

The importance of PTHrP for cancer development

Review Article

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Abbreviations: adenovirus protein E1A, (AdV E1A); adult T-cell leukemia/lymphoma, (ATLL); calcium-sensing receptor, (CaR); cAMP-responsive element, (CRE); epidermal growth factor, (EGF); extracellular matrix, (ECM); G-protein coupled receptors, (GPCR); human T lymphotropic virus type I, (HTLV-I); hypercalcaemia of malignancy, (HHM); interleukin-6, (IL-6); nuclear localization sequence, (NLS); parathyroid hormone 1 receptor, (PTH1R); parathyroid hormone, (PTH); Parathyroid hormone-related protein, (PTHrP); protein kinase A, (PKA); protein kinase C, (PKC); receptor activator of NF- κ B ligand, (RANKL); transforming growth factor 2, (TGF 2); urokinase type plasminogen activator, (uPA); vascular smooth muscle, (VSM)

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Summary

Parathyroid hormone-related protein (PTHrP) is expressed by many cells and usually acts as an autocrine, paracrine and/or intracrine factor to play numerous roles in embryonic development and normal physiology. Evidence has been accumulated suggesting that PTHrP may also serve important functions in tumor development. PTHrP has the potential to cause humoral hypercalcaemia of malignancy and is able to induce local osteolysis which facilitates growth of tumor cells that have metastasized to bone. Furthermore, PTHrP has been shown to stimulate proliferation as well as invasiveness of cancer cells and to protect cancer cells from apoptosis. In this review, I summarize the current knowledge about the role of PTHrP in cancer development and about the factors that control PTHrP expression in cancer.

I. Discovery of PTHrP

PTHrP was originally discovered as a systemic humoral factor that is released by tumor cells and causes hypercalcaemia of malignancy (HHM) (Suva et al, 1987; Wysolmerski and Broadus, 1994; Rankin et al, 1997; Grill et al, 1998). The hypercalcaemic activity of PTHrP is based on its partial homology to parathyroid hormone (PTH) (Horiuchi et al, 1987; Kemp et al, 1987), a protein that regulates calcium homeostasis. By being able to bind to the parathyroid hormone 1 receptor (PTH1R) with equal affinity as PTH (Juppner et al, 1991), PTHrP mimics PTH action and stimulates cAMP production in bone and kidney (Mannstadt et al, 1999). This results in bone resorption and renal calcium retention eventually leading to HHM.

It became clear that PTHrP is also expressed by non-transformed cells in almost all tissues (dePapp and Stewart, 1993) where it serves specific functions as an autocrine or paracrine factor (Moseley and Gillespie; 1995, Philbrick et al, 1996; Strewler, 2000). In embryogenesis, PTHrP plays an essential role in

mammary gland and bone development (Vortkamp et al, 1996, Wysolmerski et al, 1998). Disruption of the PTHrP gene in mice leads to fatal skeletal dysplasia (Karaplis et al, 1994; Karaplis and Deckelbaum, 1998). Rescued PTHrP k.o. mice, carrying a transgenic PTHrP gene under the control of a bone-specific promoter, lack mammary epithelial ducts (Wysolmerski et al, 1998). The actions of PTHrP in the developing bone and breast are paracrine in nature and depend on PTH1R. In the developing bone, PTHrP secreted from periarticular perichondrium activates PTH1R on chondrocytes, thereby preventing premature ossification (Vortkamp et al, 1996). In the developing mammary gland, PTHrP from embryonic mammary epithelial cells stimulates the mammary mesenchyme via interaction with PTH1R to differentiate into mammary-specific mesenchyme which then triggers ductal morphogenesis (Dunbar et al, 1998).

II. The functional domains of PTHrP

The PTHrP transcripts are translated into three different isoforms, PTHrP (-36/139), PTHrP (-36/141) and

