Phosphorylation at the Cyclin-dependent Kinases Site (Thr\textsuperscript{85}) of Parathyroid Hormone-related Protein Negatively Regulates Its Nuclear Localization*

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Parathyroid hormone-related protein (PTHrP) is expressed by a wide variety of cells and is considered to act as a secreted factor; however, evidence is accumulating for it to act in an intracrine manner. We have determined that PTHrP localizes to the nucleus at the G\textsubscript{1} phase of the cell cycle and is transported to the cytoplasm when cells divide. PTHrP contains a putative nuclear localization sequence (NLS) (residues 81–94) similar to that of SV40 T-antigen, which may be implicated in the nuclear import of the molecule. We identified that Thr\textsuperscript{85} immediately prior to the NLS of PTHrP was phosphorylated by CDC2-CDK2 and phosphorylation was cell cycle-dependent. Mutation of Thr\textsuperscript{85} to Ala\textsuperscript{85} resulted in nuclear accumulation of PTHrP, while mutation to Glu\textsuperscript{85} to mimic a phosphorylated residue resulted in localization of PTHrP to the cytoplasm. Combined, the data demonstrate that the intracellular localization of PTHrP is phosphorylation- and cell cycle-dependent, and such control further supports a potential intracellular role (10, 34, 35) for PTHrP.

Parathyroid hormone-related protein (PTHrP)\textsuperscript{1} is widely expressed (1–3) and acts as a paracrine, and possibly an autocrine and intracrine, factor. However, its intracellular roles have not been fully defined. Recently a nuclear localization signal (NLS) was identified within PTHrP and deletion of this motif prevented PTHrP from entering the nucleus, maintaining it as a cytoplasmic protein (4). Nucleolar localization of PTHrP delays apoptosis in chondrocytes (4) and increases smooth muscle cell proliferation (5). PTHrP has also been linked to the ras signaling pathway (6) and the hedgehog signaling pathways (7, 8), indicating its importance in regulating growth and differentiation. PTHrP expression is cell cycle-dependent (9, 10) and PTHrP mRNA expression responds to mitogenic factors only at the G\textsubscript{1} phase of the cell cycle (10). Furthermore, PTHrP localizes to the nucleolus at the G\textsubscript{1} phase of the cell cycle (10).

Cyclin-dependent kinases control the progression of the various phases of the cell cycle (11). CDKs are activated at different phases of the cell cycle by the formation of cyclin-CDK complex and deactivated when their cyclin partner is degraded. The prototype cyclin is CDC2, which associates with cyclin B and regulates the transition between the G\textsubscript{2} and M phases of the cell cycle. Cyclin E-CDK2 and cyclin A-CDK2 complexes are involved in the G\textsubscript{1} to S transition, while CDK4 and CDK6 associated with the D-type cyclins are involved in the progression through G\textsubscript{2} (12). In addition to direct regulation of the cell cycle, cyclins and CDKs have functions in other biological processes such as transcriptional control (13), and protein phosphorylation by CDC2 and CDK2 results in increase of affinity for the cytoplasm of some molecules containing an NLS (14).

A stretch of basic residues, or a pair of basic residues separated by a 10–12 amino acid spacer to form a bipartite NLS, characterizes NLSs. The archetypal protein used in nuclear localization studies is the SV40 T-antigen where nuclear/cytoplasmic localization is regulated by phosphorylation. CK2 and/or CDC2-CDK2 phosphorylations at sites near the NLS determine the rate and amount of localization within the nucleus. PTHrP at Thr\textsuperscript{85}, which is immediately prior to its NLS, with the use of wild type and mutant green fluorescent protein
GFP-PTHrP fusion proteins overexpressed in HaCaT cells, it was found that phosphorylation of Thr85 resulted in cytoplasmic retention/nuclear exclusion. The results of these studies indicate a cell cycle-dependent nuclear exclusion of PTHrP from the start of S phase to mitosis, providing support for a tightly regulated nuclear function for PTHrP at G1.

