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Cleavage of the ER-Targeting Signal Sequence of Parathyroid Hormone-related Protein is Cell-Type-Specific and Regulated in *Cis* by its Nuclear Localization Signal

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Prepro-parathyroid hormone-related protein (ppPTHrP) has two targeting signals, an N-terminal signal sequence and a nuclear localization signal (NLS). In fact, the protein is not only secreted from the cell but also found in the nucleus and/or nucleolus. In order to understand the function of the PTHrP signal sequence for the dual localization, the signal sequence cleavage of a series of ppPTHrP deletion mutants fused to *Escherichia coli* leader peptidase was analysed *in vitro* and in several cell lines. Efficiency of the PTHrP signal sequence cleavage was intrinsically low in the *in vitro* reconstitution system. In cultured cells, cleavage efficiency of the PTHrP signal sequence varied significantly, being lowest in COS-1 cells, but rising in HeLa, HEK293 and CV-1 cells. However, virtually complete signal sequence cleavage was observed in CHO cells. In addition, the NLS of PTHrP had a negative effect on its own signal sequence cleavage, which could be enhanced by deletion of the spacer sequence between the signal sequence and the NLS. There was a roughly inverse relationship between the signal sequence cleavage and the nuclear localization of PTHrP. Thus, the final destination of PTHrP could be regulated at the ER membrane.

Key words: dual localization, endoplasmic reticulum, nuclear localization signal, parathyroid hormone-related protein, signal sequence.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; GL, α_{2u}-globulin; GST, glutathione-*S*-transferase; LP, leader peptidase; MBP, maltose binding protein; NLS, nuclear localization signal; PMSF, phenylmethylsulphonyl fluoride; PNGase, peptide: *N*-glycosidase; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; ppPTHrP, prepro-parathyroid hormone-related protein; SRP, signal recognition particle.

Parathyroid hormone-related protein (PTHrP) is a secretory protein that was initially identified as a tumour-derived, adenylate cyclase-stimulating factor associated with hypercalcaemia of malignancy, an endocrine neoplastic syndrome. PTHrP has structural and functional homology with parathyroid hormone (PTH), and acts on the same pathways in malignant hypercalcaemia. Further investigations have revealed that various normal cells produce PTHrP, which serves as an important paracrine/autocrine regulator of growth, differentiation, transepithelial calcium transport and smooth muscle relaxation, as well as many other cellular functions. Most of these functions are mediated through the G protein-coupled PTH/PTHrP receptor (*1*, *2*).

Rat PTHrP is synthesized as a 177-amino-acid prepro-form (ppPTHrP) that is cleaved to generate the bioactive mature PTHrP (mPTHrP), of which the N-terminal half is homologous to PTH, as well as other poorly characterized peptides (Fig. 1A). The prepro-form has two targeting signals, an N-terminal signal sequence for ER targeting and a nuclear localization signal (NLS) in the mid region of the protein. Although the prepro-form enters the classical secretory pathway, it is also found in the nucleus and/or nucleolus where it also appears to be active in some function other than the G protein-coupled receptor-mediated pathway (*3*). It has been reported that the nuclear/nucleolar translocation of PTHrP depends on the cell cycle in chondrocyte and keratinocyte cells (*4*). A cyclin-dependent kinase has been suggested to be a regulator of the nuclear/nucleolar translocation of PTHrP through regulation of its interaction with importin β by phosphorylation at Thr85 (*5–7*). However, the molecular mechanisms underlying the regulation of its dual localization have not yet been fully investigated.

The nuclear/nucleolar localization is surprising since the NLS is located in the downstream of the ER-targeting signal sequence and is not expected to be exposed to the cytosol according to the signal recognition particle (SRP) dependent co-translational translocation mechanism. The NLS of ppPTHrP could never encounter nuclear import receptors such as importin β, and the

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protein could not enter the nucleus. However, several potential pathways have been proposed that might explain how PTHrP gains access to the cytoplasm for nuclear/nucleolar localization (*8*).

Firstly, secreted PTHrP might re-enter the cell through endocytosis at the cell surface in a receptor-mediated manner (*9*). Secondly, translational initiation of PTHrP mRNA from an alternative initiation codon (CUG) downstream of the initiator methionine (AUG) can generate a prepro-form with a shorter signal sequence that is non-functional for ER targeting and translocation. When a mutant PTHrP cDNA lacking the initiator methionine codon was introduced into cells, PTHrP was shown to be translocated exclusively to the nucleolus (*10*, *11*). However, targeting of PTHrP to the nucleolus has also been shown in CHO cells transfected with a mutant ppPTHrP cDNA, in which possible internal initiation codons for Leu (CUG) are completely replaced with non-initiation codons for Leu (UUG) to avoid internal translation initiation (*11*).

A third alternative is that a specific regulatory mechanism exists to translocate PTHrP across the ER membrane. There is evidence that the pro-PTHrP (pPTHrP) within the ER interacts with luminal BiP (*12*) and undergoes ubiquitination followed by proteasome-dependent proteolysis (*13*). Moreover, pPTHrP associated with microsomal vesicles is not completely protected from trypsin digestion (*12*), suggesting that a significant portion of the pro-form does not reside in the lumen of the ER but is exposed to the cytoplasmic face of the ER membrane. These observations suggest that regulated translocation of PTHrP precursors across the ER membrane is involved in determination of the final localization of the protein. For the ppPTHrP nascent chain, the ER targeting is the first possible step for the regulation of its localization.

In this study, cleavage of PTHrP signal sequence was analysed by expressing a series of ppPTHrP deletion mutants fused to *Escherichia coli* leaderpeptidase (LP) or its glycosylation-deficient mutant (LPΔg) in rabbit reticulocyte lysate in the presence of dog pancreas microsome and in several lines of cultured cells. Our results indicate that the cleavage of PTHrP signal sequence was cell type specific and that the internal NLS of PTHrP had an inhibitory effect on signal sequence cleavage. This inhibitory effect was enhanced by deletion of the spacer sequence between the signal sequence and the NLS. The efficiency of nuclear localization of PTHrP was roughly inversely related to the signal sequence cleavage. These results suggest that the final destination of PTHrP is being regulated at the ER membrane. Effects of importin β on cleavage of the PTHrP signal sequence and the nuclear/nucleolar localization are also discussed.

MATERIALS AND METHODS

Plasmid Construction—Plasmids encoding the entire length of ppPTHrP or its deletion mutants (Fig. 1) fused to *E. coli* leader peptidase (LP) were constructed by the insertion of the HindIII/BamHI fragments of PTHrP cDNA fragments into HindIII and BamHI-digested

