J. Biochem. 2016;159(4):393–406 doi:10.1093/jb/mvv111

JB THE JOURNAL OF BIOCHEMISTRY

Evolutionary well-conserved region in the signal peptide of parathyroid hormone-related protein is critical for its dual localization through the regulation of ER translocation

Received July 30, 2015; accepted September 29, 2015; published online November 3, 2015

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Parathyroid hormone-related protein (PTHrP) has two different targeting signals: an N-terminal signal peptide for the endoplasmic reticulum (ER) targeting and an internal nuclear localization signal. The protein not only functions as a secretory protein, but is also found in the nucleus and/or nucleolus under certain conditions. PTHrP signal peptide is less hydrophobic than most signal peptides mainly due to its evolutionarily well-conserved region (QQWS). The substitution of four tandem leucine residues for this conserved region resulted in a significant inhibition of the signal peptide cleavage. At the same time, proportion of nuclear and/or nucleolar localization decreased, probably due to tethering of the protein to the ER membrane by the uncleaved mutant signal peptide. Almost complete cleavage of the signal peptide accompanied by a lack of nuclear/nucleolar localization was achieved by combining the hydrophobic h-region and an optimized sequence of the cleavage site. In addition, mutational modifications of the distribution of charged residues in and around the signal peptide affect its cleavage and/or nuclear/nucleolar localization of the protein. These results indicate that the well-conserved region in the signal peptide plays an essential role in the dual localization of PTHrP through ER targeting and/or the membrane translocation.

Keywords: endoplasmic reticulum/nuclear localization signal/parathyroid hormone-related protein/protein conducting channel/signal peptide.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; GL, α_{2u}-globulin; LP, leader peptidase; NLS, nuclear localization signal; PCC, protein conducting channel; PNGase, peptide: N-glycosidase; PTHrP, parathyroid hormone-related protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SRP, signal recognition particle.

The prepro-form of parathyroid hormone-related protein (ppPTHrP) has two targeting signals: an N-terminal signal peptide for endoplasmic reticulum (ER) targeting and a nuclear localization signal (NLS) in its mid-region. The protein enters the secretory pathway, and is cleaved to generate the bioactive mature PTHrP (mPTHrP), in which the N-terminal half is homologous to the parathyroid hormone, and other poorly characterized peptides (*1*). PTHrP is also found in the nucleus and/or nucleolus where it may play a different role from that of the secreted mPTHrP (*2*). According to the signal recognition particle (SRP)-dependent cotranslational mechanism, the nuclear/nucleolar localization of PTHrP is surprising, since the NLS is located downstream of the ER-targeting signal peptide and is not expected to interact with nuclear transport machineries. In spite of that, several possibilities which could explain nuclear/nucleolar localization of PTHrP have been proposed (*3*).

The first possibility is reuptake of the once secreted PTHrP through endocytosis in a receptor mediated manner (*4*). To accomplish this, pro-PTHrP (pPTHrP) must escape from proteolytic processing for maturation during secretion by an unknown regulatory mechanism, since mPTHrP lacks the NLS. Whether some regulation(s) exists for pPTHrP processing between the mPTHrP and NLS by the prohormone convertase is still unclear.

The second possibility is alternative translational initiation from one of the four CUG codons of PTHrP mRNA located downstream of the authentic initiator methionine codon (AUG). The translation product is predicted to have a shorter signal peptide that is nonfunctional for ER targeting and/or translocation. In support of this possibility, it was shown that expression of a mutant PTHrP cDNA lacking the authentic initiator codon in the cell results in exclusive nuclear localization of the protein, although the translation is much less efficient compared with that from the authentic initiator codon (*5*, *6*). A shunt mechanism for the alternative translation initiation must thus be elucidated.