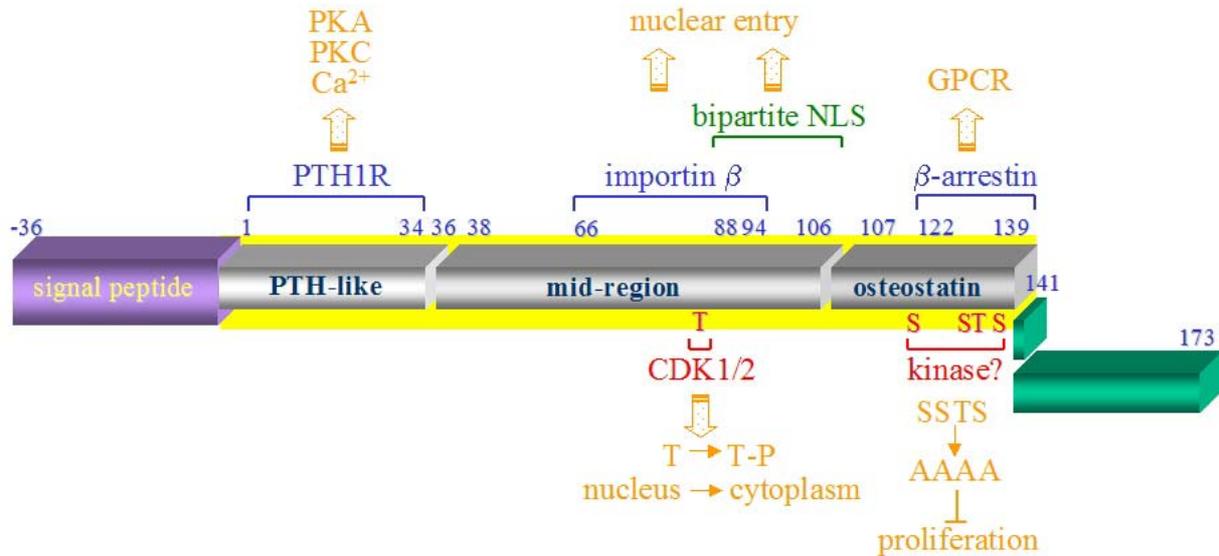


Figure 1. The functional domains of the human PTHrP protein and its interaction partners. Details are given in the text. T-P denotes phosphorylation of Thr⁸⁵. SSTS stands for residues Ser¹¹⁹, Ser¹³⁰, Thr¹³² and Ser¹³⁸ and AAAA denotes their replacements by alanines. CDK = cyclin-dependent kinase, GPCR = G-protein coupled receptor. Green bars show extension of the PTHrP protein in splicing variants -36/141 and -36/173.

PTHrP (-36/173) (**Figure 1**). They all contain the N-terminal signal sequence for entrance into the endoplasmic reticulum and the coding region between residues 1 and 139 (Martin et al, 1991; Philbrick et al, 1996; Strewler, 2000). The first 111 amino acids of the coding region are highly conserved between mice, rats and humans suggesting that they are crucial for PTHrP function. In contrast, the C-terminus is highly variable. The isoforms PTHrP (-36/141) and the human-specific PTHrP (-36/173) product feature extended C-termini. The PTHrP protein is post-translationally cleaved at a number of dibasic sites (Diefenbach-Jagger et al, 1995; Dittmer et al, 1996; Wu et al, 1996) leading to the removal of the pre-pro sequence between -36 and -1 and to a limited fragmentation of the protein. These fragments contain one or more of the three functional domains which are the N-terminal (PTHrP 1-36), the mid-region (PTHrP 38-94) and the C-terminal domain (PTHrP 107-139).

The N-terminal domain, PTHrP (1-36), is responsible for the PTH-like activity of PTHrP and is able to bind and activate PTH1R. Activation of this G-protein coupled receptor (GPCR) leads to the stimulation of the protein kinase A (PKA), protein kinase C (PKC) and/or calcium-dependent pathways (Mannstadt et al, 1999; Cataisson et al, 2000; Maioli and Fortino, 2004a).

Many of the PTH1R-dependent PTHrP effects can be mimicked by cAMP indicating that PKA is a major target of activated PTH1R.

The mid-region domain is able to enter the nucleus. It contains a bipartite nuclear localization sequence (NLS) consisting of residues 88-91 and 102-106 (Massfelder et al, 1997). This sequence also allows PTHrP to accumulate in the nucleolus (Henderson et al, 1995) and to bind to RNA (Aarts et al, 1999). Nuclear targeting can be further achieved by residues 66-94 which is recognized by importin β (Lam et al, 1999a; Cingolani et al, 2002). The

midregion sequence also holds a CDK1(cdc2)/CDK2 phosphorylation site (Lam et al, 1999b). Following its phosphorylation, PTHrP is retained in the cytoplasm suggesting that the activity of nuclear PTHrP is regulated by the cell cycle.

The C-terminal domain, also called osteostatin, is able to inhibit bone resorption and, thereby, antagonizes the action of the N-terminal domain of PTHrP (Fenton et al, 1994; Cornish et al, 1997). This inhibitory effect of osteostatin may be based on the ability of this protein to physically interact with β -Arrestin (Conlan et al, 2002). β -Arrestins are known to regulate internalization and desensitization of ligand-stimulated GPCRs, such as PTHrP-activated PTH1R (Ferrari et al, 1999). The C-terminal domain also harbors four potential targets for kinases at residues 119, 130, 132 and 138 whose mutation from a serine or threonine to an alanine abrogated the mitogenic activity of PTHrP in vascular smooth muscle cells (Fiaschi-Taesch et al, 2004)

Another domain seems to be located in the extended C-terminus of the PTHrP (-36/173) transcriptional product. The sequence between residues 140 and 173 has been shown to interfere with the nuclear localization of PTHrP (Goomer et al, 2000) and to raise the cAMP level (Hastings et al, 2004).

Synthesized in the endoplasmic reticulum, PTHrP is a secretory protein which needs to interact with specific cell membrane receptors in order to exert its function. So far, one such receptor, PTH1R, has been identified that recognizes the PTHrP N-terminal domain. Receptors that specifically interact with one of the other PTHrP domains may exist as well. This is indicated by the observations that fragments not containing the N-terminal domain are present outside of cells (Soifer et al, 1992; Wu et al, 1996) and that those fragments are able to interfere with cellular function when added exogenously (Massfelder et al, 1997;

Luparello et al, 2001). In particular, the mid-regional PTHrP (67-86) peptide, devoid of a functional NLS, has been shown to mobilize calcium through a phospholipase C-dependent pathway in squamous carcinoma cells (Orloff et al, 1996). For NLS-containing mid-region fragments, an intracrine way of action has been discussed. In order for PTHrP to enter the nucleus, PTHrP is supposed to be either synthesized directly in the cytosol or produced in the endoplasmic reticulum and then re-translocated to the cytosol (Fiaschi-Taesch and Stewart, 2003).