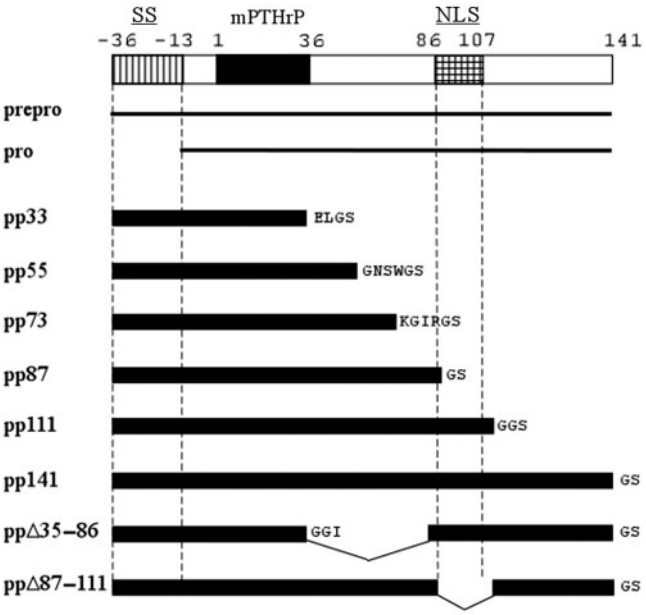

Fig. 1. **PTHrP precursor and its deletion mutants.** Functional domains of the rat PTHrP precursor are indicated as follows. SS, N-terminal ER-targeting signal sequence; mPTHrP, mature PTHrP domain; NLS, nuclear localization signal. Numbers indicate the positions of the residues relative to the N-terminus of mature PTHrP. Lines below indicate regions corresponding to the prepro (ppPTHrP) and the pro (pPTHrP) forms of PTHrP precursors. Entire length of ppPTHrP or a series of its deletion mutants used in this study are shown below. HA-tagged *E. coli* leader peptidase (LP) or its glycosylation-deficient mutant (LPΔg) reporter proteins were fused to their C-termini as described in MATERIALS AND METHODS section. Inserted amino acid residues encoded by linker sequences between PTHrP and LP are indicated by capital letters.

pcDNA-LP-3-N, encoding the 182 C-terminal residues of LP and an additional 9 residues (YPYDVPDYA) of human influenza haemagglutinin (HA) tag at the C-terminus (*14*). Additional amino acid residues encoded by linker DNA sequences are indicated in Fig. 1. Plasmids encoding CDC2/CDK2 phosphorylation site mutants (Thr85Ala and Thr85Glu) were constructed by site-directed mutagenesis of the plasmid encoding the pp141-LP fusion protein using Quick Change (Stratagene) according to the manufacturer's instructions.

To construct a plasmid-encoding rat α_{2u}-globulin (GL) (*15*) fused to LP (pGL46-LP), HindIII/ClaI fragment of GL was inserted into pBluescript KS(−). The resulting plasmid, pBS-pGL46 was digested with EcoRV and BamHI. After T4 polymerase treatment, the DNA fragment was self ligated to create pBS-pGL46ΔBE lacking BamHI, SmaI, PstI, EcoRI and EcoRV sites at the multiple cloning site. NotI/SalI fragment of this plasmid was inserted into pGEX-5X-1 to create pGEX-5X-1-pGL46. HindIII/BamHI fragment of this plasmid was inserted into pcDNA-LP-3-N as for PTHrP fusion proteins above. This fusion protein contains a nine-residue (TVDPGIRGS) insertion derived from the multiple cloning site of pGEX-5X-1 between the GL and LP sequences.

A glycosylation-deficient mutant of the LP reporter (LPΔg) was constructed by replacing the AAG codon for Asn215 with CAG (Gln) using the Quick Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Plasmids encoding GL-LPΔg and PTHrP-LPΔg fusion proteins were constructed as for LP fusion proteins described above.

To construct an expression plasmid of maltose binding protein (MBP) fusion protein [MBP-PTHrP(87-141)], a cDNA fragment containing PTHrP(87-141) region was inserted into the BamHI/SalI site of pMal-c2 (New England Biolabs). To construct MBP-PTHrP(35-141) and MBP-PTHrP(35-86) fusion protein expression plasmids, cDNA fragments containing PTHrP(35-141) or PTHrP(35-86) regions were inserted into BamHI/SalI digested pMal-c2ΔE, of which EcoRI site of pMal-c2 had been deleted by EcoRI digestion, T4 polymerase treatment and re-ligation to adjust open reading frame. The MBP-PTHrP fusion proteins were purified using amylose resin (New England Biolabs) essentially according to the supplier's protocol.

A XhoI/PstI cDNA fragment which encoding the N-terminal half human importin β(7-485) was inserted into the XhoI/HindIII site of pGEX-KG to construct a expression plasmid for GST-importin β(7-485) fusion protein. The C-terminus of the fusion protein contains additional 12 amino acid residues (ARGIRRAQAYSS) derived from a linker sequence. The fusion protein was purified using Glutahione-Sepharose 4B (GE Healthcare) essentially according to the supplier's protocol.

To construct a PTHrP expression plasmid for culture cells (pcDNA-rPTHrP), the EcoRI fragment of rat PTHrP cDNA including upstream 39 base and downstream 20 base non-coding regions was inserted into the EcoRI site of pBluescript II KS (−) in a KpnI to SacI orientation (pBS-rPTHrP5). The BamHI/HindIII fragment of pBS-rPTHrP5 containing the cDNA fragment was inserted into pcDNA3.1/Zeo (+). To construct an expression plasmid for the PTHrP deletion mutant [pcDNA-rPTHrP(Δ35-86)], the HindIII-MscI fragment of pcDNA-rPTHrP was replaced with the HindIII-MscI fragment of the expression plasmid encoding the ppPTHrP(Δ35-86)-LP fusion protein.

In Vitro Transcription and Translation/Translocation—The plasmids encoding the fusion proteins were digested with XbaI to prepare templates for transcription. The linearized plasmids were transcribed *in vitro* from the T7 promoter located upstream of the multiple cloning site of pcDNA3.1(+) using T7 RNA polymerase (Stratagene) according to the manufacturer's instructions. The transcribed mRNAs were used for translation *in vitro* using a rabbit reticulocyte lysate in the presence or absence of dog pancreas microsome membranes prepared as described previously (*14*). Immunoprecipitation and SDS–PAGE were carried out as described previously (*14*). Radioactive protein bands were detected by BAS2500 (Fujifilm) and efficiency of signal sequence cleavage, expressed as (pro-form)/[(prepro-form) + (pro-form)], was calculated using the manufacturer's analysis software supplied with BAS2500 (Fujifilm).

Cell Culture, Transfection, and Labelling—COS-1, HeLa, HEK-293, CV-1 and CHO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum or newborn calf serum, penicillin and streptomycin. Cells (1×10^5) were transfected with indicated plasmids using LipofectAMINE 2000 (Life Technologies, Inc.) in a 24-well plate according to the manufacturer's instructions. After 20 h incubation at 37°C, 5% CO_2, culture medium was replaced with 0.25 ml of DMEM (-Met, -Cys) supplemented with 10% dialysed calf serum, and the cells were incubated for 4 h. The cells were labelled with 10 μCi of [^{35}S]-Met/Cys for 15 min. The labelling reaction was stopped with 0.25 ml of 2× immunoprecipitation buffer [10 mM Tris–Cl (pH 7.4), 0.2 mM EDTA, 0.1% Triton X-100, 0.1% SDS] supplemented with protease inhibitors (1 mM PMSF, 20 μM each of chymostatin, pepstatin, leupeptin and antipain) and 2 mM each of cold Met and Cys. The lysates were treated with DNase I (0.04 mg/ml), 2 mM $MgCl_2$ and RNase A (0.04 mg/ml) for 1 h on ice. Immunoprecipitation and SDS–PAGE were performed as previously described (*14*). Where indicated, the N-glycan chain of the LP reporter was cleaved by peptide: *N*-glycosidase (PNGase, New England Biolabs) treatment according to the manufacturer's instructions.

