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The Nucleus: A Target Site for Parathyroid Hormone-Related Peptide (PTHrP) Action

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Abstract It is becoming increasingly apparent that parathyroid hormone-related peptide (PTHrP) modulates cellular function in a dual mode of action: first, by binding and activating its cognate cell surface G-protein-coupled receptor and, second, by direct intracellular effects following translocation to the nucleus and/or nucleolus of the target cell. Little is presently known about the mechanisms and events that determine the timing and degree of PTHrP nuclear translocation or the role it may serve in normal or dysregulated cellular function. Clarifying the nuclear actions of PTHrP would add significantly to our present understanding of this protein as a signaling molecule during embryonic development and as an oncoprotein whose expression in many tumors correlates with increased tumor aggressiveness and propensity for metastasis. J. Cell. Biochem. 70:193–199, 1998.

Key words: parathyroid hormone-related peptide; nucleus; nucleolus; intracrine actions

Parathyroid hormone-related peptide (PTHrP) was initially identified as a tumor-derived, secretory protein with structural similarity to parathyroid hormone (PTH), the major regulator of calcium homeostasis [for review, see Philbrick et al., 1996]. PTH and PTHrP bind to a common G-protein-coupled cell surface receptor (PTH/PTHrP or type 1 receptor) that recognizes the N-terminal (1–34) region of these peptides [Jüppner et al., 1991; Abou-Samra et al., 1992]. Hence, when tumor-derived PTHrP enters the circulation, it activates receptors in classic PTH target organs such as bone and kidney and elicits PTH-like bioactivity that gives rise to the common paraneoplastic syndrome of malignancy-associated hypercalcemia.

Although initially discovered in malignancies, PTHrP expression was subsequently demonstrated in a variety of normal adult and fetal tissues. Unlike PTH, PTHrP does not circulate in appreciable amounts in normal subjects. Rather, it is thought to exert its biological effects locally [Goltzman et al., 1989; Philbrick et al., 1996] by binding and activating the type 1 cell surface receptor, which mediates at least some of the local paracrine/autocrine actions of PTHrP and the endocrine effects of PTH.

While examining the subcellular distribution of transiently expressed PTHrP, it was noted that in ~5–10% of transfected cells the peptide also localized to the nucleolus. This finding was unexpected because native PTHrP includes a prepro sequence (leader sequence) that would normally target the nascent protein to the endoplasmic reticulum for secretion. An explanation for this unanticipated observation was provided by further examination of the PTHrP sequence. Amino acids 87–107 of the mature form of the protein encode a bipartite nuclear localization signal (NLS) consisting of two basic clusters separated by a spacer region, analogous to the prototypical nucleoplasmin NLS [Henderson et al., 1995]. This sequence also bears homology to sequences in human retroviruses (HTLV-1 and HIV-1) shown to target viral regulatory proteins to the nucleolus. Subsequent studies have shown that elimination of the NLS from preproPTHrP effectively abolished intranuclear localization of the recombinant protein. Moreover, the NLS alone was capable of translocating a heterologous cytoplasmic protein, β-galactosidase, to the nucleolus

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when expressed as a fusion protein in COS-7 cells.

In addition, experimental evidence has been presented showing that endogenous PTHrP localizes to the nucleolus of murine bone cells in vitro and in situ [Henderson et al., 1995]. Immunogold labeling for the endogenous peptide was observed over the dense fibrillar component of nucleoli, a subnucleolar structure thought to represent the major site of transcription of genes coding for rRNA. These findings provided, for the first time, unequivocal evidence for a nuclear site of PTHrP action. Subsequent studies have confirmed these initial observations in a human keratinocyte cell line (HaCaT) [Lam et al., 1997] and in cultured vascular smooth muscle cells [Massfelder et al., 1997], although, in the latter case, the intranuclear localization of endogenous PTHrP was diffuse within the nucleoplasm and not exclusively nucleolar.