III. PTHrP and cancer growth

There is evidence that PTHrP has a tumor growth effect. Mammary gland specific overexpression of PTHrP led to a higher incidence of tumor formation in mice (Wysolmerski et al, 2002). Also, a polymorphic PTHrP variant is associated with increased incidence of skin cancer in mice (Manenti et al, 2000). Furthermore, the growth of rat pituitary cancer cells in the brain of rats was found to be decreased upon treatment with anti-sense oligonucleotides against PTHrP-RNA (Akino et al, 1996). Similarly, the tumor volume formed by H-500 Leydig cells inoculated into rats was reduced after PTHrP anti-sense RNA had been administered to the animals (Rabbani et al, 1995). And, treatment of tumor-bearing mice with PTHrP-specific antibodies was shown to suppress growth of human breast cancer metastasized to bone and renal carcinoma injected into the skin (Guise et al, 1996; Massfelder et al, 2004). Furthermore, PTHrP overexpressing prostate cancer cells grew faster in MatLyLu rats than control cancer cells (Dougherty et al, 1999) while, in athymic mice, the level of PTHrP expression in human squamous cancer cells increased with tumor growth (Yamato et al, 1995).

As for the value of PTHrP as a prognostic marker for cancer, especially for breast cancer, the data are conflicting. On the one hand, a study of Martin and colleagues showed that, in a cohort of 367 breast cancer patients, immunoreactivity against N-terminal PTHrP in paraffin sections of the primary tumor tissues correlated with improved survival (Henderson et al, 2001). In contrast, Linforth et al reported that, in a cohort of 176 breast cancer patients, positive immunohistochemical staining for N-terminal PTHrP in primary tumors was associated with a reduced disease-free survival (Linforth et al, 2002). In the same study, it was shown that the RNA level of PTH1R correlated with a decreased survival as well and, interestingly, that co-expression of PTHrP with its receptor predicted the worst clinical outcome. In another study including 177 breast cancer patients, tumoral PTHrP protein expression was found to be a marker of poor prognosis (Yoshida et al, 2000). The reason for the discrepancy between the outcomes of these studies is not yet known. In other human cancers, PTHrP expression seems to correlate with advanced disease. E.g., a study on a cohort of 108 colorectal tumor patients showed that positive staining for PTHrP in the tumor was associated with an increased incidence of lymph nodes and liver metastasis (Nishihara et al, 1999). Increased PTHrP serum levels in cancer patients were also found to

correlate with increased mortality (Hiraki et al, 2002; Truong et al, 2003).

IV. PTHrP and metastasis

It is generally accepted that PTHrP plays a role in bone metastasis. By inducing local osteolysis PTHrP facilitates growth of osteotropic tumors, such as breast cancer, in the dense bony tissue (Goltzman et al, 2000; Guise, 1997; Kakonen and Mundy, 2003). PTHrP triggers osteolysis by stimulating osteoblasts to produce osteoclastogenesis-activating factors, such as receptor activator of NF- κ B ligand (RANKL) or interleukin-11 (Morgan et al, 2004; Thomas et al, 1999). However, PTHrP does not appear to directly interfere with the metastatic potential of tumor cells, at least not in mice (Wysolmerski et al, 2002).

The importance of PTHrP for bone metastasis has been demonstrated by a number of studies. A correlation between PTHrP expression and formation of bone metastasis was shown for breast and lung cancer cell lines in nude mice (Guise et al, 1996; Miki et al, 2000). Moreover, colonialization of bone tissue by MDA-MB-231 breast cancer cells could be inhibited in nude mice by PTHrP-specific antibodies (Guise et al, 1996). Similarly, the formation of bone metastases, but not metastases in other organs by SBC-5 small-lung cancer cells could be reduced by anti-PTHrP antibodies in immunocompromised SCID mice (Miki et al, 2004). The propensity of metastatic tumors in bone to express PTHrP could further be shown for human breast cancer: the highest frequency of PTHrP expression (73-92%) was found in bone metastatic lesions, whereas only a minority (17-20%) of breast cancer metastases at non-bone sites produced PTHrP (Powell et al, 1991; Vargas et al, 1992).

PTHrP induces osteolysis in cooperation with other factors, such as TGF β (Yin et al, 1999). TGF β , a factor that can either inhibit or promote tumor growth (Blobe et al, 2000; Roberts and Wakefield, 2003), is present in the bone matrix and is activated upon PTHrP-induced osteolysis. The activation of TGF β initiates a vicious cycle as active TGF β stimulates MDA-MB-231 cells to produce more PTHrP. This, in turn, leads to more osteolysis and, thus, higher levels of activated TGF β (Yin et al, 1999). Another study compared the features of bone-seeking and brain-seeking MDA-MB-231 sublines. The brain-seeking subline expressed less PTHrP than the bone-seeking one and also showed a much higher sensitivity to the growth-inhibitory activity of TGF β (Yoneda et al, 2001). The latter feature may have precluded survival of the brain-seeking subline in the TGF β -rich environment of the bone. Another support for a link between PTHrP and bone metastasis comes from two studies with MCF-7 breast cancer cells. Both down- and upregulation of the endogenous PTHrP production interfered with the ability of this cell line to form metastatic lesions in the bone (Kitazawa and Kitazawa, 2002; Thomas et al, 1999).