Interaction Between MBP-PTHrP and GST-importin β—GST-importin β(7-485) (5 μg) was incubated in 50% Glutathione-Sepharose 4B suspension in 100 μl of PBS containing 1 mM PMSF for 1 h at 4°C. After brief washing, the fusion protein bound beads were suspended in 100 μl of 50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1% Triton X-100. MBP or MBP-PTHrP fusion proteins (5 μg) were added to the suspension and the mixture was incubated for 1 h at 4°C. Unbound proteins (F) were recovered as a supernatant of brief centrifugation (15,000*g* for 2 min at 4°C). The precipitated beads were washed and the bound proteins (B) were eluted by incubation in PBS containing 5 mM glutathione (100 μl) for 30 min at 4°C. Unbound (F) and bound (B) fractions were subjected to SDS–PAGE.

Indirect Immunofluorescence—Cells cultured on coverslips were washed twice with PBS, fixed and made permeable for 3 min in ice-cold methanol-acetone (1:1), re-hydrated for 15 min in PBS and blocked with 2% BSA in PBS for 30 min. After being washed with PBS containing 0.2% Triton X-100 (PBST), the cells were incubated for 1 h at room temperature with anti-PTHrP diluted in PBST containing 2% BSA. Then the cells were washed three times with PBST, and incubated for 1 h with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody diluted in PBST containing 2% BSA. Coverslips were then washed three times with PBST and fluorescence was observed using IX-71 inverted microscope equipped with epifluorescence apparatus (Olympus).

Antibodies—Rabbit antiserum against LP (14) was prepared as described previously. Rabbit anti-PTHrP antiserum was raised against a recombinant PTHrP (1-141) protein prepared using the IMPACT kit (New England Biolabs) by cleavage from a fusion protein with chitin-binding protein (CBB) expressed in *E. coli* according to the supplier's protocol.

RESULTS

Cleavage of the Signal Sequence of the PTHrP-LPΔg Fusion Protein—Since prepro-PTHrP contains only a single methionine at the N-terminus and no cysteine, labelling with [^{35}S]methionine and/or [^{35}S]cysteine is impractical. The low specific radioactivity of [^{3}H] or [^{14}C] renders them unsuitable for short-term labelling experiments as required for precise analyses of the ER translocation. To overcome this, *E. coli* LP was selected as a reporter protein. Expression plasmids encoding entire ppPTHrP or its various deletion mutants fused to LP were constructed to examine the cleavage of the ER-targeting signal sequence itself and effects of pPTHrP region, especially its NLS, on it (Fig. 1). An expression plasmid encoding a Leu-rich signal sequence (Fig. 2A) and 46 residues of the mature portion of pre-α_{2u}-globulin (pGL) fused to LP (pGL46-LP) was also constructed for a control of signal sequence cleavage. However, resolution between the prepro-form and the glycosylated pro-form of the fusion protein was not sufficient in our SDS–PAGE system. To improve this, a glycosylation-deficient mutant of LP with an Asn215 to Gln replacement (LPΔg) was used as a reporter protein.

In vitro transcribed mRNAs for these fusion proteins were translated in rabbit reticulocyte lysate in the absence (−) or presence (+) of dog pancreas microsomes (Fig. 2B). In a fusion protein bearing the N-terminal portion of pGL (pGL46), the efficiency of the signal sequence cleavage was up to 80% (Fig. 2B and C). In contrast, the efficiency of the cleavage in a fusion protein bearing the signal sequence of PTHrP (pp33) was only 30%. This poor efficiency was also seen in four other fusion proteins which lacked the NLS region (pp55, pp73, pp87 and ppΔ87-111) as pp33. These results suggest that cleavage of the PTHrP signal sequence is intrinsically inefficient compared to that of the GL signal sequence in the *in vitro* system composed of rabbit reticulocyte lysate and dog pancreas microsome. The efficiencies of the cleavage of PTHrP signal sequence were further reduced to 10–15% in fusion proteins bearing the NLS (pp111, pp141 and ppΔ35-86), implying that the NLS region of PTHrP functions as a further negative regulator for the cleavage of its own signal sequence.

The fusion proteins were also expressed in COS-1 cells. The signal sequence of GL was almost completely cleaved in these cells (Fig. 3A, upper panel, lane 1). Like the *in vitro* experiments, cleavage of the signal sequence of ppPTHrP was significantly less efficient (70–75%) in the fusion proteins lacking the NLS region (Fig. 3, upper panel lanes 2–5 and 9) as compared to GL signal sequence (Fig. 3, upper panel lane 1). The efficiencies of the cleavage were further reduced to 55–60% in the fusion proteins bearing the NLS (Fig. 3, upper panel lanes 6 and 7). However, the apparent effect of the NLS on the cleavage of the signal sequence was less pronounced than in the *in vitro* experiment. On the other hand, the effect of the removal of the spacer region between the signal sequence and the NLS (ppΔ35-86) was more prominent such that the signal sequence cleavage was reduced to 25% (Fig. 3, upper panel lane 8). These results confirm that the inefficient cleavage of the PTHrP signal sequence and the negative effect of the NLS region on cleavage are not artefacts of the *in vitro* reconstitution system. Furthermore, reducing the distance between the two targeting signals had further enhanced the inhibitory effect of the NLS on the cleavage of the ER-targeting signal sequence in COS-1 cells.

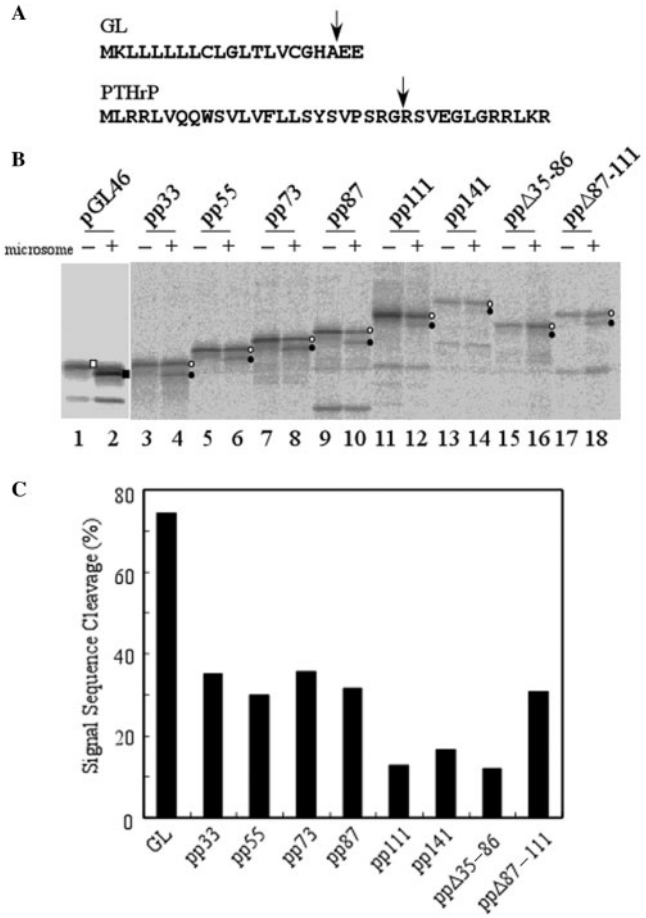

Fig. 2. **Cleavage of the ER-targeting signal sequence of PTHrP–LPΔg fusion proteins *in vitro*.** (A) Primary structures of the signal sequences of rat GL and rat PTHrP used in this study. Arrows indicate a cleavage site of GL signal sequence and a predicted cleavage site (Swiss-Prot: accession P13085) of PTHrP signal sequence. (B) mRNAs encoding the PTHrP-LP fusion proteins were translated in rabbit reticulocyte lysate in the presence (+) or absence (−) of dog pancreas microsomes at 26°C for 30 min. The fusion proteins were recovered by immunoprecipitation using anti-LP antiserum and separated by SDS–PAGE. The labelled protein bands were detected by BAS2500 (Fujifilm). An open square and a closed square indicate precursor and mature forms of the GL-LPΔg fusion protein, respectively. Open circles and closed circles indicate prepro-forms and pro-forms of the PTHrP-LPΔg fusion proteins, respectively. (C) Efficiency of the ER- targeting signal sequence cleavage of the fusion proteins, (pro-form)/[(prepro-form) + (pro-form)] × 100%, was calculated after corresponding protein bands were quantified using BAS 2500 (Fujifilm). The values are the average of two independent experiments.