EXPERIMENTAL PROCEDURES

Cell Culture—The spontaneously immortalized human keratinocyte cell line, HaCaT, which expresses PTHrP, was a kind gift from Professors Y. Fuesang and E. Poone (in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% FBS at 37 °C and equilibrated with 5% CO2, as described previously (19). The human T lymphoid cell line (CEM) was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 units/ml of penicillin, and 50 μg/ml streptomycin.

Fluorescence Immunocytochemistry—70% confluent HaCaT cells fixed and stained with a fluorescein-labeled monoclonal antibody against amino terminus PTHrP as described previously (10, 20). The fixed and stained cells were allowed to grow for 48 h before labeling experiments were performed. The cells were detached, washed twice with 10 ml of phosphate-free DMEM, then placed into phosphate-free DMEM containing 5% CO2, as described previously (19). The human T lymphoid cell line, HaCaT, which expresses PTHrP, was a kind gift from Professors Y. Fuesang and E. Poone (in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% FBS at 37 °C and equilibrated with 5% CO2, as described previously (19). The human T lymphoid cell line (CEM) was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 units/ml of penicillin, and 50 μg/ml streptomycin.

Protein Kinase Assays—Protein kinase assays were performed in a buffer containing 50 mM MOPS, pH 7.0, 10 μM magnesium acetate, 0.25 mM γ-32P-ATP (500 cpm/pmol), 0.1% (v/v) Tween 80, and varying concentrations of substrate and diluted enzyme (5 μg/ml final concentration) made up to 40 μl total volume. The enzyme was diluted in a buffer containing 50 mM MOPS, pH 7.0, 0.1% (v/v) Tween 80, and 1 mM dithiothreitol. The reactions were incubated for 20 min at 30 °C, then 20 μl of the reaction mixture spotted onto Whatman P-81 paper, washed and dried, and then quantitated as described in Pearson et al. (21).

For comparison of PTHrP phosphorylation by various cyclin-CDK combinations (Table I) and kinetic studies (Table II), 10 μl of varying concentrations of PTHrP and 10 μl of appropriately diluted cyclin-CDKs were added together. Reactions were initialized by 50 mM HEPES, pH 7, 1 mM dithiothreitol, 30 mM MgCl2, 750 μM ATP, and 5 μCi of γ-32P-ATP. All reactions were performed at 30 °C. For the cyclin-CDK comparison studies, 1 μg of PTHrP (50–69) and PTHrP (67–94) were used, together with 3 μg of histone H1. For cyclin E-CDK2 and cyclin A-CDK2, reactions were carried out for 10 min. For cyclin B-CDK1, the reaction was for 30 min. For kinetic studies cyclin E-CDK2 and cyclin A-CDK2 reactions were for 5.5 min and cyclin B-CDK1 was for 15 min. Reactions were stopped by spotting 20 μl of the 30-μl reaction onto P-81 paper, washed and dried, and then quantitated as described in Pearson et al. (22).

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Phospho-peptide Analysis—Peptides and proteins were phosphorylated as described in the protein kinase assay protocol above. γ-32P-ATP (500 cpm/pmol) for phospho-peptide mapping experiments and unlabeled ATP were used for HPLC and mass spectrometry experiments. Phospho-peptides were carried out in a total volume of 320 μl, incubated for 1 h at 30 °C, and terminated with 100 mM EDTA and 50 μg/ml trypsin. Removal of free γ-32P-ATP from phosphorylated peptides and proteins was performed by application to a Ag1-X8 column (Bio-Rad Laboratories, Hercules, CA) that was equilibrated with 5 bed volumes of 0.1% (v/v) trifluoroacetic acid. Unbound PTHrP (67–94) was eluted using 6 ml of 0.1% (v/v) trifluoroacetic acid, while PTHrP (1–141) was eluted using 10 ml of 0.1% (v/v) trifluoroacetic acid. Radioactive eluates were pooled and applied to a Sep-Pak C18 cartridge (Waters, Milford, MA) activated previously according to manufacturer’s instructions. Phospho-proteins and phospho-peptides bound to the Sep-Pak cartridges were washed twice with 10 ml of 0.1% (v/v) trifluoroacetic acid, eluted with 6 ml of 60% (v/v) acetonitrile, then vacuum-dried. HPLC and Mass Spectrometry of PTHrP—PTHrP (67–94) was phosphorylated as described above but using unlabeled ATP and chromatographed on a Vydac 208TP52, C8 reversed phase column using a SMART™ HPLC system (Amersham Pharmacia Biotech), with a linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid at a gradient rate of 1% acetonitrile/min and a flow rate of 100 μl/min (monitored at 214 and 280 nm). As control, an equivalent amount of unlabeled phosphorylated PTHrP was reacted and subject to identical elution protocols. Peaks corresponding to phosphorylated and unphosphorylated PTHrP were collected and their identity confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on a Voyager DE (PerSpective Biosystems, Farmingham, MA) mass spectrometer using a-cyano-4-hydroxycinnamic acid as the matrix.