In addition to TGF β , also interleukin-6, tumor necrosis factor or transforming growth factor α , have been shown to be able to enhance the bone destructive effect of PTHrP (de la Mata et al, 1995; Guise et al, 1993;

Tumber et al, 2001; Uy et al, 1997). In some cases, PTHrP may not be the major factor that facilitates colonialization of breast cancer cells in the bone. Prostaglandine E₂, interleukin-6 and interleukin-8 may well substitute for PTHrP (Bendre et al, 2003; Martin, 2002). E.g., interleukin-8 has been shown to mediate osteolysis of the highly metastatic MDA-MET cell line that produces less PTHrP, but higher amounts of interleukin-8 than the MDA-MB-231 parental cell line (Bendre et al, 2002). On the other hand, PTHrP and interleukin-8 expression may be connected. This was shown for prostate cancer cells, where PTHrP increased interleukin-8 production via its intracrine pathway (Gujral et al, 2001). In contrast to PTHrP, interleukin-8 can directly activate osteoclast formation.

V. Biological effects of PTHrP on cancer cells

Numerous studies have been conducted to analyze the impact of PTHrP on proliferation, invasiveness and resistance to apoptosis, biological activities that are crucial for survival and growth of cancer cells. The results of these studies are discussed below.

A. PTHrP and cancer proliferation

During murine endochondrial ossification PTHrP serves an important function by preventing chondrocytes to prematurely differentiate into hypertrophic cells (Vortkamp et al, 1996). In a positive feedback loop, prehypertrophic chondrocytes secrete Indian hedgehog (Ihh) that, by activating transforming growth factor 2 (TGF 2) (Alvarez et al, 2002), stimulates the periarticular perichondrium to produce PTHrP (Vortkamp et al, 1996; Karp et al, 2000; Kobayashi et al, 2002). PTHrP, in turn, induces proliferation of the chondrocytes by interacting with PTH1R. The activated receptor induces a decline in the expression of cell cycle inhibitor p57^{kip2} (MacLean et al, 2004) and an increase in the production of cyclin D1 (Beier et al, 2001). This well-studied example shows that PTHrP can play a role in the regulation of the cell cycle. This notion is further supported by a detailed study on keratinocytes showing that PTHrP expression increases when cells in G₁-Phase enter S-Phase, an event that is accompanied by relocation of PTHrP from the nucleolus to the cytoplasm (Lam et al, 1997). Strikingly, PTHrP expression in squamous cancer cells is constantly high throughout the cell cycle (Lam et al, 1997) suggesting that PTHrP expression becomes dysregulated in the course of carcinogenesis.

1. Autocrine actions via PTH1R

There are a number of reports suggesting that PTHrP may contribute to the high proliferative activity of cancer cells. One report demonstrated that, in breast cancer, PTH1R expression correlates well with the expression of the proliferation marker Ki67 (Downey et al, 1997). In another study, the mitogenic effect of PTHrP on MCF-7 breast cancer cells was found to be increased when PTH1R was overexpressed (Hoey et al, 2003). In a third study using the same cell line, the PTH1R ligand PTHrP

(1-34) alone could induce proliferation, which was accompanied by an increase in the intracellular cAMP level (Birch et al, 1995). The same peptide was also shown to be able to stimulate growth of PC-3 and LnCaP prostate cancer cells (Asadi et al, 2001) as well as of lung squamous BEN-57 cancer cells (Burton and Knight, 1992). In the latter case, the effect of PTHrP (1-34) could be reversed by addition of a PTHrP antibody. Furthermore, proliferation of clear cell renal carcinoma in nude mice could be equally inhibited by antibodies against PTHrP or by a PTH1R antagonist (Massfelder et al, 2004). These examples show that cancer cells can use the PTHrP/PTH1R interaction to stimulate their own proliferative activity.

2. Intracrine actions

Some reports also show anti-proliferative effects of PTHrP on MCF-7 breast cancer and vascular smooth muscle (VSM) cells (Massfelder et al, 1997; Falzon and Du, 2000; Luparello et al, 2001; Pasquini et al, 2002). Interestingly, in two of these cases, the anti-proliferative activity of PTHrP was only observed when PTHrP peptides (1-34, 1-36, 1-86, 1-108, 1-139, 1-141) were exogenously administered to the cells (Massfelder et al, 1997; Falzon and Du, 2000). When PTHrP (1-139) was transfected into the cells instead, proliferation was increased (Massfelder et al, 1997; Falzon and Du, 2000; Tovar Sepulveda et al, 2002). This mitogenic effect required the integrity of the NLS suggesting that here the mitogenic activity of PTHrP was entirely dependent on the intracrine nuclear pathway of PTHrP. In VSM cells, the mitogenic effect of PTHrP via the intracrine pathway was also dependent upon three serines and one threonine residues between positions 119 and 138 of the C-terminus (Fiaschi-Taesch et al, 2004) suggesting that certain phosphorylation events are essential for this PTHrP activity.

The results by Falzon and Du (2000) showing an anti-proliferative effect of the PTHrP (1-34) peptide on MCF-7 breast cancer cells contradict the data obtained by two other groups demonstrating a mitogenic effect of the same peptide on these cells (Birch et al, 1995; Hoey et al, 2003). This discrepancy may be explained by the genetic variability in MCF-7 sublines (Nugoli et al, 2003). In different MCF-7 sublines, PTH1R may activate PKA, PKC and the Ca²⁺ pathway to a different extent which may lead to different proliferative activities (Maioli and Fortino, 2004b). Alternatively, the PKA/cAMP pathway may have different effects on proliferation in different MCF-7 sublines. It is noteworthy in this respect that B-Raf is able to convert cAMP from an anti-mitogenic to a mitogenic factor (Fujita et al, 2002).