Cell Type Specificity in the Cleavage of the Signal Sequence of PTHrP—To assess cell type specificity of the cleavage of the PTHrP signal sequence, the fusion proteins were also expressed in HeLa cells. In the fusion proteins lacking the NLS, the efficiencies of the

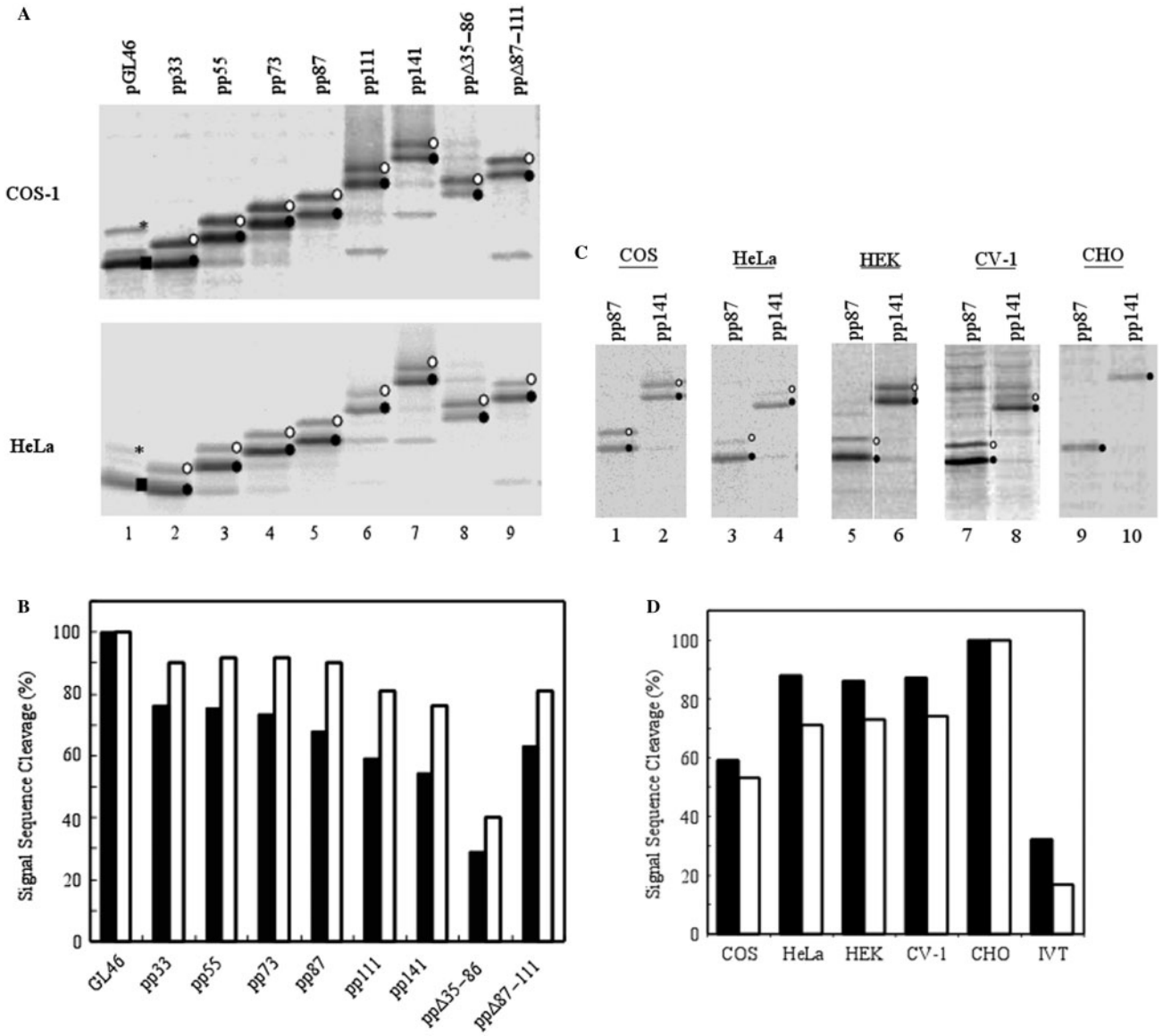

Fig. 3. **Cleavage of the signal sequence of PTHrP–LPΔg fusion proteins in culture cells.** (A) Cleavage of the signal sequence of PTHrP–LPΔg fusion proteins was assessed in COS-1 cells (upper panel) and HeLa cells (lower panel). Plasmids encoding the fusion proteins were transfected into COS-1 cells or HeLa cells. After 24 h, the cells were labelled for 15 min with [^{35}S] Met/Cys. The cells were immediately solubilized as described in MATERIALS AND METHODS section. The fusion proteins were recovered and detected as in Fig. 2. Closed squares indicate the mature form of the GL–LPΔg fusion protein. Open circles and closed circles indicate prepro-forms and pro-forms of the PTHrP-LPΔg fusion proteins, respectively. Asterisks indicate an unknown protein band usually co-precipitated with the pGL46-LPΔg fusion protein. (B) Efficiency of the signal sequence cleavage of the fusion proteins was calculated as in Fig. 2C. The values are the average of three independent experiments. Closed bars and open bars represent COS-1 cells and HeLa cells, respectively. (C) Cleavage of the signal sequence of the ppPTHrP–LPΔg fusion protein lacking the NLS (pp87) and bearing the NLS (pp141) was compared among COS-1, HeLa, HEK293, CV-1 and CHO cells as in (A). Cell type is indicated above the panel. Open circles and closed circles indicate prepro-forms and pro-forms of the PTHrP–LPΔg fusion proteins, respectively. (D) Efficiency of the signal sequence cleavage of the fusion proteins was calculated as in Fig. 2C. Closed and open bars represent pp87 and pp141, respectively.

signal sequence cleavage were significantly improved (Fig. 3A, lower panel lanes 2–5 and 9) when compared to that in COS-1 cells (about 90% *vs* 70–75%). In the fusion proteins bearing the NLS, the efficiencies of the cleavage were reduced to 75–80% (Fig. 3, lower panel lanes 6 and 7). The removal of the spacer region between the signal sequence and the NLS also resulted in significant reduction of the efficiency of the cleavage to 40% as observed in COS-1 cells (Fig. 3A upper panel lane 8).

