Importin β Recognizes Parathyroid Hormone-related Protein with High Affinity and Mediates Its Nuclear Import in the Absence of Importin α*

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Parathyroid hormone-related protein (PTHrP), expressed in a range of tumors, has endocrine, autocrine/paracrine, and intracrine actions, some of which relate to its ability to localize in the nucleus. Here we show for the first time that extracellularly added human PTHrP (amino acids 1–108) can be taken up specifically by receptor-expressing UMR106.01 osteogenic sarcoma cells and accumulate to quite high levels in the nucleus and nucleolus within 40 min. Quantitation of recognition by the nuclear localization sequence (NLS)-binding importin subunits indicated that in contrast to proteins containing conventional NLSs, PTHrP is recognized exclusively by importin α and not by importin β. The sequence of PTHrP responsible for binding was mapped to amino acids 66–94, which includes an SV40 large tumor-antigen NLS-like sequence, although sequence determinants amino-terminal to this region were also necessary for high affinity binding (apparent dissociation constant of ~2 nM for importin β). Nuclear import of PTHrP was assessed in vitro using purified components, demonstrating that importin β, together with the GTP-binding protein Ran, was able to mediate efficient nuclear accumulation in the absence of importin α, whereas the addition of nuclear transport factor NTF2 reduced transport. The polypeptide ligand PTHrP thus appears to be accumulated in the nucleus/nucleolus through a novel, NLS-dependent nuclear import pathway independent of importin α and perhaps also of NTF2.

Parathyroid hormone-related protein (PTHrP)1 is expressed in a range of tumors and is an endocrine agent in humoral hypercalcemia of malignancy. It is also expressed in many normal tissues where it exerts autocrine/paracrine or intracrine actions (1–6). The structural similarities to parathyroid hormone (PTH) at the amino terminus of PTHrP are sufficient to confer functions similar to those of PTH, mediated by the shared PTH/PTHrP receptor and its ability to activate adenylate cyclase. Although both PTH and PTHrP promote bone resorption and reduce renal calcium excretion (1, 7), roles in the regulation of placental calcium transport to the fetus (1, 7, 8), osteoclast inhibition (9, 10), and the regulation of cell growth and apoptosis (2, 11, 12) have been ascribed to distinct regions of PTHrP.

We and others have recently shown that PTHrP is expressed in a cell cycle-dependent manner (13, 14) as well as being localized to the nucleus/nucleolus at G1 (14). Regulation of the nuclear localization of PTHrP appears to be mediated through phosphorylation by the cyclin-dependent kinases p34<sup>cdk2</sup> and p34<sup>cdk2</sup>.2 These observations are consistent with the idea that cell cycle-dependent regulation of nuclear localization of PTHrP is central to the control of growth and apoptosis (2, 4).

Of significance in this regard is our observation2 that within amino acids 61–93, PTHrP retains a putative CCN motif, originally described for the SV40 large tumor antigen (T-ag; Refs. 16 and 17), comprising consensus protein kinase CK2 (S<sup>61</sup>DDE and ET<sup>86</sup>KYE–<sup>C</sup>) and confirmed cyclin-dependent kinase (K<sup>2</sup>TPGK–<sup>c</sup>) phosphorylation sites in the vicinity of a T-ag-like nuclear localization sequence (NLS-GKKGKGGKW<sup>93</sup>−<sup>N</sup>), as well as a putative nuclear localization sequence (NOS-PKRKEQKRRTR<sup>109</sup>) (Fig. 1A). In the case of T-ag, the CCN motif CK2 and cyclin-dependent kinase sites strongly regulate NLS function and the kinetics of nuclear import (16, 17); with respect to PTHrP, transfection studies indicate that deletion of amino acids 87–107, removing both NLS and NOS, results in completely cytoplasmic localization of PTHrP and concomitant impaired PTHrP-conferred resistance to apoptosis on the part of CFK2 chondrocytes (2). Based on the observation that nuclear PTHrP affects an increase in mitogenesis in vascular smooth muscle cells, Massfelder et al. (5) suggested that the clusters of basic amino acids (88–91 and 102–106) in PTHrP might be recognized by the NLS-binding importin/kyropherin subunit complex of the cellular nuclear protein import machinery.

