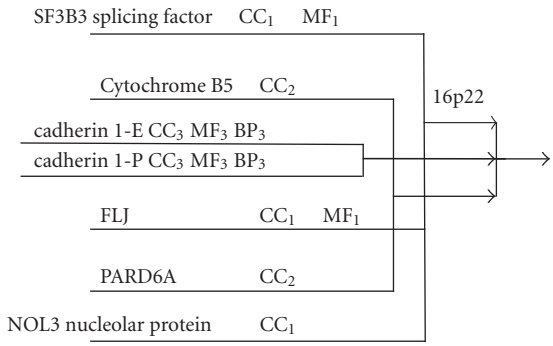
(a) CECC on chromosome 6 (Figure 2). The CECC is located on the boundary of the bands 6.22 and 6.23 and



where CC_1—nuclear localization, CC_2—plasma membrane localization, MF_1—Zn binding, MF_2—MHC class I receptors, BP_2—immune response.

Here two separate CECCs appear to overlap each other flanked by Ring protein and ZNF305 in one case and by DDR1 and butyrophilin in the other. For the simplicity of explanation, we have considered it as a single CECC.

(b) CECC1 on chromosome 16 (Figure 3).



where CC_1—nuclear localization, CC_2—membrane localization, CC_3—integral to membrane, MF_1—DNA binding, MF_3—cell adhesion, calcium ion binding, BP_3—cell adhesion.

A group of genes within the CECC are not related by ontology to the flanking genes, but per definition are considered members of the CECC. They most probably are "switched on" by the same transcription activation factor as the rest of CECC.

RESULTS

Transfection of DU 145 cells with PTHrP 1-173 and PTHrP 33-173 expression plasmids increased the concentration of immunoreactive PTHrP that the cells released into the media by two- to fivefold compared to the amount secreted by cells transfected with vector. In prostate cancer cells, 17 genes were upregulated twofold or more by transfection with PTHrP 1-173 compared to vector control but the same genes were unaffected by the PTHrP 33-173 construct (Table 1). The most striking change involved three members of the aldo-keto reductase family, C1, C2, and C3, which were increased from 8- to 13-fold. This finding is notable because only 9 of the genes probed by the U133A chip, approximately 0.04% of the total, code for proteins with aldo-keto reductase activity, yet 19% of the genes that had GO terms and were upregulated by PTHrP 1-173 fell into that category. The aldo-keto reductase genes are all located on chromosome 10p15 (Figure 1). The PTHrP 1-173-upregulated group also included four genes involved in cell adhesion or cytoskeletal organization: cysteine-rich with EGF-like domains 1 (CRELD1), syndecan 2, nebulette, and junction plakoglobin. Finally, four genes out of the collection of 17 have been implicated in growth and/or apoptosis regulation, transforming growth factor $\beta 2$ (TGFB2), interferon induced protein 16 (IFI16), caspase 10 (CASP10), and p8 protein (candidate of metastasis) (P8).

The full-length PTHrP molecule downregulated 22 genes to less than 40% of control levels. These genes were not affected by the construct lacking the amino-terminal portion of PTHrP. The group was notable for a large number of genes involved in development or differentiation: pre-B-cell leukemia transcription factor 1 (PBX1), kinesin family member 5C (KIF5C), somatostatin (SST), diphtheria toxin receptor (DTR), gap junction protein alpha 1 (GJA1), connective tissue growth factor (CTGF), adipose differentiation-related protein (ADFP), myotubularin-related protein 2 (MTMR2), high mobility group AT-hook 2, and MAGEA8. PBX1 and HMGA2 mediate transcription factor-promoter interactions, and PBX1 and SST have interactions with Hox proteins. Several downregulated genes, including tumor necrosis factor receptor superfamily 25 (TNFRSF25), SST, diphtheria toxin receptor DTR, intestinal cell kinase (ICK), GJA1, ADFP, MTMR2, and interleukin 23 (IL23A) are involved in signal transduction.