Analysis of Tryptic Fragments of PTHrP—PTHrP (67–94)—5 μg of proteins or peptides phosphorylated with γ-32P-ATP were digested at 37 °C for 24 h with 1 μg of trypsin (Promega) in 100 μl of trypsin digest buffer (50 mM NH4HCO3, pH 8.3, and 10% (v/v) acetonitrile). The peptide fragments were purified using HPLC through a Vydac 208TP52, C8 reversed phase column and confirmed with MALDI-TOF mass spectrometry as described above. Theoretical masses of each tryptic digest product were used to compare with the experimental masses.

Two-dimensional Phospho-peptide Mapping—Two-dimensional phospho-peptide mapping of trypsin-digested phosphorylated PTHrP was performed as described by Beemon and Hunter (24). The plates were exposed in a PhosphoImager cassette or an x-ray cassette for visualization.

32P-labeling of Cells Transiently Transfected with GFP-PTHrP—10–20 μg of DNA (see below for plasmid constructs) was made up in 150 mM NaCl to a final volume of 50 μl and then added to COS-1 cells that were grown to 80% confluence in a 76-cm² tissue culture plate, trypsinized, pelleted, and resuspended in 200 μl of fresh DMEM 10% FBS. The cells were electroporated in 0.4-cm gap size cuvettes at 975 microfarads, 200 V with the time constant between 40 and 42 ms using a Gene Pulser. Half of the wells were then replaced into 10 ml of DMEM 10% FBS and allowed to recover for 4–6 h. The medium was changed once the cells reattached. The cells were allowed to grow for 48 h before labeling experiments were performed. Transiently transfected COS-1 cells were washed three times in phosphate-free DMEM, then placed into phosphate-free DMEM containing 1% FBS for 2 h, 1 μCi of 32P in the form of orthophosphoric acid in water (NEN Life Science Products) was added to the medium and left for 5 h. The cells were washed three times in phosphate-buffered saline, and harvested in 0.8 ml of lysis buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 50 mM NaF) plus protease inhibitors (Complete™, Roche Molecular Biochemicals GmbH, Mannheim, Germany). Lysates were diluted in 1 volume of the same buffer and precleared with Pansorbin (Calbiochem) for 1 h at 4 °C. The precleared lysates were then centrifuged (13,000 rpm, 5 min, 4 °C), and 3 μl of rabbit polyclonal anti-GFP antibody (CLONTECH) was added to the supernatant and incubated overnight at 4 °C. Immune complexes were then collected with 10 μl of protein A-Sepharose CL-4B (Amersham Pharmacia Biotech), washed five times with lysis buffer, and resolved by SDS-polyacrylamide gel electrophoresis. Proteins were visualized following Blue-staining. The gel was then dried, and radioactivity was detected using autoradiography. Bands that corresponded in size to those of GFP-PTHrP (1–141) (45 kDa) were excised from the gel, digested with trypsin, and analyzed by two-dimensional phospho-peptide mapping.

Centrifugal Elutriation—Elutriation of CEM cells was performed in...
the Beckman J6 ME centrifuge with a JE-5 elutriation rotor, at 1500 rpm, 23 °C. The elutriation was carried out in RPMI medium with 5% fetal bovine serum, 2 mM dithiothreitol, and 5 mM EDTA. The G1 cell population was plated in RPMI medium with 10% fetal bovine serum, and fractions were collected at required time points.

Flow Cytometry—Cell cycle distributions of fractions of elutriated cells were determined by staining with propidium iodide and analysed performed using the CellFIT program resident in a FACScan flow cytometer (Becton Dickinson, Lincoln Park, NJ).