This review explores the nuclear actions of PTHrP by addressing the following questions: (1) How does PTHrP, a secretory protein, gain access to the cytoplasm? (2) Once in the cytoplasm, how is its transport to the cell nucleus regulated? (3) How does nuclear/nucleolar PTHrP modulate cellular function? and (4) What are the in vivo consequences of nuclear signaling by PTHrP?

GAINING ACCESS TO THE CYTOPLASM

It is rather intriguing that PTHrP can localize to the cell nucleus because it contains a signal sequence that would direct the nascent protein to the secretory pathway. How could a secreted protein gain access to the cytoplasmic compartment for subsequent translocation to the nucleus? Cytoplasmic localization of PTHrP, and for that matter any other secretory protein, could be accomplished by a number of distinct and not necessarily mutually exclusive pathways. One potential mechanism may involve alternative splicing leading to the generation of *PTHrP* mRNA species missing the exon encoding for the signal peptide. However, such *PTHrP* transcripts have not been described. In addition, nucleolar localization of the peptide has been observed in cells transfected with the *PTHrP* cDNA.

A number of peptide growth factors (bFGF [Bouche et al., 1987; Baldin et al., 1990; Hill et al., 1992], aFGF [Imamura et al., 1990], FGF3 [Kiefer et al., 1994], PDGF [Maher et al., 1989], and angiogenin [Moroianu and Riordan, 1994]) are known to modulate cellular function in a dual mode of action: first, by activation of classical signal transduction pathways, and, second, by nuclear actions that critically rely on an endocytosis-dependent pathway. It would appear that, after binding to cell surface receptors, the ligand–receptor complex is internalized and the ligand translocates to the nucleus and/or nucleolus of the target cells (Fig. 1a). In a number of systems, nuclear translocation is an absolute requirement for the mitogenic activity and induction of angiogenesis by growth factors [Imamura et al., 1990; Moroianu and Riordan, 1994]. Although endocytosis following ligand binding has been documented for G-protein-linked receptors such as the type 1 PTH receptor, it is usually associated with receptor downregulation and ligand degradation [Sibley and Lefkowitz, 1985; Mitchell et al., 1990; Huang et al., 1995). However, the novel splice variant of the type 1 PTH receptor, which is primarily found intracellularly, may represent a form of the receptor used to internalize the intracellularly targeted PTHrP ligand [Joun et al., 1997]. Alternatively, endocytosis could be mediated by a receptor that recognizes the N-terminal domain of PTHrP but is distinct from the classic type 1 receptor. The presence of high-capacity, low-affinity binding sites for PTHrP (1–36), in association with high-affinity receptors on squamous carcinoma cells, gives further credence to this pathway [Orloff et al., 1992, 1995]. Similarly, a surface binding protein may recognize other regions of the protein, including the NLS, and internalize the peptide, as has been reported for the HIV-1 Tat protein [Weeks et al., 1993]. Irrespective of the nature of such a "receptor," lack of firm experimental support makes this highly attractive mechanistic alternative only speculative.

Studies in other systems have advanced the notion that secreted proteins can be diverted from the default secretory pathway and targeted to the nucleus by alternative initiation of translation at non-AUG codons, in particular CUG (Fig. 1b). Hence, the choice of initiation codon changes the subcellular fate of the protein. In the notable example of FGF-3, the subcellular fate of the N-terminally extended product arises from the competition between secretory and nuclear targeting signals within the same molecule [Kiefer et al., 1994]. Nevertheless, the structural features in FGF-3 that facilitate this dual targeting are absent from