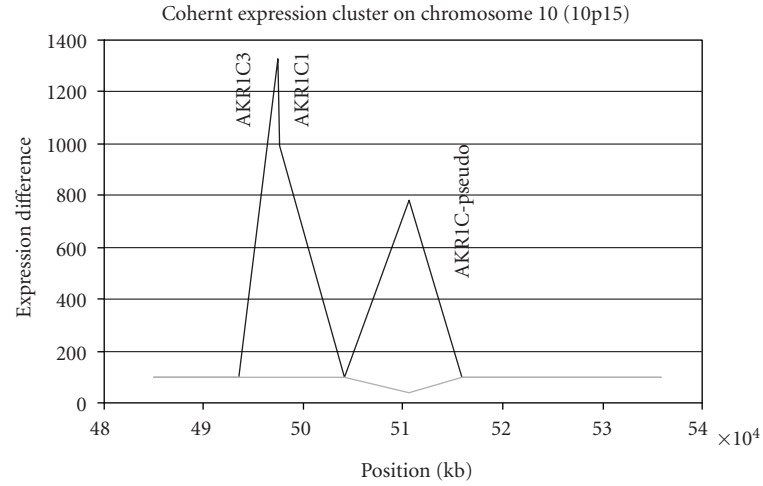


Figure 1. Cluster of functionally similar genes induced by PTHrP 1-32 on chromosome 10. RNA from DU 145 prostate carcinoma cells that were stably transfected with PTHrP 1-173 (in gray), PTHrP 33-173 (in black), or vector was probed with an Affymetrix U133A array and analyzed for differential changes in gene expression between vector and PTHrP-transfected cells. The graph demonstrates a coherent expression cluster, a chromosomal region containing functionally related coregulated genes. The ordinate shows mRNA levels from the PTHrP-transfected cells expressed as a % of the levels measured in the vector-transfected cells. In this example from chromosome 10p15, the genes are members of the aldo-keto reductase family that are involved in the metabolism of prostaglandin D2 to prostaglandin F2a and in conversion of androgens to inactive forms. PTHrP 1-173 transfection caused an (8–13)-fold increase in levels for the three genes.

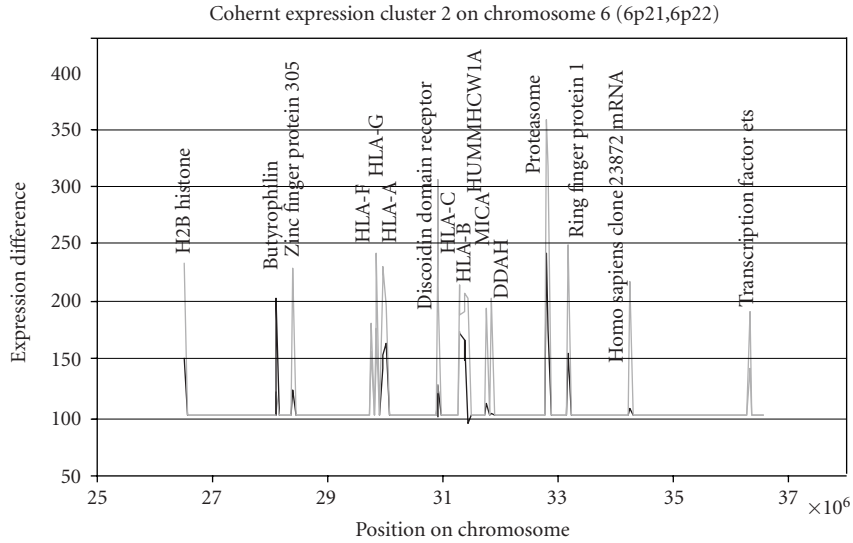


Figure 2. Functionally coherent expression induced by PTHrP 33-173. This graph represents data for chromosome 6p21-22 from the same experiment described in Figure 1. HLA-A, HLA-B, HLA-C, HLA-F, and HLA-F are all upregulated by PTHrP 33-173 in black (gray PTHrP 1-173). Ring finger protein 1 and two proteasome beta subunits, which are also upregulated, may work with the HLA antigens in a common pathway, since the proteasome is responsible for processing intracellular proteins to be presented on the cell surface with the HLA antigens.