In the present study we use recombinant and synthetic PTHrP peptides encompassing various regions of PTHrP to demonstrate for the first time that PTHrP can be internalized by receptor-expressing UMR106.01 cells and localize rapidly in the nucleus and nucleolus. In addition we show that in contrast

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1 The abbreviations used are: PTHrP, parathyroid hormone-related protein; PTH, parathyroid hormone; T-ag, SV40 large tumor antigen; NLS, nuclear localization sequence; NOS, nuclear localization sequence; HIV, human immunodeficiency virus; TCPTP, T-cell protein tyrosine phosphatase; FITC, fluorescein isothiocyanate; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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to conventional NLS-containing proteins, PTHrP is recognized with high affinity by the $\beta$ and not the $\alpha$ subunit of the importin heterodimer. We map the specific region of PTHrP recognized by importin $\beta$ and show that it alone, in concert with the GTP-binding protein Ran, can mediate nuclear accumulation of PTHrP in a reconstituted nuclear import assay system. We conclude that PTHrP localizes in the nucleus through a novel import pathway, possibly shared by other importin $\beta$-recognized proteins such as HIV Rev (18), the T-cell protein tyrosine phosphatase (TCPTP; Ref. 19), and the yeast transcription factor GAL4 (20).

**MATERIALS AND METHODS**

**Chemicals and Peptides—**Reagents were from the sources previously described (14, 20–25). Peptides PTHrP 1–34, 50–69, 66–86, 66–94, 67–84, 67–86, 67–94, and 83–108 were synthesized using the Merrifield solid phase procedure and purified and characterized as described (26). Recombinant PTHrP 1–106 and 55–141 were expressed in Escherichia coli and purified as described previously (27). Peptide and protein concentrations were determined by quantitative amino acid analysis following acid hydrolysis. Bacterially expressed human NTF2 (28) was provided by Bryce Paschal (University of Virginia, Charlottesville, VA).

**Cell Culture—**Cells of the HTC hepatoma tissue culture and UMR106.01 osteoblast lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (16, 21).

**Fluorescent Labeling—**PTHrP 1–108 was conjugated to Oregon Green™ (FITC) (Molecular Probes, Eugene OR), whereby 10 mg/ml PTHrP 1–108 in 0.1 M sodium bicarbonate, pH 9.0, was incubated with a one-tenth volume of 10 mg/ml FITC (in MeSO) for 1 h at room temperature in the dark. Unbound chromophore was removed by passage through a PD-10 column (Pharmacia Biotech AB, Uppsala, Sweden) using phosphate-buffered saline, pH 7.0, or 0.1 M acetic acid, pH 2.0. Fractions of the eluant containing $>0.1$ mg/ml protein were pooled, and the concentration of the pooled fraction was determined to be 627 $\mu$g/ml PTHrP 1–108.

**ELISA-based Binding Assay—**An ELISA-based assay (22–25) was used to examine binding between importin subunits (with and without GST moieties) and NLS-containing proteins or peptides. The latter were coated onto 96-well microtiter plates and hybridized with increasing concentrations of importin subunits, and detection of bound importin-GST was performed using goat anti-GST primary and alkaline phosphatase-coupled rabbit anti-goat secondary antibodies and the substrate $p$-nitrophenyl phosphate (22). Absorbance measurements were performed over 90 min using a plate reader (Molecular Devices, Sunnyvale, CA), and values were corrected by subtracting absorbance both at 0 min and in wells incubated without importin (22).

**In Vitro Nuclear Transport—**Nuclear import kinetics were analyzed at the single cell level using mechanically perforated HTC cells in conjunction with confocal laser scanning microscopy (16, 21, 23–25). Experiments were performed for 40 min at room temperature in a 5-$\mu$L volume containing 30 mg/ml bovine serum albumin, 2 mM GTP, an ATP regenerating system (0.125 mg/ml creatine kinase, 30 mM creatine-phosphate, 2 mM ATP), transport substrate (375 ng/ml FITC-PTHrP), and where indicated, 4 $\mu$M RanGDP, 0.15 $\mu$M NTF2, and 1 $\mu$M importin $\alpha$ and $\beta$ subunits. In experiments where PTHrP peptides were tested for their ability to compete FITC-PTHrP transport, 0.5 $\mu$M mIrgP and 4 $\mu$M RanGDP were used, together with a 100-fold molar excess of peptide. In some experiments, reticulocyte lysate (45 mg/ml) was used in place of purified importins/Ran/NTF2 (21, 23–25) in the absence or presence of the nuclear envelope permeabilizing detergent CHAPS (0.025%); in the latter case, nuclear/nucleolar accumulation can only occur through binding to nuclear/nucleolar components (24, 25). Image analysis and curve fitting was performed as described (23–25).