Overall, PTHrP seems to predominantly act as a mitogenic factor on cancer cells. However, under certain conditions (certain type of tumor, certain features of the individual cell clone, the particular way PTHrP was administered) PTHrP may also inhibit proliferation. How easily PTHrP can switch from a mitogenic to an anti-mitogenic agent is nicely demonstrated for a C-terminal PTHrP peptide (Whitfield et al, 1992). This peptide was found to inhibit proliferation of dividing keratinocytes, yet

it was shown to trigger cell cycle entrance of quiescent cells.

B. PTHrP and invasion

Invasive behavior is a hallmark of metastasizing cancer cells. For the acquisition of an invasive phenotype, cancer cells need to coordinate the interaction of many proteins involved in adhesion, migration and proteolysis of the extracellular matrix (ECM) (Price et al, 1997). PTHrP has been found to interfere with the expression of some of those proteins. In MCF-7 breast cancer cells and PC3 prostate cancer cells, overproduction of PTHrP induced the expression of a number of integrins, in particular integrins α_6 and β_4 (Shen and Falzon, 2003; Shen et al, 2004). Elevated levels of these integrins correlated with an enhanced ability of PTHrP-treated MCF-7 cells to migrate on the integrin α_6/β_4 ligand laminin and to invade extracellular matrix. Integrin α_6/β_4 has also been shown to increase invasiveness of MDA-MB-435 breast cancer cells (Shaw et al, 1997). Modulation of invasiveness and integrin expression by PTHrP in PC-3 and MCF-7 cells required the integrity of the PTHrP-NLS suggesting that PTHrP regulates invasiveness in these cells through the intracrine pathway.

Effects of PTHrP on cellular invasiveness and on proteins involved in this process were also observed when PTHrP peptides were added exogenously. Administered to chondrocytes, PTHrP (1-141) and (1-84) peptides induced an increased expression of matrix metalloproteases MMP2, MMP3 and MMP9 (Kawashima-Ohya et al, 1998). Added to 8701-BC breast cancer cells, the PTHrP (67-86) peptide increased invasion and, at the same time, upregulated urokinase type plasminogen activator (uPA) (Luparello et al, 2003). This serine protease is involved in cancer mediated ECM degradation (Price et al, 1997) and has prognostic value for the survival of breast cancer patients (Harbeck et al, 2002). On the other hand, PTHrP (38-94) was found to reduce the ECM degrading activities of a number of breast cancer cell lines (Luparello et al, 2001).

A single-nucleotide polymorphism in the C-terminal region of the murine PTHrP revealed that also the C-terminal part of PTHrP is important for invasion. Mice carrying the Pthlh^{Pro} allele at amino acid 130 of the mature protein showed a higher susceptibility to skin tumorigenesis than mice harboring the Pthlh^{Thr} allele (Manenti et al, 2000). When transfected into the human squamous cell carcinoma line NCI-H520, Pthlh^{Pro} conferred to these cells a much greater ability to migrate than Pthlh^{Thr} (Benelli et al, 2003).

C. PTHrP and apoptosis

Escaping apoptosis enables tumor cells to survive and proceed in the neoplastic process (Naik et al, 1996). By interfering with the apoptotic machinery, PTHrP may contribute to this important step in carcinogenesis. Overexpression of rat PTHrP rendered chondrocytes resistant to serum starvation-induced apoptosis (Henderson et al, 1995). Similarly, ectopic expression of PTHrP (-5/139) protected MCF-7 breast cancer cells from apoptosis which was accompanied by a rise in the

expression of anti-apoptotic proteins Bcl-2 and Bcl-x_L (Tovar Sepulveda et al, 2002). In both cases, the anti-apoptotic PTHrP effect was mediated by the nuclear pathway of PTHrP. Also exogenous PTHrP peptides are potent anti-apoptotic factors. Treatment of chondrocytes with PTHrP (1-37) stimulated the expression of Bcl-2 in a PKA-dependent manner (Amling et al, 1997). PTHrP (1-34) and PTHrP (140-173), but not PTHrP (38-64), PTHrP (67-86) or PTHrP (107-139), were shown to protect lung cancer cells from UV-induced caspase 3 activation and apoptosis (Hastings et al, 2003). PTHrP (140-173) also prevented Fas-dependent apoptosis in these cells. Both PTHrP (1-37) and PTHrP (140-173) exerted their anti-apoptotic effects by activating PKA (Amling et al, 1997, Hastings et al, 2004). PTH1R-interacting peptides, namely PTH (1-34), can also promote apoptosis. This was demonstrated for confluent PTH1R-expressing mesenchymal stem cells (Chen et al, 2002). Interestingly, at lower cell density, the same peptide induced the inverse effect. Both effects were dependent upon cAMP demonstrating again the dual character of the cAMP signaling system. Also Ca²⁺ can be involved in pro-apoptotic effects of PTH1R ligands, as was found for the apoptosis-inducing PTH effect on PTH1R overexpressing human embryonal kidney 293 cells (Turner et al, 2000).