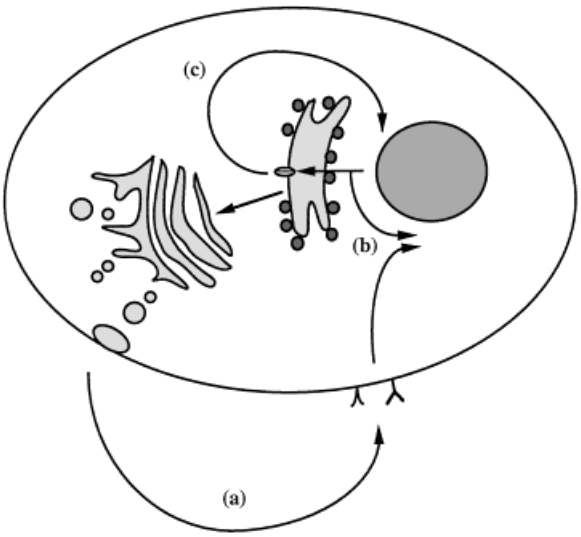

Fig. 1. Potential pathways used by PTHrP to gain access to the cytoplasm. **a:** Secreted PTHrP undergoes endocytosis at the cell surface in a "receptor"-mediated manner. Endocytosis could be mediated by the type 1 receptor or a binding protein distinct from the receptor that recognizes either the N-terminal domain or other regions of the PTHrP protein. **b:** Initiation of translation of *PTHrP* mRNA downstream from the initiator methionine generates a protein with a shorter signal peptide. Such a protein localizes within the cytoplasmic compartment because translocation through the membrane of the endoplasmic reticulum is now impaired. **c:** PTHrP that has translocated into the ER lumen "dislocates" back to the cytoplasm by the translocation unit or another transporter system.

PTHrP. However, initiation of translation occurring downstream from the initiator ATG at any of the four CUG codons within the prepro sequence of PTHrP would result in the formation of a shorter signal peptide. The interaction of such a truncated signal sequence with signal recognition particles and its subsequent translocation through the membrane of the endoplasmic reticulum would undoubtedly be impaired. As such, the translated protein would be localized within the cytoplasmic compartment whereby it could gain access to the cell nucleus on the strength of its intact and fully functional NLS.

The notion that secretory proteins can be reverse translocated or "dislocated" from the endoplasmic reticulum (ER) lumen to the cytoplasm has emerged recently from studies on protein degradation and challenges the established concept that translocation of proteins into the ER is an irreversible process. Emerging from these studies is the radically novel notion that misfolded proteins within the ER are exported to the cytosol for presentation to the cytoplasmic ubiquitin-proteosome machinery for degradation [Kopito, 1997]. Although initially described for mutant integral membrane proteins (CFTR and SEC61) and newly synthesized class I heavy chains following human CMV infection, the cytoplasmic degradation pathway has been observed for a number of secretory proteins. Mutant forms of yeast secretory protein prepro alpha factor, α_1-antitrypsin and carboxypeptidase Y (CPY), are proposed to undergo retrograde translocation from the ER for degradation by the cytoplasmic ubiquitin-proteosome machinery. In all of these cases, improper folding appears to serve as the signal for dislocation, ubiquitination, and degradation. Is this applicable to wild-type proteins? Perhaps, because PTHrP itself has been reported to serve as a substrate for the ubiquitin proteolytic system [Meerovitch et al., 1997]. In fact, PTHrP is the first wild-type secretory polypeptide shown to undergo ubiquitin-dependent proteolysis, and it raises the possibility that additional signals must exist to identify wild-type proteins targeted for dislocation. This notion is further substantiated by the observation that toxins such as ricin and Shiga are capable of traveling retrograde through the secretory pathway to the ER and translocating through the ER membrane to the cytoplasm [Sandvig and Deurs, 1994]. It is conceivable, therefore, that under certain circumstances secreted proteins may undergo recruitment, via interactions with sequestering proteins, chaperones, or "receptors", and subsequent translocation to the cytoplasmic compartment (Fig. 1c). At present, it is unclear as to whether this may occur by reversal of the translocation machinery or by a distinct transport system and whether it always leads to proteolytic degradation. Irrespective of the operative mechanism, reversal of the early steps of protein commitment to secretion makes this pathway a plausible mechanistic alternative for the translocation of PTHrP from the ER lumen to the cytoplasm.