**RESULTS**

**PTHrP Localizes in the Nucleus after Endocytosis by Intact UMR106.01 Cells—**We initially tested whether PTHrP could localize in the nucleus/nucleolus subsequent to receptor-mediated endocytosis. FITC-PTHrP 1–108 was incubated with UMR106.01 osteoblast cells without or with (nonspecific binding) preincubation/coincubation with an excess of unlabeled ligand, and subcellular localization was visualized and quantitated using confocal laser scanning microscopy (Fig. 2). Nuclear/nucleolar uptake reached maximal levels (4- and over 13-fold, respectively) by about 40 min (Fig. 2B). It could be blocked by an excess of either unlabeled PTHrP 1–108 (Fig. 2) or unlabeled PTHrP 1–34 (not shown), which is responsible for PTHrP binding to the PTHrP receptor, indicating that the uptake was receptor-mediated and thus comparable with that of other nuclear localizing ligands such as interleukin-5 (35) and growth hormone (36).

**PTHrP Is Recognized Specifically by $\beta$-Importins—**The ability of PTHrP to localize in the nucleus in intact cells implied that PTHrP may possess a functional NLS (Fig. 1A). We accordingly set out to quantitate the binding of mouse importin subunits to purified polypeptides and synthetic peptides encompassing various parts of PTHrP using an ELISA-based assay (22, 23), which we have used successfully to determine the binding affinities (apparent dissociation constants ($K_d$)) of...
importin subunits for different NLSs (20, 24, 37). Surprisingly, we found that PTHrP 1–108 and 35–141 were recognized with high affinity by importin \( \beta \) and not importin \( \alpha \) (Fig. 3, top panels, and Table I). Maximal binding by importin \( \alpha \) was only about 50% that of importin \( \beta \), whereas the \( K_d \) values for PTHrP 1–108/35–141 and importin \( \beta \) were 3.5 and 1.8 nM, respectively, about 2 orders of magnitude lower than the corresponding values for importin \( \alpha \) (Table I). The unexpected finding that PTHrP was recognized by mImp \( \beta \) and not Imp \( \alpha \) was confirmed using importin subunits from yeast (31), results clearly indicating high affinity recognition of PTHrP 1–108/35–141 by yImp \( \beta \) and not yImp \( \alpha \) (Table I and data not shown). This high affinity recognition by importin \( \beta \) was in direct contrast to our observations using the same assay system for the conventional NLS-containing proteins T-ag (22), Rb (24), and Dorsal (37), all of which require importin \( \alpha \) for NLS recognition and are not directly recognized by importin \( \beta \).

**Mapping of the PTHrP NLS**—Shorter peptides were used to define the specific region of PTHrP recognized by importin \( \beta \). PTHrP 50–69, 67–94, and 83–108 were initially tested with results clearly indicating binding to 67–94 and not to either 50–69 or 83–108 (Fig. 3, middle panels, and Table I). The latter, although encompassing the NOS together with the NLS (Fig. 1A), maximally bound only about 40% the amount of importin \( \beta \) bound by 67–94, with a \( K_d \) well above (over 300-fold in the case of mImp \( \beta \)) that of the latter (Fig. 3, middle panels; Table I; and data not shown), implying that residues 95–108 were not required for high affinity binding, whereas sequences amino-terminal to the NLS (KKKKGK\(^{93}\)) clearly were. Of other peptides tested, PTHrP 66–94, 67–84, and 67–86, only 66–94 bound importin \( \beta \) (\( K_d \) of around 2 nM; Fig. 3, bottom panels, and Table I), consistent with this. The fact that the \( K_d \) values for recognition of PTHrP 66–94 by importin \( \beta \) were significantly smaller (3-fold in the case of mImp \( \beta \)) than those for 67–94 and entirely comparable with those for PTHrP 1–108 (Table I) implied that Arg\(^{66}\) was involved in binding to...