VI. Regulation of PTHrP expression in cancer

Given the evidence that links PTHrP expression to cancer progression, it is important to understand the mechanism(s) by which PTHrP is(are) regulated in cancer cells. PTHrP expression is mainly regulated on the transcriptional level (Inoue et al, 1993; Wysolmerski et al, 1996; Falzon, 1997; Lindemann et al, 2001). In humans, transcription of the PTHrP gene can be driven by three different promoters, P1, P2 and P3 (**Figure 2**). Of these promoters, the distal (P1) and proximal promoters (P3) were identified first (Suva et al, 1989; Mangin et al, 1990) and subsequently called P1 and P2, respectively. Later, when a third GC-rich promoter was found in between P1 and P2 (Vasavada et al, 1993), the GC-rich promoter became P2 and the proximal was renamed P3. The PTHrP transcripts that are generated by each promoter can easily be distinguished by certain non-coding exons that they specifically contain (Southby et al, 1995; Lindemann et al, 2001). This allows to assess the contribution of each promoter to the PTHrP expression in a given cell population. In solid cancers, the P3 promoter was found to be always active (Southby et al, 1995) and to increase its activity when breast cancers metastasize (Bouizar et al, 1999).

A. Regulation by Ets transcription factors

One of the first proteins that have been shown to activate the P3 promoter was HTLV-I Tax₁ (Dittmer et al, 1993). HTLV-I Tax₁ is a unique viral protein encoded by the human T lymphotropic virus type I (HTLV-I) that causes adult T-cell leukemia/lymphoma (ATLL) (Franchini, 1995). In almost all ATLL patients, the PTHrP

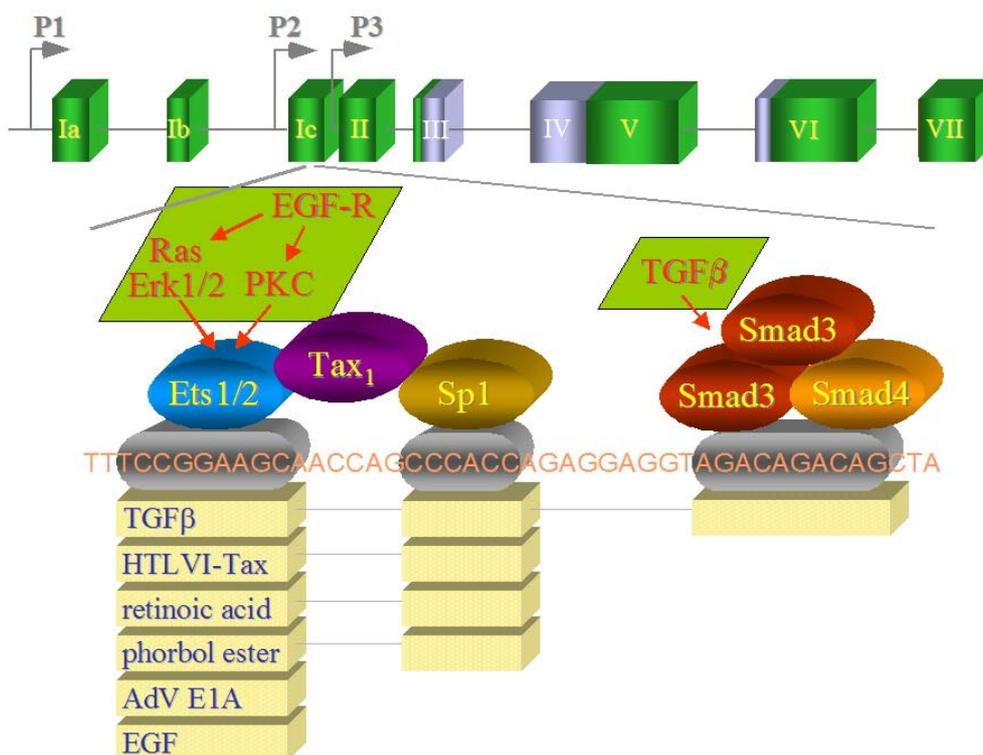


Figure 2. Organization of the human PTHrP gene. The magnified area shows the major functional elements of the PTHrP P3 promoter and the transcription factors that have been shown to interact with these DNA sequences. The yellow bars indicate which DNA binding motif/ves is/are essential for regulation of the P3 promoter by TGF β , HTLV-I Tax, retinoic acid, phorbol ester, adenovirus protein E1A (AdV E1A) or EGF. Details are given in the text.

protein level in the blood is increased (Yamaguchi et al, 1994) and PTHrP is detectable in the leukemic cells (Watanabe et al, 1990). Tax₁ is a transcriptional activator that by itself is unable to bind to DNA and, instead, interacts with transcription factors to manipulate the transcriptional machinery (Gitlin et al, 1993; Lenzmeier and Nyborg, 1999). We found that Tax₁ tethers to Ets1, a member of the Ets family of transcription factors (Dittmer, 2003), to activate the PTHrP P3 promoter. The interaction of Ets1 with the promoter takes place at a typical Ets GGAA-containing binding site in close proximity to a CCCAC element. The CCCAC element was shown to recruit Sp1 to the promoter to form an Ets1/Sp1 composite element together with the Ets recognition motif allowing Ets1 and Sp1 to cooperatively activate the promoter (Dittmer et al, 1994). Tax₁ is able to form a ternary complex with both transcription factors to further stimulate transcription from the P3 promoter (Dittmer et al, 1997).