The number of genes regulated by the non-amino-terminal portion of PTHrP was much larger than the number affected by amino-terminal PTHrP. PTHrP 33-173 upregulated 253 genes in DU 145 cells to levels > 200% of control cells and downregulated 103 genes. Table 2 presents a sampling of genes identified and organized by function. Several human leukocyte antigen (HLA) class I molecules, all located in close proximity on chromosome 6p21 (Figure 2), were upregulated by PTHrP 33-173 twofold or more. Interestingly, the 6p21-22 region also

Table 1. Genes regulated by PTHrP 1-173 but unaffected by PTHrP 33-173.

Gene	Unigene no	Region	Expression with PTHrP 1-173 transfection*(%)
Upregulated to >200% of control			
Transforming growth factor, beta 2	Hs.169 300	1q41	265
Interferon, gamma-inducible protein 16	370 873	1q23	264
ALL1-fused gene from chromosome 1q	75 823	1q21	271
Interferon-induced protein 44	82 316	1p31	344
Caspase 10, apoptosis-related cysteine protease	5353	2q33	255
Zinc finger protein 288	436 987	3q13	281
Cysteine-rich with EGF-like domains 1	9383	3p25	253
I factor (complement)	312 485	4q25	494
Rab coupling protein	96 125	8p11	292
Syndecan 2	1501	8q22	253
Aldo-keto reductase family 1, member C1	306 098	10p15	991
Aldo-keto reductase family 1, member C2	201 967	10p15	1328
Aldo-keto reductase family 1, member C3	78 183	10p15	785
Hypothetical protein FLJ12616	287 537	10q26	260
Nebulette	5025	10p12	277
p8 protein (candidate of metastasis 1)	418 692	16p11	263
Junction plakoglobin	2340	17q21	298
Downregulated to <40% of control			
Tumor necrosis factor receptor superfamily, member 25	299 558	1p36	20
Pre-B-cell leukemia transcription factor 1	40 822	1q23	23
Kinesin family member 5C	6641	2q23	39
Butyrylcholinesterase	422 857	3q26	21
Somatostatin	12 409	3q28	31
Hypothetical protein FLJ23235	211 501	4p14	34
Hypothetical protein FLJ23548	22 895	4q26	39
Hypothetical protein DKFZp434N1235	149 030	4q28	12
Potassium intermediate/small conductance			
Calcium-activated channel, subfamily N, member 2	98 280	5q22	16
Lysyl oxidase	102 267	5q23	32
Diphtheria toxin receptor	799	5q23	36
Intestinal cell kinase	417 022	6p12	37
Connexin 43	74 471	6q21	29
Connective tissue growth factor	410 037	6q23	33
Adipose differentiation-related protein	3416	9p22	34
Myotubularin-related protein 2	181 326	11q22	38
Interleukin 23, alpha subunit p19	98 309	12q13	33
High mobility group AT-hook 1	57 301	6p21	36
Heat shock 105kD	36 927	13q12	36
Glycine amidinotransferase	75 335	15q15	39
Ubiquitin-like 5	386 532	19p13	16
Melanoma antigen, family A, 8	37 109	Xq28	39

*Expression is presented as a % of the expression by the vector-transfected control cells.

includes three upregulated genes coding for a ring finger protein (RING1) and two components of the proteasome, beta subunits 8 and 9 (PSMB8,PSMB9). These proteins are functionally related to the major histocompatibility complex proteins because the proteasome processes peptides for presentation with HLA I proteins [21]. Furthermore, RING1 is involved in monoubiquitination of proteins targeted for proteasomal processing [22]. Thus,