REGULATION OF NUCLEAR IMPORT

Selective protein import into the nucleus proceeds through the nuclear pore complex and requires the interaction between the NLS and an importinlike NLS receptor complex [for review, see Görlich and Mattaj, 1996]. The NLS is recognized and bound by the 60-kDa subunit of importin, importin α. The amino terminus of importin α then binds to the second subunit of

the complex, the 90-kDa importin β protein, which in turn mediates docking of the NLS-importin complex with the nuclear pore complex. Translocation of the NTS-importin complex through the nuclear pore requires energy that is derived from hydrolysis of GTP by the GTPase Ran/TC4. Following translocation, the importin α subunit accompanies the NLS-containing protein into the nucleus, and importin β remains associated with the inner side of the nuclear envelope. Whether this pathway is operative in translocating cytoplasmic PTHrP to the nucleus remains to be determined. However, the absolute requirement for the NLS in targeting PTHrP to the nucleus argues strongly for the classic pore route as being operative in this process.

Whereas the NLS determines the specificity, the timing and the rate of import into the nucleus is often modulated by covalent modification of the transported proteins. Specifically, the redistribution of nuclear proteins between cytoplasmic and nuclear compartments has been associated with a change in phosphorylation status. Phosphorylation proximal to nuclear localization sequences by p34^{cdc2} kinase has been demonstrated for a number of nuclear proteins such as SV-40 T antigen, p53, c-abl, and lamin A/C and likely serves as an element of nuclear import regulation [Jans et al., 1991]. Interestingly, a consensus motif (K-T^{85}-P-G-K) for phosphorylation by p34^{cdc2} kinase lies immediately upstream, and overlaps, the PTHrP NLS (Fig. 2). Its presence, therefore, suggests that phosphorylation at this position may modulate the timing of PTHrP nuclear import.

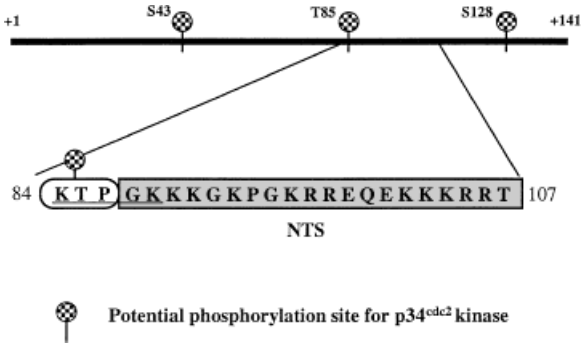

Fig. 2. Potential phosphorylation sites for p34^{cdc2} in PTHrP. The PTHrP nucleolar targeting sequence (NTS), encompassing amino acids 87 and 107 of the mature protein, is preceded by a consensus sequence (underlined) for p34^{cdc2} kinase phosphorylation (T85). S43 and S128 constitute other potential sites for phosphorylation by this kinase.

Is PTHrP a substrate for phosphorylation by p34^{cdc2} kinase and is phosphorylation associated with regulation of nuclear translocation? Recent studies have provided convincing in vitro evidence that PTHrP is indeed a phosphorylation substrate for p34^{cdc2} kinase [Lam et al., 1997, Nguyen et al., 1997]. Phosphorylation occurs at T85 and likely on serine residues of PTHrP, which comprise imperfect consensus sites [Nguyen et al., 1997]. The role of phosphorylation of T85 on nuclear translocation has been examined by using site-directed mutagenesis [Lam et al., 1997]. The PTHrP(T85A) mutant that precludes phosphorylation is associated with nuclear and nucleolar localization of the protein, whereas the T85E mutation that functionally simulates phosphorylation causes nuclear exclusion. Inhibition of nuclear import by phosphorylation of T85 can be ascribed to either inactivation of the NLS or to induction of cytoplasmic anchoring.