**RESULTS**

Fluorescence immunocytochemistry and CLSM of PTHrP expression in a asynchronous proliferating culture of HaCaT cells showed that PTHrP was localized to the nucleolus and the cytoplasm in cells that show a G1 phase phenotype (Fig. 1, A and B: i). In cells that show a G2 and M phenotype, which have a more intense nuclear stain, PTHrP was not detected in the nucleolus but was expressed at higher levels in the cytoplasm (Fig. 1, A and B: ii and iii). PTHrP expression was greatest in actively dividing cells (Fig. 1, A and B, iii). Because of this interesting observation, we looked at the sequence of the mature PTHrP protein compared with that of various nuclear proteins. The amino acid sequence of PTHrP (Fig. 2) showed similarities with the Ccn motifs of SV40 T-antigen, p53, c-Myc, and A-MYB (13, 17). The Ccn motif included a putative NLS with the amino acid string of basic residues Lys84-Thr-Pro-Gly-Lys88. The NLS lies within amino acids 85 and 94, thereby implicating Thr85, the only phosphorylated amino acid present.

To ascertain the stoichiometry of phosphorylation, PTHrP-(67–84) phosphorylated with recombinant cyclin A-CDK2 was chromatographed on a Vydac C-8 reversed phase column and the eluted peaks analyzed by mass spectrometry. The masses of the HPLC peaks were in agreement with the theoretical masses of phosphorylated PTHrP-(67–94) phosphorylated only PTHrP proteins and peptides containing the putative phosphorylation motif Lys84-Thr-Pro-Gly-Lys88. These results indicate that the phosphorylation site of PTHrP lies within amino acids 85 and 94, thereby implicating Thr85, the only phosphorylated amino acid present.

In order to determine whether specific cyclin-CDK combinations phosphorylated PTHrP, a panel of expressed and activated cyclin-CDKs were used to phosphorylate PTHrP-(67–94) in vitro (Table I). Cyclin E-CDK2, cyclin A-CDK2, and cyclin B-CDK2-phosphorylated PTHrP-(67–94). The G1-specific cyclin-CDK combinations tested, namely cyclin D1-CDK4, cyclin D1-CDK4, and cyclin D1-CDK6, did not phosphorylate PTHrP-(67–94) (results not shown), suggesting that PTHrP could be phosphorylated in all stages of the cell cycle except G1/G0.

Kinetic studies comparing the efficiency of PTHrP-(67–94) as a substrate for various cyclin-CDK complexes indicated that PTHrP-(67–94) was more efficiently phosphorylated by PTHrP-(67–94) phosphorylated with (Fig. 3). Cyclin A-CDK2 phosphorylated PTHrP-(67–94) in vitro.

**FIG. 1. Fluorescence immunocytochemistry of PTHrP in asynchronous HaCaT cells.** Identical field of a confocal laser scanning micrograph showing HaCaT cells stained for PTHrP with a monoclonal antibody, 3F5 (A), and DNA with propidium iodide (B). (b) with G1 and M phenotypes, demonstrating condensed and more intensely stained (B) nuclei. PTHrP is no longer in the nucleus, and there is increased cytoplasmic PTHrP staining (A). Cells at mitosis demonstrate the highest PTHrP expression.
The single letter amino acid code is used with the phosphorylated residues in bold. The proposed CcN motif (residues 61–91) consisting of a CK2 phosphorylation motif, a CDC2-CDK2 phosphorylation motif, and a NLS is consistent with SV40 T-antigen and other nuclear localized proteins (reviewed in Ref. 14) and NoS (residues 93–106) consisting of an arginine hinge flanked by basic amino acids are indicated. Alternatively, the combination of (Lys88-Lys-Lys-Lys91) and (Lys 102-Lys-Lys-Arg-Arg-Arg-Arg) can be viewed as a bipartite NLS.

As expected, comparisons could not be made with the efficiency of PTHrP-(50–69) as substrate compared with a known CK2 substrate. PTHrP-(50–69) was used as a negative control and was not phosphorylated by any of the cyclin-CDK complexes as expected. Comparisons could not be made between each of the cyclin-CDK assays as the purified cyclin-CDKs were of different concentrations and also different activation states.