Efficiencies of the cleavage of PTHrP signal sequence of the fusion proteins lacking the NLS (pp87) and containing the NLS (pp141) were further compared in several cell lines (Fig. 3C). The efficiency of the signal sequence cleavage of the fusion protein lacking the NLS was 59% in COS-1 cells, and was improved to 86–88%

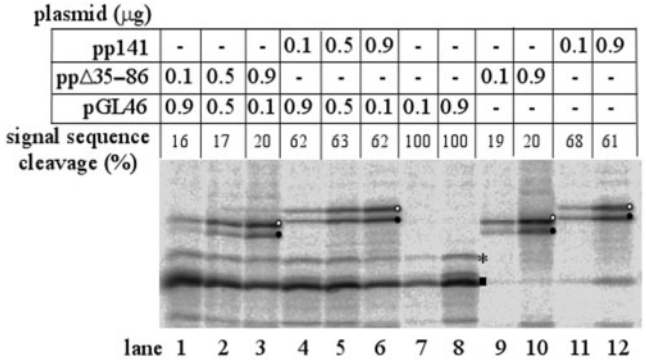

Fig. 4. **Dose-independent efficiency of PTHrP signal sequence cleavage.** COS-1 cells (1×10^5 cells) were transfected with the indicated amount of plasmids encoding GL-LPΔg or PTHrP–LPΔg fusion proteins. After 24 h, the cells were labelled for 15 min with [^{35}S] Met/Cys. The radio-labelled fusion proteins were recovered and detected as in Fig. 3. Efficiency of the signal sequence cleavage of the fusion proteins was calculated as in Fig. 2C and indicated above the panel. A closed square indicates the mature form of the GL–LPΔg fusion protein. Open circles and closed circles indicate prepro-forms and pro-forms of the PTHrP–LPΔg fusion proteins, respectively. An asterisk indicates the unknown protein band usually co-immunoprecipitated with the GL–LPΔg fusion protein.

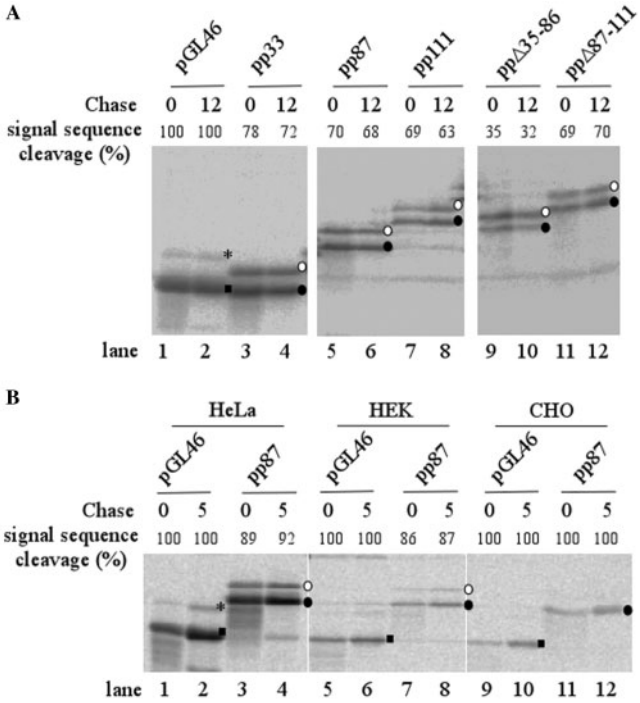

Fig. 5. **Pulse-chase experiment of PTHrP fusion proteins.** (A) Plasmids encoding PTHrP-LPΔg fusion proteins were introduced into COS-1 cells as described. The cells were pulse labelled for 5 min with [^{35}S] Met/Cys and chased for 12 min. The radio-labelled fusion proteins were analysed as in Fig. 3. (B) Plasmids encoding PTHrP–LPΔg fusion proteins indicated above the panel were introduced into HeLa, HEK293 or CHO cells. The cells were pulse labelled for 1 min with [^{35}S] Met/Cys and chased for 5 min. The radio-labelled fusion proteins were analysed as in (A). Closed squares indicate the mature form of the GL–LPΔg fusion protein. Open circles and closed circles indicate prepro-forms and pro-forms of the PTHrP–LPΔg fusion proteins, respectively. Asterisks indicate the unknown protein band usually co-precipitated with the GL–LPΔg fusion protein. Efficiency of the signal sequence cleavage of the fusion proteins was calculated as in Fig. 2C and is shown above the panel.

in HeLa, HEK293 and CV-1 cells. The negative effect of the NLS region on the cleavage of signal sequence was observed in all of those cells. In contrast, the signal sequence of PTHrP was almost completely cleaved in CHO cells. Furthermore, the negative effect of the NLS on the signal sequence cleavage was not observed in CHO cells. These results indicate that the efficiency of PTHrP signal sequence cleavage is cell-type-specific. It seems likely that the inefficient cleavage of the signal sequence itself in the cells is a prerequisite for the apparent negative action of the NLS domain.

The expression levels of the fusion proteins in HeLa and HEK-293 cells were comparable to those of COS-1 cells. However, CV-1 and CHO cells expressed less than one-tenth as much recombinant protein. To assess possible dose dependence on the efficiency of cleavage of the signal sequence, varying amounts of pp141 and ppΔ35-86 fusion proteins were expressed in COS-1 cells with or without co-expression of the pGL46-LPΔg fusion protein (Fig. 4). When the total amount of the plasmids encoding PTHrP fusion proteins and the GL fusion protein was kept to 1 μg for transfection, the efficiency of the signal sequence cleavage was independent of the expression levels of either of the two fusion proteins (Fig. 4, lanes 4–6). Also, complete cleavage of the signal sequence of the GL fusion protein was not affected by the presence of the PTHrP fusion proteins at any expression levels. The efficiencies of the signal sequence cleavage were also unchanged when varying amounts of GL or PTHrP fusion proteins were expressed separately (Fig. 4, lanes 7–12). These results indicate that the inefficient cleavage of the signal sequence in COS-1 cells is not due to the capacity of the translocation machinery but is instead a cell type-specific property.

Uncleaved ppPTHrP Fusion Proteins are Segregated from the Secretory Pathway—pGL46 and PTHrP fusion proteins were pulse labelled for 5 min and chased for 12 min in COS-1 cells (Fig. 5A). The GL fusion protein was completely converted to the mature form during the pulse time period. In contrast, the uncleaved prepro-forms of all of the PTHrP fusion proteins observed in the pulse period were not converted to their pro-forms during the chase time period irrespective of the presence of the NLS or of distance between the ER-targeting signal sequence and the NLS. There was neither obvious delay in cleavage of the PTHrP signal sequence nor evidence of rapid degradation of the fusion proteins. Similar results were obtained in HeLa and HEK293 cells, even with a shorter period (1 min) of the pulse labelling (Fig. 5B, lanes 1–8). The uncleaved prepro-form was neither converted to the pro-form nor subjected to rapid degradation during a 5 min chase period. In contrast, the pp87 fusion protein was almost completely converted to the mature form during 1-min pulse period in CHO cells (Fig. 5B, lanes 9–12). Efficiency and time course of the cleavage were indistinguishable between GL and PTHrP signal sequences in CHO cells.