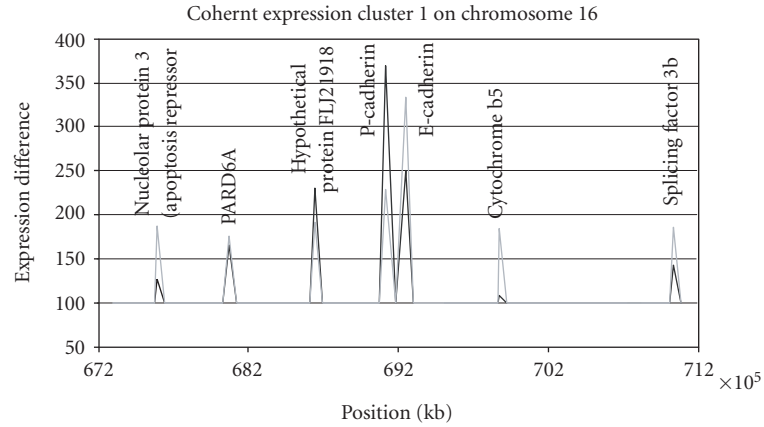


FIGURE 3. Functionally coherent expression induced by PTHrP 33-173. This graph represents data for chromosome 6 from the same experiment described in Figure 1. All proteins are all upregulated by PTHrP 33-173 in black (gray PTHrP 1-173). Coherent expression cluster 1 on chromosome 16 (p22). E-cadherin (CDH1) and P-cadherin (CDH3) are both involved in cell-cell interaction and lie within a 0.15-megabase region on chromosome 16p22. PTHrP 33-173 downregulated several genes coding for proteins involved in cell-matrix or cytoskeletal interaction and also decreased expression of DICRI, a Dcr-1 homolog. DICER1 codes for a ribonuclease that produces active small RNA components involved in repressing gene expression.

6p21-22 contains an amplicon of genes with related functional properties that share responsiveness to PTHrP 33-173, a feature we term a coherent expression cluster. Chromosome 16 (Figure 3) also contains a coherent expression cluster for PTHrP 33-173 consisting of a pair of genes. E-cadherin (CDH1) and P-cadherin (CDH3), which are both involved in cell-cell interaction [23, 24, 25], lie within a 0.15 megabase region on chromosome 16p22. PTHrP 33-173 downregulated several genes coding for proteins involved in cell-matrix or cytoskeletal interaction and also decreased expression of DICRI, a Dcr-1 homolog. DICER1 codes for a ribonuclease that produces active small RNA components involved in repressing gene expression [26].

PTHrP regulated a total of 245 annotated genes in DU 145 cells. Of this group, 219 were found in CECCs and 26 were not. Other investigators have estimated that 20% of the expressed annotated genes on the Affymetrix U133A chip may fall into functional clusters [27]. Thus, the number of PTHrP-regulated genes included in CECCs was much greater than expected on the basis of chance alone (Chi square $P < .0001$).

DISCUSSION

This work utilized a novel approach to microarray analysis to demonstrate distinct pathway- or process-specific differences in the pattern of gene expression induced by amino-terminal versus distal sequences of PTHrP. We compared microarray data from human prostate carcinoma cells transfected with full-length PTHrP 1-173 versus cells expressing a non-amino-terminal peptide, PTHrP 33-173, which lacks the amino-terminal sequence. We have attempted to generate smaller PTHrP peptides on the order of PTHrP 1-32 with transfected expression plasmids. However, transfection with these plasmids does not appear to generate immunoreactive PTHrP, presumably because the molecules are too short to transverse the endoplasmic reticulum [19]. With the comparison between PTHrP 1-173 and PTHrP 33-173, a change in function that depended on amino-terminal PTHrP would be observed only after transduction with PTHrP 1-173, while effects due to distal PTHrP sequences, common to both PTHrP forms, would be apparent with either construct. Amino-terminal PTHrP comprises PTHrP 1-32, a physiologic ligand for the receptor shared with the amino-terminus of PTH. The distal region contains mid-molecule and carboxy-terminal sequences that also exert biologic activity, but have no known receptors [28]. The mid-molecule region also contains a nuclear localizing sequence (NLS) at residues 87–106 and an adjacent sequence at amino acids 107–139 that are important for intracrine effects [15, 29]. The sequence of PTHrP 65–171 is 39% homologous to CHD-4, a nuclear DNA binding protein. Structural studies are ongoing to evaluate whether a DNA binding function with this region is important for PTHrP intracrine effects. Thus, PTHrP could influence prostate cancer pathology through multiple mechanisms; these mechanisms could be modulated by protein processing, changes in secretory pattern, receptor expression, and nuclear localization.