The third possibility is the regulation of ER targeting and/or translocation, the first possible step for determining the protein localization, through the unique structural features of the PTHrP nascent chain including its signal peptide. In our previous work, we revealed, by examining the effects of a series of deletions of ppPTHrP, that the proportion of nuclear/nucleolar localization of PTHrP is roughly inversely correlated to the proportion of signal peptide

cleavage (*7*). Moreover, the targeting of PTHrP to the nucleus is still observed when a PTHrP cDNA, in which all the CTG codons in the signal peptide has been replaced with TTG codons to avoid alternative translation initiation without changing the encoded amino acids, is expressed in Chinese hamster ovary cells (*6*). In addition, the precursor forms of PTHrP associated with microsomal vesicles are not completely protected from trypsin digestion, which suggests that a significant portion of the protein is exposed to the cytoplasmic face of the ER membrane for proteasome-mediated degradation (*8*). These observations suggest that partitioning of the PTHrP nascent chain through ER targeting and/or the membrane translocation is involved in the mechanism(s) for the dual localization of the protein. Although it is known that acute stress of the ER temporarily prevents prion protein from translocating to the ER and redirects it to the cytosolic degradation pathway (*9*), the underlying mechanism for the translocational regulation of PTHrP, which is insensitive to stress conditions, seems to be different (*7*).

In this study, we focused on the unique structural features of the N-terminal region of ppPTHrP, including its signal peptide, and assessed their roles in signal peptide cleavage and in the dual localization of the protein in detail by use of mutational analysis. Our results revealed that the well-conserved region in the h-region of the signal peptide and the positively charged residues in the pro-region play important roles in the regulation of ER targeting and/or translocation of the PTHrP nascent chain and have an impact on its dual localization. In addition, we also investigated the structural requirements of the signal peptide for its efficient cleavage and for suppression of its inefficient cleavage by cycloheximide and anisomycin.

Materials and Methods

Plasmid constructions and site directed mutagenesis

Plasmids encoding the mutant proteins listed in Fig. 1B were constructed by site directed mutagenesis of the plasmid encoding the N-terminal 33 residues of rat preproPTHrP fused to a linker sequence (ELGS), the C-terminal 182 residues of *Escherichia coli* (*E. coli*) leader peptidase (LP) and an additional nine residues (YPYDVPDYA) of human influenza hemagglutinin (HA) tag (ppPTHrP33-LP) (*7*) using the Quick Change II site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Synthesis of the mutagenesis oligonucleotides (Table I) and confirmation of the sequences of the constructed plasmids encoding the mutations were performed by Fasmac Co., Ltd (Kanagawa, Japan).

An expression plasmid encoding full-length ppPTHrP-EGFP fusion protein was constructed by insertion of the *Hin*dIII/*Bam*HI fragment of the full-length PTHrP cDNA (*7*) into *Hin*dIII and *Bam*HI-digested pEGFP-N3 (Clonetech). Plasmids encoding the mutant ppPTHrP-EGFP, used in Figs 3 and 4, were constructed as described above.

Antibodies

Preparation of an antibody against *E.coli* LP (anti-LP) is described previously (*7*). An antibody against the C-terminal 20 residues of rat proPTHrP [anti-PTHrP(122-141)] was prepared as follows. A peptide consisting of the rat proPTHrP(122-141) (CDPQPHTSP TSTSLEPSSRTH) and an extra N-terminal cysteine residue was synthesized and conjugated to keyhole limpet hemocyanin through the sulfhydryl group of the N-terminal cysteine residue using MBS (m-maleimidobenzoyl-N-hydroxysuccinimide ester) (PIERCE, USA). A rabbit (female New Zealand White) was immunized with the conjugated peptide.