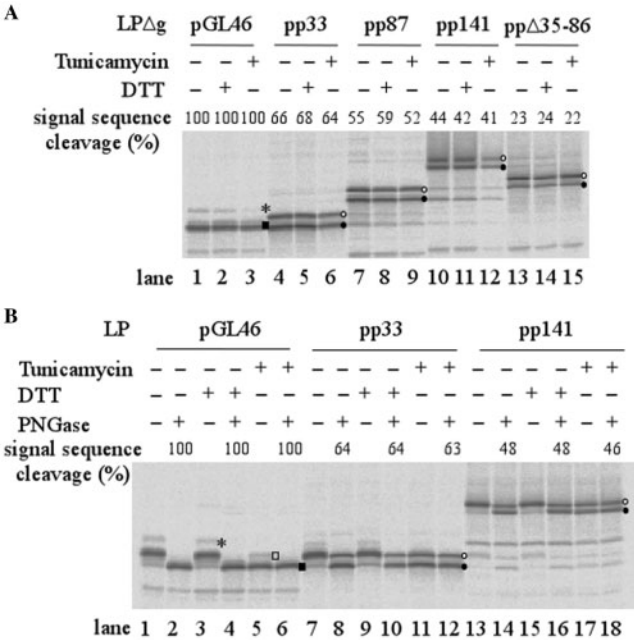

Fig. 6. **Reagents that evoke the unfolded protein response had no effect on efficiency of cleavage of the PTHrP signal sequence.** (A) Plasmids encoding glycosylation-deficient (LPΔg, panel A) or wild-type (LP, panel B) LP reporter fusion proteins were introduced into COS-1 cells. Where indicated, DTT (2 mM) or tunicamycin (5 μg/ml) was added and the cells were incubated for 1 h, and then the cells were radio-labelled for 15 min with [^{35}S] Met/Cys. The LPΔg fusion proteins (A) were recovered and analysed as in Fig. 3. The PTHrP-LP fusion proteins (B) were recovered by immunoprecipitation before further treatment. Half was treated with PNGase for 1 h and the other half was mock treated, and the fusion proteins were analysed as in Fig. 3. Closed squares indicate mature form of the GL–LPΔg or PNGase-treated GL–LP fusion protein. An open square indicates glycosylated mature form and precursor form of the GL–LP fusion protein. Open circles and closed circles indicate prepro-forms and pro-forms of the PTHrP–LPΔg or PNGase-treated PTHrP–LP fusion proteins, respectively. Asterisks indicate the unknown protein band usually co-immunoprecipitated with the GL–LPΔg fusion protein. Efficiency of the signal sequence cleavage of the fusion proteins was calculated as in Fig. 2C and is shown above the panel.

The prepro-forms uncleaved during the pulse period are most likely to be segregated from the secretory pathway irreversibly at an early phase of the co-translational translocation pathway. In addition, the result with CHO cells, showing that the cleavage of PTHrP signal sequence can be virtually complete within as short a time as 1 min of the pulse labelling, further supports cell type-specific regulation of cleavage of the PTHrP signal sequence.

Stress Conditions Did Not Affect the Cleavage of the Signal Sequence of PTHrP—To assess effects of the ER stress conditions on cleavage of the PTHrP signal sequence, COS-1 cells transfected with the plasmids encoding the PTHrP fusion proteins were subjected to stress conditions. Treatment with neither DTT nor tunicamycin, known to evoke such stress conditions, had any apparent effect on cleavage of the signal sequences of GL or PTHrP fused to the glycosylation-deficient LP irrespective of the presence or absence of the NLS (Fig. 6A).

In the LP fusion proteins, band separation between the prepro-forms and the glycosylated pro-forms of PTHrP fusion proteins was not sufficient in the SDS–PAGE system. Deglycosylation of the LP fusion proteins with PNGase treatment enabled quantification of the efficiency of the cleavage of the signal sequences of GL- and PTHrP-LP fusion proteins. The efficiency was not significantly different from that of the corresponding glycosylation-deficient LP fusion proteins (LPΔg) in any of the conditions tested. Taken together, the ER stress conditions had no apparent effect on cleavage of PTHrP signal sequence irrespective of the presence or absence of the NLS region, or of glycosylation of the reporter protein.

Effect of Importin β on Cleavage of the Signal Sequence and Nuclear/Nucleolar Localization of PTHrP—Regulation of the signal sequence cleavage may occur through cytosolic factors such as importin β, which interacts with the region between residues 67 and 94 of PTHrP (*7*). An MBP-PTHrP fusion protein containing the entire importin β interaction region [MBP-(35-141)] efficiently bound to a N-terminal half importin β fused to GST [GST-importin β(7-485)] (Fig. 7A, lane 8). Deletion of 35-86 region of PTHrP [MBP-(87-141)] almost completely abolished the interaction between the MBP–PTHrP fusion protein and the GST-importin β fusion protein (lane 6), although the protein contains the entire NLS region. MBP and MBP-(35-86) fusion protein did not interact with the GST-importin β fusion protein (lanes 2 and 4). These results indicate that the 35-86 region of PTHrP is required for stable interaction between PTHrP and importin β as expected from the previous reports (*6*, *7*).

To examine the effects of importin β on the signal sequence cleavage, the GST-importin β fusion protein was added to the *in vitro* translation mixture in the presence of microsome (Fig. 7B). As expected, addition of the improtin β fusion protein had no effect on the PTHrP signal sequence cleavage of the PTHrP–LP fusion proteins lacking the entire importin β interaction region (pp87 and ppΔ35-86). Significantly, no effect was also observed in the fusion protein containing the entire interaction region (pp111).

Phosphorylation of the threonine residue adjacent to the NLS (Thr85) has been proposed as a mechanism involved in the regulation of nuclear/nucleolar localization of PTHrP by regulating its interaction with importin β (*5*). To assess whether the phosphorylation is involved in the regulation of the PTHrP signal sequence cleavage, efficiencies of the signal sequence cleavage of the pp141 fusion proteins bearing the Thr85Glu (T85E) or Thr85Ala (T85A) mutation were examined in COS-1 cells (Fig. 7C). The results showed that neither Thr85Glu nor Thr85Ala had any significant effect on cleavage of the signal sequence. These results suggest that the phosphorylation site is not involved in the regulation of the cleavage of the signal sequence of PTHrP.

In order to assess effects of the deletion in PTHrP on its nuclear/nucleolar localization, wild-type ppPTHrP (ppPTHrP141) and ppPTHrP(Δ35-86) were expressed in