Ets and Sp1 binding sites also play a role in P3-dependent PTHrP transcription in breast cancer cells. In MDA-MB-231 cells, TGF β -induced transcription from the P3 promoter requires the responsive elements for Ets1, Sp1 and an AGAC binding site, which was found to recruit the TGF β effectors Smad3/Smad4 to the promoter (Lindemann et al, 2001). In the presence of TGF β , Ets1 was shown to synergistically activate the PTHrP P3 promoter in concert with Smad3. In agreement with this finding, stable transfection of MDA-MB-231 cells with Smad proteins were found to increase TGF β -dependent

PTHrP secretion (Kakonen et al, 2002). Conversely, preventing TGF β -mediated Smad3 nuclear import by inhibiting p38 MAP kinase abolished the TGF β effect on PTHrP expression (Kakonen et al, 2002; Lindemann et al, 2003c).

TGF β -dependent PTHrP expression could also be diminished by PKC inhibitors (Lindemann et al, 2001). Particularly, PKC α was found to be important for the TGF β -dependent activation of the P3 promoter in breast cancer cells. This kinase is required to allow Ets1 to activate the P3 promoter in breast cancer cells (Lindemann et al, 2003a) and to maintain Ets1 protein expression in a variety of cancer cells (Vetter et al, 2004). Further evidence for PKCs being involved in P3-driven PTHrP expression is provided by studies using phorbol ester (PMA) to upregulate PTHrP expression in Ets1-deficient MCF-7 cells (Lindemann et al, 2003b). A synergistic effect between Ets2, a close relative of Ets1, and PKC ϵ was found to mediate the PMA-induced activation of the PTHrP P3 promoter. Again the integrity of the Sp1 binding site was required.

Ets1 and/or Ets2 have also been found to be potent activators of the PTHrP promoter in primary human keratinocytes (Cho et al, 2004), in P19 embryonal carcinoma cells (Karperien et al, 1997) and tumorigenic breast epithelial cell line NS2T2A1 (Cataisson et al, 2003). In addition, the Ets binding site has been reported to mediate at least in part the effect of retinoic acid (Karperien et al, 1997) and adenovirus E1A on P3 promoter activity (Foley et al, 1999). Also stimulation of

PTHrP expression by epidermal growth factor (EGF)-like factors may involve the Ets binding site (Cho et al, 2004). EGF and EGF-like factors, such as transforming growth factor and amphiregulin, are potent activators of PTHrP expression in a variety of cells (Allinson and Drucker, 1992; Burton and Knight, 1992; Ferrari et al, 1994; Heath et al, 1995; Cramer et al, 1996b; Cho et al, 2004). They are ligands of the EGF receptor (EGF-R, ErbB1) which is aberrantly expressed in many cancers (Kolibaba and Druker, 1997) and plays an important role in regulating proliferation in estrogen receptor-negative breast carcinoma cells (Biswas et al, 2000).

Ets1 and Ets2 are both involved in carcinogenesis (Dittmer, 2003; Foos and Hauser, 2004) and are targets of the Ras/MEK1/Erk1/2 pathway (Yang et al, 1996; Seidel and Graves, 2002). Activation of this pathway leads to phosphorylation and superactivation of these Ets proteins. The Ras/MEK1/Erk1/2 pathway has shown to play a role in the regulation of PTHrP expression. E.g. in rat Leydig tumor H-500 cells, activation of the Ras/MEK/Erk pathway stimulated PTHrP expression (Aklilu et al, 2000) and, in keratinocytes, dominant negative versions of the Ras and Raf protein downregulated PTHrP P3 promoter activity (Cho et al, 2004). In addition, transfection with Ras alone or in combination with Src increased PTHrP production in fibroblasts (Li and Drucker, 1994; Motokura et al, 1995; Aklilu et al, 1997). Also cotransfection of fibroblasts with Ras and mutant p53 activated PTHrP expression (Motokura et al, 1995). In particular, the Ras/mutant p53 cooperative effect might have been mediated by Ets1, as mutant p53 has been shown to physically and functionally interact with this Ets protein (Sampath et al, 2001). Given the importance of Ets1 for PTHrP expression and the involvement of both proteins in invasion, it is reasonable to suggest that Ets1 may exert part of the invasion-promoting function through PTHrP.

B. Other PTHrP regulating factors

A variety of other proteins have been shown to stimulate PTHrP expression in cancer cells. In lung cancer cells, PTHrP production is increased in response to tumor necrosis factor (TNF) and interleukin-6 (IL-6) (Rizzoli et al, 1994). In HTLV-I infected MT-2 leukaemic cells and in the human lung cancer cell line BEN, PTHrP expression can be augmented by agents that raise the cAMP level (Ikeda et al, 1993b; Chilco et al, 1998). Calcitonin and cAMP have been shown to activate the P1 and the P3 promoter (Chilco et al, 1998). In the P1 promoter, a cAMP-responsive element (CRE) could be identified that mediates these effects.

Steroids, such as 1,25-dihydroxyvitamin D₃, dexamethasone and androgens, have been found to inhibit PTHrP expression in cancer cells on the transcriptional level (Ikeda et al, 1993a; Inoue et al, 1993; Glatz et al, 1994; Rizzoli et al, 1994; Falzon, 1997; Tovar Sepulveda and Falzon, 2002; Pizzi et al, 2003). Vitamin D was shown to affect P3 and upstream PTHrP promoters (Endo et al, 1994). Dexamethasone and non-calcaemic vitamin D analogues were also demonstrated to inhibit tumor-dependent hypercalcaemia and to reduce tumor burden in

mice (Endo et al, 1994; Cohen-Solal et al, 1995; El Abdaimi et al, 1999).