Is nuclear translocation of PTHrP related to the cell cycle? In the human keratinocyte cell line HaCaT, PTHrP localizes to the nucleolus at G_1 and is excluded from the nucleus from the start of the S phase to mitosis [Lam et al., 1997]. These findings tend to support a cell-cycle-dependent nuclear exclusion of PTHrP and implicate a nuclear function for PTHrP at G_1. In vascular smooth muscle cells, nuclear PTHrP has been observed in cells that are dividing or in the process of completing cell division, suggesting that nuclear translocation is associated with activation of the cell cycle [Massfelder et al., 1997]. Whether these differences are a reflection of the methodology employed or whether they reflect truly distinct nuclear actions of PTHrP remains to be defined.

NUCLEAR ACTIONS

The nucleolar localization of PTHrP implicates a role for the protein at this subnuclear organelle. Because the major function associated with the nucleolus is the biogenesis of ribosomes, it is conceivable that nucleolar PTHrP modulates cellular functions by influencing this process. The nucleolar accumulation of PTHrP could alter rRNA synthesis by modulating the activity of RNA polymerase I, as has been reported for bFGF [Kiefer and Dickson, 1995], the *Rb* gene product [Cavanaugh et al., 1995], and SV40 large T antigen [Zhai et al., 1997]. Alternatively, PTHrP could influence ri-

bosome assembly and/or function by interacting with ribosomal proteins. Irrespective of the mechanism, it remains to be established in what way these changes influence cellular function. In other systems, nucleolar isoforms of growth factor have been associated with activation of ribosomal gene transcription [Bouche et al., 1987] and inhibition of DNA synthesis and cell proliferation [Kiefer and Dickson, 1995].

Could additional actions be attributed to nuclear PTHrP? The diffuse nucleoplasmic localization of PTHrP in vascular smooth muscle cells is certainly suggestive of alternative intranuclear effects by this protein. Interaction of PTHrP with other proteins within the nucleus or with nucleic acids may be necessary for the observed cell cycle activation. Such interactions may result in altered regulation of gene transcription, as has been described for bFGF [Nakanishi et al., 1992].

PHYSIOLOGICAL CONSEQUENCES OF NUCLEAR PTHrP SIGNALING

Accumulating in vitro and in vivo evidence indicates that the secreted and nuclear forms of PTHrP have distinct effects on cellular function and that its "intracrine" effects may be different from the paracrine/autocrine response. Expression of PTHrP forms containing the NLS prolong survival of chondrocytes under conditions that promote programmed cell death (serum withdrawal) [Henderson et al., 1995]. Moreover, constitutive expression of the nucleolar (mature) form of PTHrP in stably transfected populations of cells derived from the clonal chondrocytic cell line CFK2 inhibit the expression of differentiation-associated matrix proteins. However, wild-type preproPTHrP acts as a potent mitogen and as an inhibitor of differentiation [Henderson et al., 1996]. Because the expression levels of differentiation markers are not altered by addition of PTHrP-(1–34) to the culture medium, this effect is not mediated through the common PTH/PTHrP receptor but rather through an intracellular mechanism of action, likely at the level of the nucleolus.

In cultures of vascular smooth muscle cells, addition of PTHrP forms with intact amino termini (1–36), (1–86), and (1–141) markedly inhibit proliferation ($IC_{50} \sim 1$ nM), whereas mid-region and carboxy-terminal fragments have no effect [Massfelder et al., 1997]. In contrast, stable overexpression of the mature PTHrP form that localizes to the nucleus stimulates cellular proliferation. Substitution of either or both of the bipartite NLSs dramatically reduces nuclear localization and blocks proliferation in these cells. Bromodeoxyuridine incorporation into aortic vascular smooth muscle cells in vivo is lower in PTHrP-null homozygous embryos [Karaplis et al., 1994] than in their wild-type littermates.