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**Fig. 2.** Visualization (A) and quantitation (B) of nuclear/nucleolar uptake of PTHrP by UMR106.01 osteoblasts. Cells were incubated with FITC-PTHrP 1–108 without or with (nonspecific binding) preincubation/coincubation with an excess of unlabeled ligand as indicated and visualized by confocal laser scanning microscopy at various time points. B, quantitative data for \( \text{Fn/c} \) (ratio between nuclear (\( \text{Fn} \)) and cytoplasmic (\( \text{Fc} \)) fluorescence) and \( \text{Fnu/c} \) (ratio between nucleolar fluorescence (\( \text{Fnu} \)) and cytoplasmic (\( \text{Fc} \)) fluorescence) are from a single typical experiment, representative of a series of three similar experiments. In A, phase contrast images are shown to the left of each fluorescent image; nucleoli are indicated by arrows in the bottom left panels, with a scale bar indicated. In B, each data point represents at least eight separate measurements for each of \( \text{Fn}, \text{Fnu}, \) and \( \text{Fc} \) where the S.E. was not greater than 14% the value of the mean.
importin β. Based on the results for the various peptides, the importin β binding site was concluded to comprise PTHrP amino acids 66–94.

Nuclear Import of PTHrP 1–108 Can Be Reconstituted in Vitro Using Importin β and Ran—To test whether nuclear import of PTHrP 1–108 could be mediated by importin β in the absence of importin α, we tested the ability of mLmb to mediate nuclear import in our previously described reconstituted in vitro system (16, 21) in the absence or presence of purified RanGDP and NTF2. In the absence of importins, nuclear entry but not accumulation of PTHrP 1–108 (about 12.5 kDa) was evident, in direct contrast to the strong accumulation in the presence of mImpβ together with Ran (Fig. 4 and Table II). Maximal levels of nuclear accumulation (Fn/cmax) were as high as 8-fold those in the cytoplasm, half-maximal accumulation being achieved within about 5 min (Fig. 4B; see Table II for pooled data). mLmpα could not substitute for mLmb in mediating nuclear accumulation (Fn/cmax of about 1.5), whereas the combination of α and β was not as efficient as Impβ alone but more efficient than Impα alone (Fn/cmax of about 3.5; see Table II). The inclusion of NTF2 in combination with Impβ and Ran increased the import rate (half-maximal accumulation at 1.8 min; see Table II) but reduced maximal transport by over 50%, resulting in an Fn/cmax of about 2.5. It was concluded that Impβ and Ran were sufficient to mediate nuclear import of PTHrP.

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## Table 1

### NLS binding parameters of PTHrP derivatives as measured using an ELISA-based binding assay

Importin binding parameters were determined as described under “Materials in Methods” (21–24) from experimental data fitted as shown in Fig. 3. Results for the apparent dissociation constant ($K_d$) and maximal level of binding (expressed relative to the binding by the respective β-subunit (mlmpβ or ylmpβ) to the first PTHrP derivative listed for either polypeptides or synthetic peptides represent S.E. (in parentheses); where n is not indicated, the S.E. is determined from the curve fit. ND, not able to be determined. Where no S.E. is shown, the value was 0.04.