Cell culture, transfection and labelling

COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin and streptomycin. Cells (1×10^5) were seeded into 24-well plates and transfected with the indicated plasmids using LipofectAMINE 2000 (Life Technologies, Inc.) 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 further incubated for 4 h. The cells were labelled with 10 μCi of [^{35}S]-Met/Cys for 15 min for the ppPTHrP33-LP fusion proteins or 30 min for the ppPTHrP-EGFP fusion proteins. The labelling reaction was stopped with 0.25 ml of 2 × immunoprecipitation buffer [10 mM Tris-Cl (pH 7.4), 0.2 mM ethylenediaminetetraacetic acid, 0.1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS)] supplemented with protease inhibitors (1 mM phenylmethanesulfonyl fluoride (PMSF), 20 μM each of chymostatin, pepstatin, leupeptin and antipain), and 2 mM each of cold Met and Cys. The cell 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 by the anti-LP antibody for the ppPTHrP33-LP fusion proteins or the anti-PTHrP(122-141) for the ppPTHrP-EGFP fusion proteins and SDS–PAGE (polyacrylamide gel electrophoresis) were performed as previously described (*7*). Where indicated, the N-glycan chain of the LP reporter was cleaved by peptide N-glycosidase (PNGase F, New England Biolabs, USA) treatment according to the manufacturer's instructions. The protein bands were quantified using BAS5000 (Fuji Film) or Typhoon FLA 7000 (GE-Healthcare), and the following values were calculated.

Proportion of glycosylated prepro-form in PNGase F-untreated experiments:

$$glycosylated\ prepro\,(\%) = \left[\frac{(glycosylated\ \ prepro)}{[(glycosylated\ \ prepro) + (glycosylated\ pro) + (non\text{-}glycosylated\ \ prepro)]}\right] \times 100 \quad (1)$$

Proportion of signal peptide cleavage in PNGase F-treated experiments:

$$signal\ peptide\ cleavage\,(\%) = \frac{(pro)}{(prepro) + (pro)} \times 100 \quad (2)$$

The proportion of non-glycosylated prepro-form in the PNGase F-untreated experiments was calculated under an assumption that all the glycosylated pro-form is converted to non-glycosylated pro-form by the PNGase F treatment.

Fluorescence microscopy

Cells transfected with the plasmid encoding the ppPTHrP-EGFP fusion protein were cultured on a ϕ35 mm culture dish. After the medium was changed to phosphate buffered saline (PBS), the transfected cells were observed using an IX-71 inverted microscope equipped with fluorescence apparatus (Olympus).

Results

Mutants altering the structural features of the PTHrP signal peptide and pro-region

The signal peptide of PTHrP has several characteristic structural features, which are evolutionarily well-conserved among mammalian species but are unfavourable for its ER targeting and/or translocation (Fig. 1A). To assess the functions of these structural features on the dual localization of PTHrP, we constructed mutants that alter the charge distribution, the hydrophobicity, and/or the cleavage site of the signal