To determine whether phosphorylation of PTHrP-(67–94) was cell cycle-stage-specific, whole cell lysates of CEM cells were collected at various points of the cell cycle following G1 selection by centrifugal elutriation and used to phosphorylate PTHrP-(67–94). Consistent with the kinase activity in vivo (Table I), this in vitro enzyme assay for kinase activity using PTHrP-(67–94) as substrate shows that kinase activity was low at G1 (0 h), increased 2-fold (Fig. 4A) as the cells moved on to S (4–6 h) (Fig. 4B), and increased to 4-fold (Fig. 4A) at G2 and M (8–10 h) (Fig. 4B). In contrast, the activity of CK2 with hCK2 peptide (Arg-Arg-Arg-Asp-Asp-Asp-Ser-Asp-Asp-Asp-NH2) as a substrate was constant throughout the cell cycle (Fig. 4, A and B).

To confirm that PTHrP was phosphorylated in vivo, COS-1 cells transfected with GFP-PTHrP-(1–141) were labeled with PTHrP-(67–94) is a substrate for cyclin E-CDK2, cyclin A-CDK2, and cyclin B-CDC2. 1 μg of PTHrP-(67–94) was used as an in vitro substrate for recombinant cyclin E-CDK2, cyclin A-CDK2, and cyclin B-CDC2, and 1 μg of PTHrP-(50–69) was used as a negative control, while 3 μg of histone H1 was used as positive control and also to demonstrate the relative efficiency of PTHrP as a substrate for these kinases. The G1-type cyclin-kinases combinations of cyclin D1-cdk4, cyclin D1-cdk4, and cyclin D1-cdk6 did not phosphorylate PTHrP-(67–94) (results not shown).

Table I

| Substrate          | Cyclin Ep33CDK2 | Cyclin Ap33CDK2 | Cyclin Bp34CDK2 |
|--------------------|-----------------|-----------------|-----------------|
| PTHrP-(67–94)      | 6168.22         | 6218.00         | 6110.88         |
| PTHrP-(50–69)      | 202.42          | 193.47          | 187.81          |
| Histone H1         | 37799.97        | 8673.00         | 5829.00         |

Table II

| Cyclin-kinase combination | PTHrP-(67–94) | Histone H1 |
|---------------------------|---------------|------------|
|                           | μM            |            |
| Cyclin E-p33CDK2          | 80.71 ± 7.2   | 0.28 ± 0.03 |
| Cyclin A-p33CDK2          | 4.15 ± 1.26   | 0.30 ± 0.02 |
| Cyclin B-p34CDK2          | 1.73, 2.72μM  | 0.224 ± 0.004 |

Only two assays for cyclin B-CDC2 were performed, since there was insufficient enzyme from the same batch for further replicates.
32P. Immunoprecipitation from the cell lysates with an antibody against GFP resulted in a radiolabeled protein band at 45 kDa, corresponding to GFP-PTHrP-(1–141) (Fig. 5A). When this band was excised and analyzed by two-dimensional phospho-peptide mapping, the resultant map (Fig. 5B, ii) corresponded to that of cyclin A-CDK2-phosphorylated recombinant PTHrP-(1–141) (Fig. 5B, i), implicating Thr 85 to be the site of phosphorylation. The heavy loading in Fig. 5B, ii, was necessary to enable visualization of tryptic phosphopeptides, but this resulted in a slight retardation of the phosphopeptide in the second dimension.