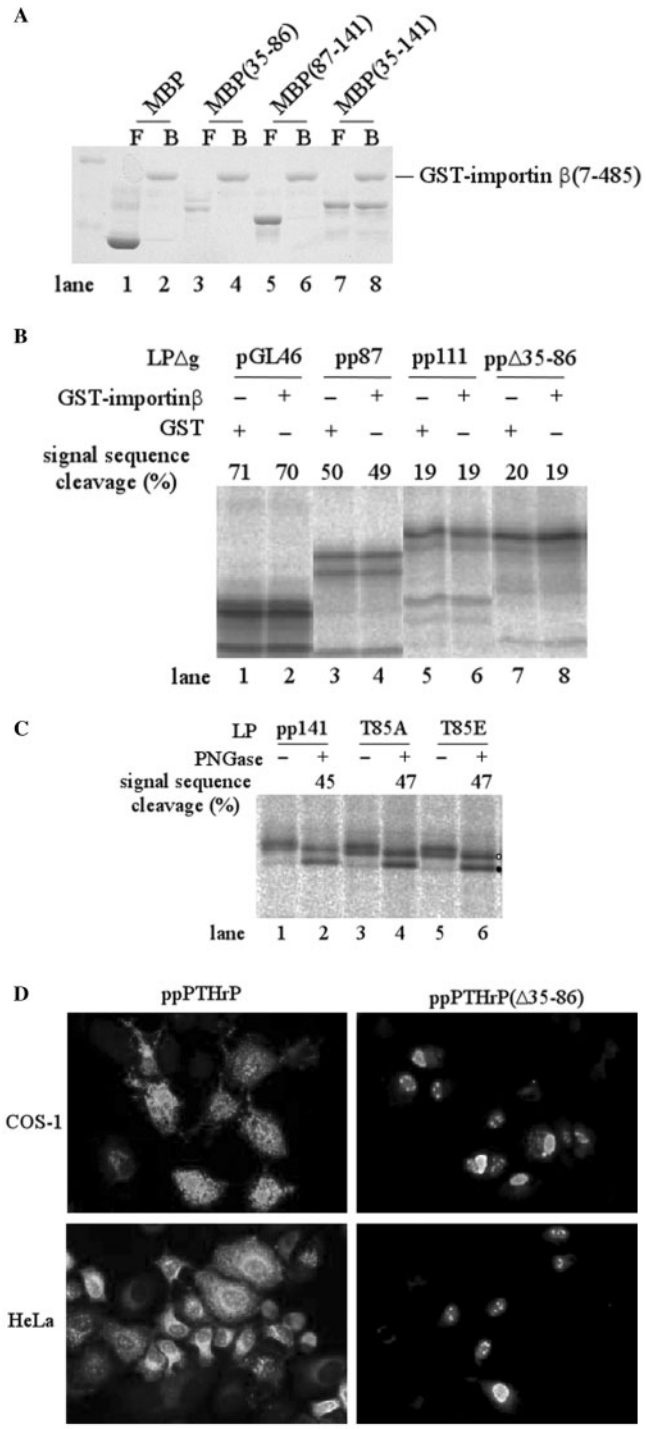

Fig. 7. **Effect of importin β on cleavage of the signal sequence and nuclear/nucleolar localization of PTHrP.** (A) GST-importin β(7-485) bound Glutathione-Sepharose 4B beads were incubated with MalE-PTHrP fusion proteins at 4°C for 1 hr, and then the reaction mixture was separated into free (F) and bound (B) fractions by brief centrifugation. The precipitated beads were washed and then incubated with PBS containing 5 mM glutathione for 30 min to release the bound proteins. The proteins of both of the fractions were subjected to SDS–PAGE and detected by Coomassie blue staining. (B) mRNAs encoding the PTHrP–LPΔg fusion proteins were translated in rabbit reticulocyte lysate in the presence of dog pancreas microsomes at 26°C for 30 min. Where indicated, GST (3 μg) or GST-importin β(7-485) (5 μg) were added to the translation mixture. The radio-labelled fusion proteins were analysed, and efficiency of the signal sequence cleavage of the fusion proteins was calculated as in Fig. 2. The values are the average of two independent experiments. (C) Plasmids encoding ppPTHrP–LP fusion proteins of the wild type (pp141) or its CDC2/CDK2 phosphorylation site mutants (T85A and T85E) were transfected into COS-1 cells. After 24 h, the cells were labelled for 15 min with [^{35}S] Met/Cys. The fusion proteins were recovered with immunoprecipitation. Half of the solubilized protein was treated with PNGase for 1 h and the other half was mock treated, and the fusion proteins were analysed as in Fig. 3. Open circles and closed circles indicate prepro-forms and pro-forms of the PTHrP–LPΔg fusion proteins, respectively. Efficiency of the signal sequence cleavage of the fusion proteins was calculated as in Fig. 2C and is shown above the panel. (D) ppPTHrP (left panels) or ppPTHrP(Δ35-86) (right panels) were expressed in COS-1 cells (upper panels) or HeLa cells (lower panels). Localization of the proteins was detected by indirect immunofluorescence using anti-PTHrP(1-141) as described in MMATERIALS AND METHODS section.

COS-1 cells and HeLa cells, and the localization of the proteins were analysed by indirect immunofluorescence (Fig. 7D). The wild-type ppPTHrP was detected in the cytoplasm, nucleus and nucleolus. On the other hand, ppPTHrP(Δ35-86) was detected almost exclusively in the nucleus and nucleolus in both COS-1 and HeLa cells. These results indicate that the efficiency of nuclear/nucleolar localization of PTHrP is roughly inversely related to efficiency of the signal sequence cleavage, and also suggest that the interaction between ppPTHrP(Δ35-86) and importin β is dispensable for the nuclear/nucleolar localization of the deletion mutant.

DISCUSSION

In this study, we have demonstrated that the cleavage efficiency of the PTHrP signal sequence is cell-type-specific, and is negatively regulated in *cis* by its own NLS domain. The uncleaved prepro-form of the fusion protein is segregated from the secretory pathway and is exposed to the cytoplasmic face of the ER membrane (unpublished data). This may facilitate interaction of ppPTHrP with importin β and/or other factors that are required for nuclear/nucleolar translocation of the protein. Roughly inverse relationship between the efficiency of the nuclear/nucleolar localization and the signal sequence cleavage of PTHrP was observed in COS-1 or HeLa cells expressing wild-type ppPTHrP or its Δ35-86 mutant.

Cell-Type-Specific Cleavage of PTHrP Signal Sequence—Inefficient cleavage of the signal sequence of PTHrP was significant both *in vitro* and in COS-1 cells, and was improved in HeLa, HEK293 and CV-1 cells. Virtually complete cleavage of the signal sequence was observed in CHO cells (Figs 3–5). Similarly, the signal sequence of HCMV US11 is more efficiently cleaved in HEK293 cells than in COS-1 cells (*16*). Segregation from the secretory pathway of the GAL4 DNA-binding domain (GAL4 BD) and nuclear factor-κB transcriptional activation domain (NF-κB AD) fusion proteins bearing the signal sequences of prion protein or osteopontin depends on cellular metabolism in HeLa and N2a cells (*17*). Similar regulation of the segregation is observed in the

fusion proteins bearing the signal sequences of calreticulin or osteopontin expressed in MDCK cells. These cellular metabolism specific patterns of localization were only observed in specific combinations of signal sequences and cell lines. The cell type- and cell metabolism-specific function of these signal sequences including that of PTHrP suggests that physiological regulation of protein destination or membrane topology of these proteins is occurring at the point of targeting to and/or insertion into the ER membrane by nature of their signal sequences.

Molecular Basis of the Regulation of Signal Sequence Cleavage—The PTHrP signal sequence had similar biochemical characteristics to the GL signal sequence in both the sensitivity of their cleavage to *N*-ethylmaleimide and in their efficiency of high salt-resistant binding of ribosome nascent chain complexes to microsomal membrane (unpublished data). These results suggest that the ER targeting of the ppPTHrP nascent chain is SRP-dependent and that the targeting efficiency of the PTHrP signal sequence is not significantly different from that of the GL signal sequence. However, it is still possible that the relatively low hydrophobicity of the PTHrP signal sequence (Fig. 2A) results in reduced binding affinity of the PTHrP signal sequence to SRP and/or to the signal binding site of the translocation channel protein, Sec61p.