| PTHrP derivative | Mouse importins | Yeast importins |
|------------------|-----------------|----------------|
|                  | Importin b       | Importin y      |
| Polypeptides     | mlmpβ | K_d | B_max | mlmpβ | K_d | B_max | mlmpβ | K_d | B_max | mlmpβ | K_d | B_max | mlmpβ | K_d | B_max |
| 1–108            | 320 ± 194 (3)   | 5.8 ± 2.6 (4)  | 98 ± 5  | 3.5 ± 0.8 (3) | 100  | 21 ± 2 | 55 ± 2 | 5.1 ± 1.5 | 90 ± 4 | 1.3 ± 0.3 | 100  |
| 31–141           | 223 ± 177 (3)   | 3.0 ± 0.1 (2)  | 104 ± 9 | 1.8 ± 0.7 (3) | 100  | 13 ± 1 | 52 ± 3 | 1.4 ± 0.2 (2) | 72 ± 6 | 1.3 (2) | 100  |
| 67–94            | 520 ± 285 (3)   | 2.9 ± 0.8 (3)  | 99 ± 20 | 2.6 ± 0.5 (2) | 100  | 96 ± 11 | 57 ± 4 | 19 ± 3 | 74 ± 5 | 7.7 ± 1.6 | 100  |
| 50–64            | >2300 ND        | >2300 (2)      | 7 ± 4   | 579 ± 326 (2) | 6.5 ± 1.2 | ND | ND | ND | ND | ND | ND |
| 83–108           | >2300 25 ± 4    | >2300 (2)      | 26 ± 5  | 845 ± 168 | 41 ± 9 | 72 ± 28 | 25 ± 5 | 53 ± 10 | 19 ± 1 | 94 ± 50 | 38 ± 13 |
| 66–94            | 75 ± 2 47 ± 1   | ND | ND | 1.8 ± 0.2 | 100  | 11 ± 0.8 | 53 ± 4 | 10 ± 2 | 81 ± 5 | 2.3 ± 0.1 | 100  |
| 66–86            | 2038 ± 44 3.7 ± 0.1 | ND | ND | 2020 ± 21 | 1.9 | 780 ± 62 | 7.7 ± 0.6 | ND | ND | ND | ND |
| 67–84            | >2300 2.8       | ND | ND | >2300 (1.8 ± 0.1) | 2.1 ± 0.2 | 990 ± 44 | 7.0 ± 3 | ND | ND | >2300 2.4 ± 0.4 |
| 67–86            | >2300 3.3 ± 0.1 | ND | ND | >2300 (2.1 ± 0.2) | 2.1 ± 0.2 | 990 ± 44 | 7.0 ± 3 | ND | ND | >2300 2.4 ± 0.4 |

DISCUSSION

A number of recent reports have established that there are multiple signal-dependent nuclear transport pathways. These include those mediated exclusively by importin β (reviewed in Refs. 35–42 and this article) and those where soluble cytosolic receptors appear not to be required at all (24, 41, 44). Importin β has been shown to mediate nuclear transport in vitro (36, 41), as well as in vivo (37–40). It has also been shown that importin β can mediate nuclear transport of proteins that do not need to be transported (35, 41–43) and that the presence of importin β is not required for transport (35, 41–43). These findings highlight the importance of importin β in mediating nuclear transport in the absence of importin α.

To demonstrate formally that residues 86–94 were responsible for nuclear transport of PTHrP 1–108, and that in contrast to the nuclear import of proteins containing conventional NLSs, such as Tag, PTHrP 1–108 is not transported by importing Impα in the absence of Impβ, we used the yeast double mutant, M12 (M12/1925), which exhibits impaired signaling (2, 5). In contrast, PTHrP 67–84, 83–108, and 50–69, all of which do not encompass the complete NLS, had negligible or minor effects on nuclear accumulation (Fig. 4A, bottom left panel). Nuclear transport of PTHrP 1–108 is dependent on the nuclear envelope-permeabilizing detergent CHAPS (Fig. 4A, bottom right panel), and data not shown). Nuclear transport of PTHrP 1–108 is also dependent on the nuclear envelope-permeabilizing detergent CHAPS (Fig. 4A, bottom right panel), and data not shown). Nuclear transport of PTHrP 1–108 is also dependent on the nuclear envelope-permeabilizing detergent CHAPS (Fig. 4A, bottom right panel), and data not shown). Nuclear transport of PTHrP 1–108 is also dependent on the nuclear envelope-permeabilizing detergent CHAPS (Fig. 4A, bottom right panel), and data not shown). Nuclear transport of PTHrP 1–108 is also dependent on the nuclear envelope-permeabilizing detergent CHAPS (Fig. 4A, bottom right panel), and data not shown). Nuclear transport of PTHrP 1–108 is also dependent on the nuclear envelope-permeabilizing detergent CHAPS (Fig. 4A, bottom right panel), and data not shown).

In conclusion, these data strongly suggest that PTHrP 1–108 is transported by a nuclear transport pathway that requires importin β, and that this pathway is distinct from the conventional NLS-mediated nuclear transport pathway.