Additional in vivo evidence supporting the dual function of PTHrP has come from gene targeting studies. Mice homozygous for PTHrP gene ablation are born alive (meeting Mendelian expectations) but die soon after birth because of a multitude of skeletal deformities that arise as a consequence of diminished proliferation and inappropriate differentiation of chondrocytes in the developing skeleton [Amizuka et al., 1994; Karaplis et al., 1994]. It contrast, animals homozygous for the cell surface type 1 PTH receptor-null allele exhibit a more severe phenotype characterized by early embryonic lethality (day 14.5 of gestation) [Lanske et al., 1996]. The embryonic lethality of the receptor-null mice is very intriguing. Does it implicate the necessity for circulating PTH to partly compensate for PTHrP deficiency? This notion is unlikely given that PTH expression does not normally occur before day 15.5 of gestation, whereas PTHrP is expressed widely in the embryo proper by that time. Alternatively, the more severe phenotype seen in mice missing the receptor gene may bear relevance to the dual role of PTHrP on cellular function. It is likely that normal embryonic development requires the coordinated activity between the amino-terminal end of the protein acting on the cell surface receptor and the intracellular form functioning at the level of the nucleus (Fig. 3). In the absence of the amino terminal receptor, the unopposed nuclear effects of PTHrP would lead to severely dysregulated cell growth and/or differentiation and thereby early demise of the receptor-negative mutants. In contrast, ablation of the ligand would eliminate both receptor-mediated and nuclear activities, leading to a more "coordinated" cellular impairment and hence a less severe phenotype.

It is clear from the foregoing discussion that consolidating a physiologic role for nuclear PTHrP will necessitate addressing the question in the intact organism. This in vivo approach will require the generation of an animal model that permits the selective study of a strategically placed mutation(s) in the PTHrP gene

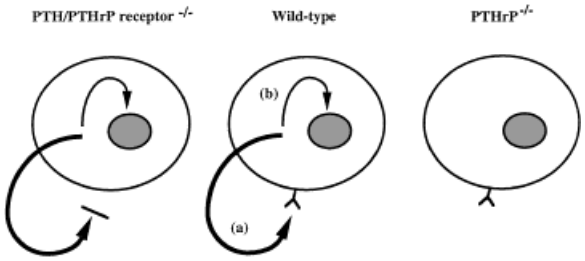

Fig. 3. PTHrP signaling in normal, PTH/PTHrP receptor-, and PTHrP-negative cells. In normal cells **(center),** the biological actions elicited by PTHrP require the coordinated activity between its **(a)** autocrine/paracrine and **(b)** intracrine actions. In receptor-deficient cells **(left),** extracellular signaling is absent, whereas the nuclear effects proceed unabated. This leads to severely dysregulated cell function and to the more severe phenotype of the receptor-null animals. In contrast, both PTHrP signaling pathways are abolished in ligand-deficient cells **(right),** leading to a more "coordinated" functional disruption.

that interferes with nuclear translocation and signaling while maintaining signaling through the cell surface amino terminal receptor when introduced into the mouse genome. Such a genetically modified mouse, with intact but mutated PTHrP alleles, would be amenable to studies addressing the physiological consequences associated with impaired nuclear localization of the protein. Generating mice expressing mutant forms of PTHrP is now feasible by resorting to modifications of the standard knockout technology that offers the opportunity to introduce subtle mutations into the gene of interest without altering the rest of the murine genome [Wu et al., 1994]. This methodology is ideally suited for examining in the intact organism how the absence of PTHrP nuclear signaling alters normal cellular function. The findings will have important implications in elucidating the intracrine actions of this protein.

CONCLUSIONS AND PROSPECTS

Previously, the effects of PTHrP were considered only in terms of its interaction with the amino-terminal receptor. Studies now indicate that the capacity of PTHrP to influence normal cellular function must also be considered in terms of its effects at the level of the nucleus. It becomes essential, therefore, to understand the mechanism whereby PTHrP translocates to the nucleus and influences cellular function. Any impairment in this interaction may have critical consequences on the role of PTHrP as a signaling molecule during fetal development and as an oncoprotein during the progressive stages of neoplasia.

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