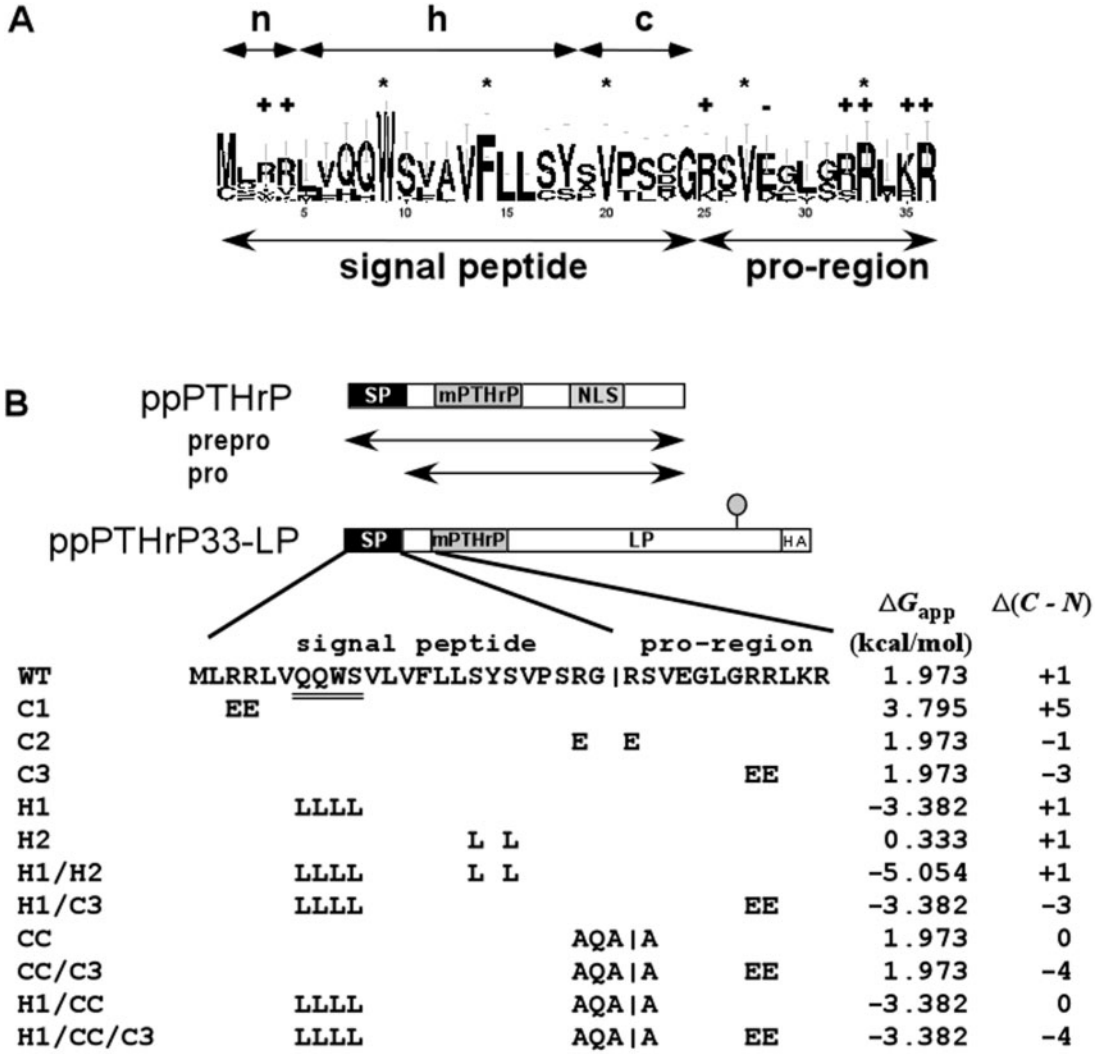

Fig. 1 Evolutionary conservation of the ER-targeting signal peptide of PTHrP (A), and the mutations used in the functional analysis of the signal peptide (B). (**A**) The amino acid sequences of the signal peptide and the pro-region of PTHrP are aligned and graphically represented by WebLogo 3 (*31*). The asterisks indicate the completely conserved residues; charged residues are indicated by the positive/negative signs; and the height of the letter indicates the relative frequency along with the degree of sequence conservation of each amino acid at that position. The regions of the signal peptide are shown above. The accession numbers for ppPTHrP sequences are P17251 (chicken), P13085 (rat), P22858 (mouse), Q9GLC7 (rabbit), P58073 (bovine), P52211 (dog), P12272 (human), CAB94712 (fugu) and AAF79073 (seabream).(**B**) Schematic diagram showing the N-terminus of rat ppPTHrP including the signal peptide, the pro-region and the N-terminal 33 residues of mPTHrP fused to the C-terminal portion of *Escherichia* LP followed by a hemagglutinin-tag (HA) at the C-terminus (ppPTHrP33-LP). The shaded circle indicates the N-linked sugar modification site of LP. The wild-type (WT) amino acid sequence of the signal peptide and the pro-region of ppPTHrP are shown below. The signal peptide cleavage site of ppPTHrP (|) was estimated by SignalP4.0 (*11*). The substituted residues and ΔG_{app} values calculated by ΔG predictor (*12*) of each mutant are indicated in the below. The well-conserved region in the h-region of PTHrP signal peptide is doubly underlined. $\Delta(C-N)$ was calculated by subtracting the total sum of the charges for the n-region (N) including a positive charge of N-terminal amino groups from that for the pro-region (C).