There are conflicting data about the effect of estrogen, an important mitogen in mammary carcinogenesis (Keshamouni et al, 2002), on the regulation of PTHrP expression in breast cancer cells. In MCF-7 cells, both estrogen and anti-estrogen tamoxifen were shown to increase PTHrP mRNA levels in MCF-7 breast cancer cells (Funk and Wei, 1998), whereas, in KPL-3C breast cancer cells, estrogen inhibited and tamoxifen stimulated PTHrP secretion (Kurebayashi and Sonoo, 1997). Estrogen has also been demonstrated to interfere with PTHrP action by inhibiting PTHrP-induced bone resorption (Kanatani et al, 1998).

PTHrP and calcium seem to be linked in several ways. Not only can PTHrP increase the blood calcium level and intracellularly activate the calcium-signalling pathway, but it also can respond to extracellular calcium (Buchs et al, 2000; Tfelt-Hansen et al, 2003). Extracellular calcium is an important regulator of proliferation and differentiation of normal cells. Deregulation of its receptor, the calcium-sensing receptor (CaR), in cancer cells can lead to cancer progression (Rodland, 2004). CaR was shown to be responsible for the calcium-dependent activation of PTHrP transcription in H-500 cells (Tfelt-Hansen et al, 2003). CaR has also been found to upregulate PTHrP synthesis and secretion in astrocytomas, meningiomas and breast cancer cells (Chattopadhyay et al, 2000; Sanders et al, 2000). Overexpression and activation of CaR in HEK293 cells revealed that MAP kinases ERK1/2 and p38 are involved in the CaR effect on PTHrP expression (MacLeod et al, 2003).

PTHrP expression is also influenced by the substratum cells are attached to. Depending on the extracellular matrix protein pancreatic adenocarcinoma cells were grown on, PTHrP expression was either up- or downregulated (Grzesiak et al, 2004). Reduced expression of PTHrP was found when cells were plated on type I and IV collagen or laminin, whereas higher expression was observed with fibronectin or vitronectin.

Gene silencing may be another way by which PTHrP abundance is regulated. Gene silencing can be epigenetically induced by CpG island methylation which appear to occur in cancer cells in an increased rate (Jones and Laird, 1999). In the PTHrP gene, a single CpG island is located upstream of the P3 promoter (Ganderton et al, 1995; Holt et al, 1993). In lung cancer biopsies, PTHrP expression was found to be independent of the methylation status of this CpG island (Ganderton and Briggs, 2000). However, Methylation of certain CpG dinucleotides upstream of the CpG island were shown to influence PTHrP expression in renal carcinoma cell lines (Holt et al, 1993).

PTHrP expression seems also be controlled on the post-transcriptional level. Von Hippel-Landau tumor suppressor gene has been demonstrated to negatively regulate PTHrP in clear cell renal carcinoma via a post-transcriptional mechanism (Massfelder et al, 2004). In oral squamous carcinoma cells, TGF β has been shown to stimulate expression of PTHrP in part by increasing the stability of its RNA (Sellers et al, 2002). In osteosarcoma

cells, serum increased PTHrP expression by both upregulation of transcription and stabilization of PTHrP RNA (Falzon, 1996). There is also evidence, that in prostate cancer, PSA inactivates PTHrP by proteolytic cleavage (Cramer et al, 1996a; Iwamura et al, 1996).

VII. Concluding remarks

Originally identified as a tumor-derived factor that induces the paraneoplastic syndrome HHM, it is now generally accepted that PTHrP also plays a role in stimulating local osteolysis, thereby, facilitating growth of metastatic cancer in the bony tissue. In addition, PTHrP has the potential to regulate proliferation, invasion and apoptosis in cancer cells in a way that is beneficial for tumor growth. On the other hand, PTHrP has shown to have anti-mitogenic effects and to inhibit angiogenesis (Bakre et al, 2002) suggesting that PTHrP may also act as an anti-tumor factor. Which of these activities of PTHrP prevail might depend on the type of tumor and tumor stage.

While the prognostic value of PTHrP in human cancer is still unclear, PTHrP may be a useful predictive marker for anti-PTHrP treatment response in bone metastasis. A number of attempts have been made to suppress PTHrP expression in cancer cells. Factors that downregulate PTHrP transcription, such as vitamin D analogues and modified guanosine nucleotides, have been successfully used to inhibit PTHrP expression, hypercalcaemia, osteolysis and bone metastasis in mice (El Abdaimi et al, 1999; Gallwitz et al, 2002). PKC inhibitors, novel anti-cancer drugs that have entered clinical trials (Roychowdhury and Lahn, 2003), may also be suitable to attenuate PTHrP synthesis on the transcriptional level (Lindemann et al, 2001). By a different mechanism, prostate secretory protein PSP-94 was found to suppress the ability of prostate cancer cells to synthesize PTHrP, to grow and to form skeletal metastases in rats (Shukeir et al, 2004). In another approach, PTHrP activity is inhibited by an anti-PTHrP antibody, originally shown by Guise et al (1996) to reduce formation of bone metastasis in tumor-bearing mice and now being humanized (Sato et al, 2003) for the use in clinical trials. Further analysis of the mechanism underlying the regulation of PTHrP expression in cancer is needed to identify further targets for an anti-PTHrP therapy. It is also important to identify the PTHrP-responsive genes and to clarify the role of nuclear PTHrP in order to understand the action of PTHrP in cancer.

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