We found that regulation of genes in several pathways and biologic processes varied in a manner dependent on specific PTHrP regions. Amino-terminal PTHrP increased mRNA levels for several aldo-keto reductase (AKR) enzymes (Table 1), genes related to cell adhesion and cytoskeleton, and genes regulating growth and/or apoptosis, effects not observed with cells transfected with the cut version of PTHrP. In addition, the amino-terminal

Table 2. Representative genes regulated by PTHrP 33-173.

Gene	Unigene no		Expression with PTHrP 33-173 transfection* (%)
Upregulated genes			
Class 1 major histocompatibility complex proteins			
HLA-A	Hs. 181 244	6p21	224
HLA-B	Hs. 77 961	6p21	200
HLA-C	Hs. 274 485	6p21	201
HLA-F	Hs. 411 958	6p21	180
HLA-G	Hs. 512 152	6p21	242
Proteasome-related proteins			
Proteasome (prosome, macropain) subunit, beta type, 8	Hs. 180 062	6p21	358
Proteasome (prosome, macropain) subunit, beta type, 9	Hs. 381 081	6p21	318
Ring finger protein 1, RING1	Hs. 202 430	6p21	249
Proteins mediating cell-cell interactions			
E-cadherin, CDH1	Hs. 194 657	16q22	334
P-cadherin, CDH3	Hs. 191 842	16q22	229
Arachidonic acid pathway proteins			
Glutathione peroxidase 1, GPX1	Hs. 76 686	3p21	200
Glutathione peroxidase 3, GPX3	Hs. 386 793	5q23	718
Nuclear proteins			
Basic leucine nuclear zipper factor Jem-1, BLZF1	Hs. 444 697	1q24	221
Kruppel-like factor 3, KLF3	Hs. 145 754	4p14	197
Zinc finger protein 305, ZNF305	Hs. 134 816	6p22	229
Downregulated genes			
Cell-Matrix/Cytoskeleton-related proteins			
Sterile alpha motif domain containing 4, SAMD4	Hs. 98 259	14q22	22
Dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 4	Hs. 439 530	12p13	33
Myosin light polypeptide kinase, MYLK	Hs. 386 078	3q21	10
Transmembrane 4 superfamily member 2, TM4SF2	Hs. 439 586	Xp11	34
Lysyl oxidase-like 2, LOXL2	Hs. 83 354	8p21	37
Dicer1, Dcr-1 homolog, DICER1	Hs. 87 889	14q32	34

*Expression is presented as a % of the expression by vector-transfected control cells.