Phosphorylation at the CDC2-CDK2 site adjacent to the SV40 T-antigen NLS decreases the rate and amount of nuclear localization of this protein (16). In order to determine whether this is the case also with PTHrP, GFP-PTHrP fusion products containing wild type and point mutations of Thr 85 were constructed. Thr 85 was mutated to Ala 85 to preclude phosphorylation at this site or to Glu 85 to mimic the negative charge of a phosphorylated threonine. Mutant PTHrP-GFP fusion proteins encompassed the NLS (amino acids 86–91) and the putative NoS (amino acids 94–106) (Fig. 6A, c–e). The vector pEGFP-C1-containing GFP without any fusion sequences was used as a vector nonfusion control (Fig. 6A, a). Mature PTHrP-(1–141) is phosphorylated in vivo. A, immunoprecipitates, using a polyclonal antibody against GFP, of 32P-labeled COS-1 cells transiently transfected with GFP (pSMR790) or GFP-PTHrP-(1–141) (pSMR792) were resolved on a 10% SDS-polyacrylamide gel electrophoresis. B, two-dimensional tryptic peptide maps of recombinant PTHrP-(1–141) phosphorylated with cyclin A-CDK2 (a) and immunoprecipitated GFP-PTHrP-(1–141) from 32P-labeled COS-1 cells transiently transfected with GFP-PTHrP-(1–141) (pSMR792) (b). Arrows indicate similarly migrating phospho-peptides.
FIG. 6. GFP fused to site-directed mutants of PTHrP Thr<sup>85</sup>. A, schematic diagram of GFP-PTHrP fusion proteins, the PTHrP-(82–141) mutants included the CDC2-CDK2 phosphorylation site, the NLS, and the NoS. The arrows and off numbers above and below each schematic indicate the primer pairs used to generate each construct. PTHrP mutants were fused at the N terminus to GFP. Schematic of full-length human PTHrP is shown on the top, pSMR790, pEGFP-C1 vector control (a); pSMR792, GFP fused to mature PTHrP-(1–141) (b); pSMR807, wild type PTHrP-(82–141) (c); pSMR808, PTHrP-(82–141), Thr<sup>85</sup> is mutated to Ala<sup>85</sup> to preclude phosphorylation (d); pSMR809, PTHrP-(82–141), Thr<sup>85</sup> is mutated to Glu<sup>85</sup> to mimic phosphorylated Thr<sup>85</sup> (e). The vectors were transfected into HaCaT cells using the calcium phosphate method as described under “Experimental Procedures.” B, confocal laser scanning micrographs of site-directed PTHrP mutants fused to GFP and transfected into HaCaT cells. Cells expressing GFP or GFP-PTHrP are green, and actin stained with Texas Red-X phallolidin is red. The bar at the bottom left indicates 50 μm. a, pSMR790; b and c, pSMR792; d, pSMR807; e, pSMR808; f, pSMR809. C, analysis of localization of GFP-PTHrP-(82–141) wild type and mutant constructs in HaCaT cells. GFP-PTHrP fusion proteins in each cell were scored according to its nuclear or cytoplasmic localization and tabulated. Each set of GFP-PTHrP results represents 10 observations from each of three transfections, Thr<sup>85</sup>, pSMR807; Ala<sup>85</sup>, pSMR808; Glu<sup>85</sup>, pSMR809.
fused at the N terminus to GFP was used as a positive control (Fig. 6A, b).

HaCaT cells transfected with the pEGFP-C1 vector (Fig. 6B, a) demonstrated diffuse fluorescence. Mature PTHrP-(1–141) fused to GFP at the N terminus (pSMR792) demonstrated clear nuclear and nucleolar localization (Fig. 6B, b) as well as cytoplasmic localization in some cells (Fig. 6B, c), indicating regulation of its localization during the cell cycle. A similar pattern of localization was observed in living cells, indicating that fixation did not alter GFP-PTHrP-(1–141) location (result not shown). HaCaT cells transfected with the wild type GFP-PTHrP-(82–141)Thr85 (pSMR807) fusion protein demonstrated both cytoplasmic and nuclear localization, with preferential localization to the nucleus (Fig. 6B, d). Cells transfected with GFP-PTHrP-(82–141)Ala85 (pSMR808) demonstrated strong nuclear localization with some punctate cytoplasmic localization (Fig. 6B, e), while cells that were transfected with GFP-PTHrP-(82–141)Glu85 (pSMR809) demonstrated cytoplasmic localization only (Fig. 6B, f).