An alternative mechanism, which could also affect signal sequence cleavage, may be the topology of the signal sequence insertion into Sec61p. Both a positive charge in the N-terminal region and a decreased number of Leu residues in the signal sequence favour the topology of binding with N-terminus towards the cytoplasm (N_{cyt}/C_{exo}) (*18*). Certain conserved, charged residues of Sec61p contribute to determine the topology preference of the signal sequence insertion (*19*, *20*). In addition, ribosomal proteins and/or RNA located at the polypeptide exit site are hypothesized to affect orientation of signal sequence insertion (*21*). In the case of ppPTHrP, the positive charges around the cleavage site (Fig. 2A) might increase the ratio of N_{exo}/C_{cyt} topology insertion with which the cleavage site would not be exposed to the active site of the signal peptidase located in the luminal face of the ER membrane.

It is also possible that the structure of ppPTHrP around the signal sequence cleavage site is not suitable for efficient substrate recognition or cleavage reaction by the signal peptidase. Although analysis of the signal sequence probability with a machine-learning approach (*22*) predicted that the N-terminal region of ppPTHrP was indeed a signal sequence, its cleavage site probability was significantly lower than that of GL. These properties of the PTHrP signal sequence may also explain its inefficient cleavage.

Delayed cleavage of the signal sequence is observed in HCMV US11 (*16*) and the amber mutant at codon 145 of prion proteins (*23*). In the latter case, the uncleaved portion of the precursor protein undergoes rapid degradation by the proteasomal pathway. In contrast to these proteins, ppPTHrP synthesized and uncleaved in the pulse period (1 or 5 min) was neither converted to pPTHrP nor rapidly degraded during the 5 or 12 min of chase period (Fig. 5). This suggests that the fate of the nascent PTHrP is determined just after translation and ER targeting. In the case of the prion protein signal sequence, stress conditions such as DTT or thapsigargin treatment resulted in translocation attenuation that had a positive effect on cell survival (*24*). Dissociation from calnexin is required for cleavage of the HIV-1 gp120 signal sequence (*25*). Contrary to these examples, stress conditions such as DTT or tunicamycin treatment had no apparent effect on accumulation of the prepro-form of the PTHrP-LP fusion proteins (Fig. 6). These results suggest that the regulation of PTHrP signal sequence cleavage differs from that of HIV gp120 (*25*) and prion protein (*24*), which depend on ER luminal component(s) for the cleavage of the signal sequence.

Effect of Importin β on Regulation of PTHrP Localization—In the present work, we have shown that the NLS region of ppPTHrP exerts a negative effect on the signal sequence cleavage (Figs 2 and 3). In support for our results, secretion of PTHrP from the cells expressing the NLS-deleted ppPTHrP was reported to be higher than that from the wild-type expressing cells (*26*, *27*). Though it is possible that another regions of the PTHrP–LP fusion protein also affect the signal sequence cleavage, artefacts of the reporter is unlikely because replacement of the LP reporter with GL or EGFP had no significant effect on cleavage of the signal sequence of PTHrP including the cell type specificity and *cis* regulation by the NLS (unpublished data).

Subcellular localization of PTHrP or PTHrP(Δ35-86) detected by indirect immunofluorescence revealed roughly inverse relationship between the efficiency of the nuclear/nucleolar translocation and the signal sequence cleavage of PTHrP when expressed in COS-1 or HeLa cells (Figs 3B and 7D). Competition between the ER targeting through the SRP–signal sequence interaction and the nuclear targeting through the importin-β–NLS interaction could occur in the cytosol, when the kinetics of the SRP–signal sequence interaction is slow enough that the NLS may emerge from the ribosome before the SRP–signal sequence interaction. Alternatively, even after targeting to the ER membrane, regulation of the signal sequence cleavage may occur through a cytoplasmic factor(s) because the ribosome-nascent chain complex of ppPTHrP33-GL fusion protein bound to the microsomal membrane is not efficiently protected from protease digestion (unpublished data). However, in either case, involvement of importin β seems unlikely as the PTHrP(Δ35-86) deletion mutant could not interact with importin β (Fig. 7A). Furthermore, addition of the GST-importin β fusion protein into the *in vitro* reconstitution system had no effect on the PTHrP signal sequence cleavage even for the fusion protein containing the entire importin β interaction region, supporting the idea that inportin β is not involved in the regulation of the PTHrP signal sequence cleavage (Fig. 7B).

Phosphorylation at Thr85 by cyclin-dependent kinase has been suggested as a possible regulator of the nuclear/nucleolar translocation of PTHrP by inhibiting the interaction between PTHrP and importin β (*5*, *7*). The ratio of nuclear staining of GFP–PTHrP fusion proteins without the signal sequence is reported to be

significantly reduced with the Thr85Glu mutation and slightly increased with the Thr85Ala mutation compared to the wild type (*5*). However, these mutations had no effect on signal sequence cleavage (Fig. 7C). These results suggest that improtin β and therefore cyclin-dependent kinase are not regulators of PTHrP signal sequence cleavage.

Poly(G) has been reported to be one of other molecules that interact with the NLS domain of PTHrP (*28*). However, addition of poly(G) into the *in vitro* translation/translocation system had no effect on cleavage of the PTHrP signal sequence as in the case of GST-importin β fusion protein (data not shown). To understand the nuclear localization of PTHrP(Δ35-86) and the regulation of the signal sequence cleavage by its NLS, effects of other candidates for the NLS interacting molecules such as other importin family proteins (*29*) and/or effects of microtubule (*30*) should be examined.

Cis-Regulation of Cleavage of the Signal Sequence by the C-terminal Region of the Nascent Chain—It is possible that the NLS itself or molecule(s) interacting with the NLS significantly affects the cleavage of the signal sequence by inhibiting N_{cyt}/C_{exo} topology insertion of the signal sequence into Sec61p. Indeed, some distant transmembrane domains can participate as *cis*-regulators in combination with certain N-terminal signal sequences. Cleavage of the signal sequence of the HCMV US11 protein is induced by its C-terminal transmembrane domain (*16*). In prion proteins, certain mutations at the transmembrane region have been shown to affect the efficiency of signal sequence cleavage (*31*). The distance between the signal sequence and an internal transmembrane domain also affects the topology of prion protein (*32*). Inefficient cleavage of the signal sequence of a fusion protein consisting of the preprolactin signal sequence and the transmembrane region of asialoglycoprotein is observed when the distance between the signal sequence and the transmembrane region is reduced (*33*). Competition between the signal sequence and the transmembrane domain for binding to SRP or inhibition of N_{cyt}/C_{exo} topology insertion of the signal sequence into Sec61p by the transmembrane region may affect the cleavage efficiency of the N-terminal signal sequence (*32*, *33*). In the case of PTHrP, competition for SRP seems unlikely as the NLS may not interact with SRP. However, the PTHrP NLS could affect the cleavage of its signal sequence through modulation of topology of the signal sequence insertion. It must be noted that negatively charged surface by rRNA elements near the polypeptide exit site could interact with nascent chain (*21*). It is possible that interaction between rRNA near the exit site and PTHrP NLS could affect the orientation of the PTHrP signal sequence insertion.

Observed functions of PTHrP within the nucleus/nucleolus include protection of serum-deprived cells from apoptosis and regulation of cell proliferation (*3*, *27*, *34*). These fundamental physiological roles must be regulated in a tissue, differentiation and/or cell-cycle-specific manner. Our results strongly suggest that nuclear/nucleolar transport of PTHrP is at least partly regulated during early phases of the ER translocation of the nascent protein by the property of its ER-targeting signal sequence.

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