region decreased expression of genes with a role in differentiation or development. In contrast, the effects of the distal 33–173 residue portion of the molecule were notable in regard to expression of genes for MHC I proteins, proteasome function, cell-cell interaction, arachidonic acid metabolism, nuclear proteins, and cell-matrix and cytoskeleton-related proteins (Table 2). Multiple genes from the same pathway were frequently regulated in a coordinated fashion. The HLA genes and proteasomal subunit genes PSMB8 and PSMN9 represent such a set, since the proteasome prepares short peptides for antigen presentation on MHC-1. Similarly, GLG1, the TGF-beta complex protein, and cadherins all participate in the TGF-beta pathway. In some cases, amino-terminal PTHrP and distal PTHrP appeared to act on related functional pathways. The effects of the amino-terminal and carboxy-terminal domains on AKR enzymes and on proteins with a role in arachidonic acid pathways exemplify this situation. The enzymes upregulated by PTHrP 1-173 are involved in prostaglandin metabolism [30], while in the PTHrP 33-173-regulated group, glutathione peroxidases 1 and 3 inhibit 12-lipoxygenase and, to a lesser degree 5-lipoxygenase [31, 32]. In addition, amino-terminal PTHrP and distal PTHrP both have effects on several cell-matrix and cytoskeleton genes, although the proximal sequences upregulate expression and the distal sequences decrease expression.

Exogenous PTHrP peptides do have biologic actions in wild-type DU 145 cells. For example, we have evaluated their effects on staurosporine-induced apoptosis. PTHrP140-173 peptide treatment decreased caspases-3 and -9 activities and nuclear condensation compared to vehicle treated cells. No significant effects on nuclear condensation and caspases-3 or -9 were observed with treatment with PTHrP 1-34 or scrambled PTHrP 140-173 peptide [32]. Since protective effects on apoptosis

were not observed for PTHrP 1-34, the human-specific 140-173 region appears responsible for the antiapoptotic properties of PTHrP in prostate cancer cells through a paracrine mechanism. These results would be consistent with effects mediated by transfection with either the PTHrP 1-173 or the PTHrP 33-173 construct.

The genes affected by PTHrP 1-173 and PTHrP 33-173 could have variable effects on prostate carcinoma progression or metastasis. In several instances, both PTHrP forms had actions that could retard cancer growth. For example, the apoptosis genes upregulated by PTHrP 1-173 only were generally proapoptotic and could conceivably promote prostate cancer cell death. There are a number of precedents for apoptosis sensitization by amino-terminal PTHrP. PTHrP 1-32 induces or augments apoptosis in human embryonic kidney (HEK) 293 cells [33], postconfluent mesenchymal cells [34], and lung alveolar type II cells [12]. Just as apoptosis enhancement would slow tumor growth, the effects of carboxy-terminal PTHrP on HLA class I expression and proteasomal function could hinder carcinoma progression. Many prostate cancer cells and other cancer cells downregulate MHC antigens, presumably as a mechanism of avoiding immune surveillance [35, 36]. HLA class I antigen expression decreases in more invasive prostate carcinomas and expression is directly related to patient survival [37]. By upregulating these molecules, PTHrP 33-173 expression could improve the ability of CD8 T cells to detect and kill prostate tumor cells [36]. Finally, increased expression of the glutathione peroxidase enzymes mediated by distal PTHrP sequences could negatively influence prostate tumor growth. By inhibiting 5- and 12-lipoxygenase, GPX1 would reduce cancer cell levels of 5-HETE and 12-HETE, mediators that promote survival, growth, and invasiveness of prostate cancer cells [38, 39, 40]. On the other hand, increased expression of the AKR enzymes could augment prostate cancer growth. AKRC13 could limit production of PPARγ ligands arising from PGJ_2 by favoring $PGF_{2\alpha}$, rather than PGJ_2, production from PGD_2. Decreasing these ligands could support tumor progression because PPARγ has antiproliferative effects in prostate cancer cells and promotes cell death [41, 42]. It should be noted that upregulation of the AKR enzymes could also be significant in prostate cancer in terms of effects on androgen levels. The AKR1C isoforms are involved in inactivating male and female sex hormones [43]. For example, they catalyze the conversion of 5α-dihydrotestosterone to 5α-androstane-3α, 17β-diol, a much less potent androgen [44], thereby regulating occupancy and activation of the androgen receptor. Increased expression of E-cadherin and P-cadherin would also favor prostate carcinoma growth. E-cadherin has a positive relationship with tumor invasiveness. Thus, the differentially expressed genes regulated by PTHrP may either stimulate or retard cancer progression. The overall effect of amino- and non-amino-terminal PTHrP on the pathophysiology of prostate cancer will require direct experimental evaluation.