A total of 30 cells from three independent experiments were scored, with the cells graded for GFP-PTHrP localization to the nucleus or cytoplasm (Fig. 6C). For the unmutated GFP-PTHrP-(82–141)-transfected cells, 23 out of 30 cells had nuclear localization, and 27 out of 30 cells also showed cytoplasmic localization. 26 out of 30 cells with the GFP-PTHrP-(82–141)Ala85 mutant demonstrated nuclear localization, while 19 out of the 30 cells had cytoplasmic localization. In contrast, all cells with the GFP-PTHrP-(82–141)Glu85 construct demonstrated cytoplasmic localization and only 3 of these 30 demonstrated nuclear fluorescence. These data indicate that when phosphorylation of Thr85 occurs, it results in decreased nuclear accumulation of PTHrP, while in an unphosphorylated state (e.g. Ala85), PTHrP is preferentially nuclear localized.

DISCUSSION

This is the first report of an endocrine/paracrine factor that possesses a phosphorylation-regulated NLS and displays differential cellular localization (nuclear/nucleolar versus cytoplasm), inferring that it acts intracellularly. To date, proteins shown to possess such motifs have intracellular functions only, such as viral proteins (e.g. SV40 T-antigen), oncoproteins (e.g. c-MYC) and tumor suppressors (e.g. p53) (14, 33).

Within PTHrP we identified a putative CcN motif similar to those of SV40 T-antigen, c-MYC, p53, c-ABL, and A-MYB. Like these “CcN motifs,” PTHrP contains a consensus CDC2-CDK2 phosphorylation site near the putative NLS. We showed that PTHrP was phosphorylated by CDC2-CDK2 specifically at Thr85 in the CcN motif and that this phosphorylation occurred in cells overexpressing PTHrP.

Phosphorylation of residues in the vicinity of the NLS in other CcN-containing proteins, such as SV40 T-antigen, alters their cellular distribution (14, 16). Phosphorylation at the CDK site N-terminal to the NLS redistributes these proteins from a nuclear/nucleolar location and confines them to the cytoplasm (14, 16). To address whether phosphorylation of PTHrP similarly results in a cellular redistribution of PTHrP, we employed GFP-PTHrP expression constructs in which Thr85 was mutated to Ala85 or Glu85. These residues were chosen to preclude phosphorylation or to mimic a phosphorylated residue, respectively. These studies indicate that PTHrP in a dephosphorylated state is maintained in the nucleus, while in the phosphorylated state, PTHrP is excluded from the nucleus and is essentially cytoplasmic. In common with proteins possessing a Cdk phosphorylation site immediately adjacent to a NLS (14, 16), phosphorylation at this site inhibited NLS function (14), and this also appeared to be true for PTHrP.

CDC2-CDK2 phosphorylation activity was found to begin at S phase and peaked at the G2/M phase of the cell cycle, coincident with the localization of cyclin A and B1 to the nucleus (32), suggesting that PTHrP might be phosphorylated and thus excluded from the nucleus at these phases. In agreement, we observed that PTHrP was localized to the nucleus during G1, further supporting the notion that cell cycle-related nuclear localization of PTHrP may be regulated by CDK phosphorylation.

To date, evidence has indicated that PTHrP acts as a hormone in certain patients with cancer and possibly also in lactating women, but it acts as a paracrine factor in several normal tissues (2, 3). The demonstration that PTHrP is localized to the nucleus and nucleolus indicates that PTHrP may have intracellular roles (10, 34, 35). A nuclear/nucleolar location for PTHrP may arise as a result of two distinct mechanisms: (i) as a normal consequence of PTHrP production within a cell expressing PTHrP or (ii) in a target cell as a result of binding to its cognate receptor and internalization of the complex with consequent nuclear localization. The control mechanisms for determining intracellular trafficking of PTHrP, whether via the trans-Golgi network and secretory vesicles versus a nuclear localization is not known.

Since PTHrP demonstrates phosphorylation and cell cycle-dependent nuclear localization, it is conceivable that PTHrP may have a defined nuclear role, possibly to regulate growth and differentiation. Support for this proposition results from the demonstration that intracellular targeted PTHrP increases proliferation of vascular smooth cells (5). It is likely that other secreted growth factors, such as acidic and basic fibroblast growth factors, platelet derived growth factor, and angiogenin (36, 37), which possess basic amino acid sequences analogous to those of a NLS, may also display regulated intracellular localization similar to that of PTHrP.

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