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well as recognize NLS-containing proteins such as HIV-1 Rev (18), GAL4 (20), and TCPTP (19), implying that importin β may be able to function independently as a nuclear import receptor. The findings of the present study not only establish that PTHrP is similar to the above proteins in being recognized by importin β alone but also demonstrate conclusively for the first time that importin β, in concert with Ran, is sufficient to mediate nuclear import of PTHrP in the absence of importin α. Clearly, although the details are not fully understood, novel NLS-dependent nuclear import pathways exclusively mediated by importin β exist that are presumably analogous to those mediated by transportin and other importin β homologs (39). Based on the results here showing that NTF2 is able to reduce importin β/Ran-mediated transport in our reconstituted system, it appears that, in contrast to the more conventional importin α/β-mediated pathways (Refs. 28, and 45; see also Refs. 33 and 46), NTF2 may be a negative regulator of this pathway, as has been described for in vivo nuclear import/export (47). Interestingly, importin β and Ran did not appear to be sufficient to effect nucleolar accumulation of PTHrP in vitro, which was, however, observed in vitro in the presence of cytosol (Fig. 4A, bottom panels), and in intact cells subsequent to endocytosis of ligand (Fig. 2), implying that other factors may be required. Based on the results of Tiganis et al. (19) where both importin β and a 116-kDa protein (p116) appear to recognize the NLS-containing region of TCPTP, it could be speculated that p116 may be an additional component of the pathway shared by proteins recognized exclusively by importin β. Since extracellularly added PTHrP can be targeted to the nucleus subsequent to receptor-mediated endocytosis (see also below), the potential importance of this novel nuclear import pathway should not be underestimated.

This study defines the 29-amino acid PTHrP sequence (amino acids 66–94) that confers high affinity binding to importin β.
Importin β-mediated Nuclear Import of PTHrP

Table II
Dependence of in vitro nuclear import of PTHrP derivatives on transport factors

| Addition | Nuclear import parameter<sup>b</sup> | t<sub>1/2</sub> | F<sub>nuc/c<sub>max</sub></sub> | t<sub>1/2</sub> |
|----------|----------------------------------|--------|----------------|--------|
| None     |                                  | ND     | 1.19 ± 0.09    | ND     |
| Ran + NTF2 |                                | ND     | 1.54 ± 0.19    | ND     |
| mImpβ + Ran |                              | ND     | 5.74 ± 1.40    | 5.11 ± 1.06 |
| mImpβ + Ran + NTF2 |                        | ND     | 2.53 ± 0.54    | 1.79 ± 0.53 |
| mImpα + Ran |                              | ND     | 1.47 ± 1.50    | ND     |
| mImpα + Ran + NTF2 |                        | ND     | 1.48 ± 0.11    | ND     |
| mImpαβ + Ran |                             | ND     | 3.63 ± 0.68    | 9.98 ± 3.94 |
| mImpαβ + Ran + NTF2 |                        | ND     | 1.80 ± 0.09    | ND     |
| yImpβ + Ran |                                | ND     | 3.28 ± 0.18    | 3.03 ± 0.58 |
| yImpβ + Ran + NTF2 |                        | ND     | 2.17 ± 0.17    | 1.45 ± 0.54 |
| yImpα + Ran |                                | ND     | 1.08 ± 0.09    | ND     |
| yImpαβ + Ran |                             | ND     | 1.72 ± 0.02    | ND     |
| Cytosol   |                                  | ND     | 3.41 ± 0.07    | 1.03 ± 0.39 |
| CHAPS/cytosol |                             | ND     | 3.71 ± 0.20    | 7.64 ± 0.62 |
| CHAPS/cytosol: no ATP |                    | ND     | 5.12 ± 0.64    | 12.2 ± 0.99 |
| CHAPS    |                                  | ND     | 4.52 ± 0.89    | 10.3 ± 2.01 |

<sup>a</sup> All additions include an ATP regenerating system, unless otherwise indicated (“Materials and Methods”).

<sup>b</sup> Fits to a single point from experiments (see Fig. 4B and not shown) performed as described in “Materials and Methods,” represent the mean ± SEM (n in parentheses). ND, not able to be determined.