The changes in gene expression induced by PTHrP were notable for including several coherent expression clusters, including the AKR enzymes on chromosome 10p15, the HLA class I antigens and proteasomal pathway proteins on chromosome 6p21 and the E-cadherin/P-cadherin pair on chromosome 16q22. These findings are not unusual because genes with similar functions tend to occur in adjacent positions along chromosomes. For example, the individual members of pairs of adjacent yeast genes shared the same functional category in 387 of 2018 pairs (19%), significantly more than would be expected by chance [45]. Clustering of genes with organ-specific function has been observed in humans, as well [46]. Chromosomal clustering of differentially expressed genes could result from either genomic changes or transcriptional mechanisms. In our experiments involving transduction with a single gene, widespread genomic changes are unlikely and transcriptional control is a more probable explanation for regionally coherent changes in expression patterns. This control could take the form of common responses to transcription factors among functionally similar genes. As an example, E-cadherin and P-cadherin have homology in their 5′-promoter regions, which are both recognized by Sp1-related nuclear factors [47]. Similarly, expression of HLA class I genes is mediated by conserved cis-acting regulatory elements, including the enhancer A, the ISRE module, and the SXY module [48]. Alternatively, coherent expression clusters could arise from epigenetic mechanisms involving changes in chromatin structure. Thus, histone deacetylase inhibitors have been shown to enhance expression of MHC class I antigens in tumor cells [49].

The observation that non-amino-terminal PTHrP caused differential expression of many more genes than amino-terminal PTHrP was somewhat unexpected. The result is understandable, however, given the multifunctional nature of PTHrP. Genes regulated solely by PTHrP 1-173 and not PTHrP 33-173 are under the control of paracrine interactions of PTHrP 1-32 with its receptor. In contrast, genes regulated by PTHrP 33-173 may be affected by paracrine interactions with any of the downstream biologically active portions of the PTHrP molecule, as well as intracrine mechanisms. Furthermore, the effect of PTHrP 33-173 transfection on expression of DICER1 could be an additional explanation for the augmented number of differentially expressed genes. Dicer is an RNase that produces RNAi activity. Depletion of Dicer reduces such activity in drosophila cells, while *C elegans* lacking functional Dicer are impaired in RNAi-mediated gene suppression [50, 51]. Thus, a reduction in Dicer expression after PTHrP 33-173 transduction in prostate cancer cells could contribute to a general increase in gene expression by reducing RNAi activity.

Our studies introduce a novel method to identify effects of PTHrP that are specific to functional peptide domains contained within this polypeptide. Our results show that PTHrP can exert nonoverlapping biological effects in prostate carcinoma cells that are attributable

to amino-terminal and non-amino-terminal domains. Amino-terminal PTHrP regulated groups of genes involved in growth/apoptosis, prostaglandin and androgen steroid metabolism, cell-matrix interactions, and development/differentiation. Large changes were seen in aldo-keto reductase genes that could play a role androgen response. On the other hand, PTHrP 33-173 effects were significant for increases in expression of HLA class I antigens and the proteasomal proteins that could be involved in antigen presentation. These changes could render prostate carcinoma cells more susceptible to immune surveillance and might constitute a rationale for a PTHrP-based therapy for prostate cancer. Finally, our work has identified coherent expression clusters, chromosomal regions of increased differential gene expression after PTHrP transduction. Two interesting clusters involved the aldo-keto reductase genes on chromosome 10p15 and the HLA genes on chromosome 6p21. Comparing microarray analysis results from cells transduced with different forms of a regulatory protein provides a mechanism for elucidating domain-specific effects in gene expression. Further application of this novel approach to microarray analysis with other PTHrP species can help to elucidate the complex effects that PTHrP exerts on cancer cells.

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