FIG. 5. Competition of PTHrP 1–108 nuclear import in vitro by PTHrP peptides. Maximal nuclear accumulation of FITC-PTHrP 1–108 above background (transport in the absence of mImpβ) mediated by mImpβ and Ran was measured in the absence and presence of a 100-fold molar excess of the peptides indicated as indicated under “Materials and Methods.” The results represent averages from three separate experiments (measurements performed as described in the legend to Fig. 4B), with the S.E. indicated.

as the region specific for targeting PTHrP to the nucleus. The data indicate that although a consensus bipartite NLS sequence can be identified in PTHrP comprising the clusters of basic amino acids 88–91 and 102–106 (2, 5), the region for importin binding and nuclear targeting appears to require only amino acids 66–94. That it is directly responsible for the nuclear transport of PTHrP is supported by in vitro studies (2, 5) and the peptide competition experiments in this study (Fig. 5). Comparison of PTHrP amino acids 66–94 with the importin β-recognized NLS of TCPTP (20), shown in Fig. 1B, indicates a shared carboxyl-terminal cluster of five basic amino acids, as well as a number of other conserved/similar residues (shown in bold type), and predicted secondary structure where an aminoterminal α-helix of 11–15 residues (including N/Q/KV/EQ) flanks a β turn, adjacent to the basic cluster. Significantly, although the minimal sequences for importin β-binding on the part of Rev and GAL4 have not been determined, similar sequences within these proteins can be identified (Fig. 1B). Clearly, although it contains a carboxyl-terminal cluster of basic residues similar to the T-ag NLS, the importin β binding NLS is quite distinct from importin α-recognized NLSs (17, 48); site-directed mutagenesis should enable the key sequence elements of the PTHrP NLS to be defined more precisely. Our preliminary characterization of the region of importin β responsible for binding PTHrP indicates that, as for TCPTP (19), amino acids 380–463 are involved, meaning that binding is mediated by a region of importin β distinct from that recognizing importin α.

Perhaps most significantly, this study demonstrates for the first time that the polypeptide ligand PTHrP is able to accumulate rapidly in the nucleus/nucleolus of intact UMR106.01 cells subsequent to receptor-mediated uptake; that uptake is receptor-mediated is indicated by the fact that accumulation can be competed by an excess of unlabeled ligand (Fig. 2). The precise details of the pathway by which PTHrP is able to localize in the nucleus subsequent to receptor-mediated endocytosis are incomplete at this stage, but the speed of nuclear/nucleolar accumulation (detectable within 35 min) implies that a lysosomal pathway is unlikely to be involved, as is the case for receptor-mediated uptake/nuclear localization of growth hormone (36), interleukin-5 (35), and other polypeptide ligands (49, 50). In analogous fashion to growth hormone and interleukin-5, it seems possible that PTHrP is internalized, rapidly escapes from the endosomal vesicle by an as yet undetermined mechanism (see Ref. 36), and then undergoes initial high affinity interaction with importin β (K<sub>D</sub> of 2 nM) as well as other factors of the cellular nuclear import machinery including RanGDP to accumulate rapidly in the nucleus/nucleolus, with binding to nuclear components (Fig. 4A) possibly contributing to the latter as well.

Although its functional importance has been demonstrated in diverse cell types (2, 5), the exact signaling role of nuclear
targeting of PTHrP is largely unclear at this stage. By analogy with other nuclear targeting polypeptide ligands, possibilities include PTHrP-mediated piggy back co-transport of the PTH/PTHrP receptor to the nucleus (shown for interleukin-5–36), where it may activate signaling components (15, 38), or the modulation of gene transcription through binding of PTHrP, with or without bound receptor, to nuclear factors or chromatin itself (49, 50). The ability of PTHrP to accumulate in the nucleus/nucleolus in vitro in the absence of an intact nuclear envelope (Fig. 4A) implies that the latter is feasible, but this will of course require confirmation using a variety of approaches. Determination of the precise nature of signaling by nuclear PTHrP, as well as the details of its novel nuclear import pathway, should enable insight into the importance of nuclear targeting to the unique endocrine, autocrine/paracrine, and intracrine roles of PTHrP in malignancy.

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