Table I. Oligonucleotides used for mutagenesis

Mutation	Direction	Sequence
C1	Sense	cgagcggcacgatgctggaggagctggttcagcagtgg
	Antisense	ccactgctgaaccagctcctccagcatcgtgccgctcg
C2	Sense	cagctactccgtgccctccgaggggagtcggtggaggggcttgg
	Antisense	ccaagcccctccaccgactccccctcggagggcacggagtagctg
C3	Sense	ctcggtggaggggcttggtgaggagctaaaacgcgcggtgtctg
	Antisense	cagacaccgcgcgttttagctcctcaccaagcccctccaccgag
H1	Sense	ctgcggaggctggttctgctgctgctggtcctggtgttcctgctc
	Antisense	gagcaggaacaccaggaccagcagcagcagaaccagcctccgcag
H2	Sense	gtcctggtgttcctgctcctgtacctggtgccctcccgcgggcgc
	Antisense	gcgcccgcgggagggcaccaggtacaggagcaggaacaccaggac
CC	Sense	ctactccgtgccctccgcccaggccgcggtggaggggcttgg
	Antisense	ccaagcccctccaccgcggcctgggcggagggcacggagtag

peptide (Fig. 1B). Because the C-terminal portion of ppPTHrP including the NLS affects cleavage of the signal peptide (*7*), the N-terminal region of rat ppPTHrP (ppPTHrP33) that does not contain the NLS was used to assess the intrinsic activity of the signal peptide including the pro-region. *Escherichia coli* LP was used as a reporter protein. Glycosylation of the reporter domain indicates its translocation across the ER membrane.

The difference of the total sum charges of up to 15 residues in each side flanking the first internal signal-anchor domain is reported to determine its orientation, with the more positive portion facing the cytosol (*10*). According to this criteria, the net charge difference calculated for the wild-type rat ppPTHrP by subtracting the sum of the charges of the n-region (N) including a positive charge from the N-terminal amino group of the signal peptide from that of the pro-region (C) seems to be unfavourable for translocation of its C-terminal portion across the ER membrane. The difference [$\Delta(C-N)$] for the rat PTHrP signal peptide is +1 (Fig. 1B), suggesting that the signal peptide is preferentially inserted into the ER membrane in an N-terminal exoplasmic/C-terminal cytosolic (N_{exo}/C_{cyto}) orientation that might preclude access of the cleavage site to the active site of the signal peptidase located in the lumen of the ER. The well-conserved charged residues in the pro-region (R32 and R33) are functionally unknown, although the C-terminal two residues (R35 and R36) are likely required for cleavage by pro-hormone convertase (*1*, *11*). A C1 mutant was constructed to increase the $\Delta(C-N)$ value from +1 to +5. To invert the charge difference in favour of insertion with an N_{cyto}/C_{exo} orientation, the positively charged arginines in the c-region (R23 and R25) and in the pro-region (R32 and R33) are replaced with the negatively charged glutamic acids in C2 and C3 mutants, respectively.

The h-region of the PTHrP signal peptide is less hydrophobic than typical h-regions. The predicted apparent free energy (ΔG_{app}) of insertion of a transmembrane helix into the ER membrane has been presented as a biological hydrophobicity scale (*12*). We calculated ΔG_{app} for the N-terminal 20 residues of wild-type and mutant PTHrP signal peptide (Fig. 1B). For example, whereas ΔG_{app} for the control α_{2u} globulin (GL) signal peptide is −1.221 kcal/mol, ΔG_{app} for the PTHrP signal peptide is 1.973 kcal/mol. The conserved region (QQWS) significantly contributes to the lower hydrophobicity. Relatively well-conserved hydroxyl residues located on the boundary between the h- and the c-regions also contribute to the lower hydrophobicity.

The conserved region (QQWS) was replaced with four tandem leucine residues (LLLL) in the H1 mutant. The ΔG_{app} of the H1 mutant signal peptide was predicted to be −3.382 kcal/mol (Fig. 1B). To increase the hydrophobicity of the boundary between the h- and the c-regions, two serine residues were replaced with leucine residues in the H2 mutant. The ΔG_{app} of the H2 mutant was predicted to be 0.333 kcal/mol, while the predicted ΔG_{app} was markedly decreased (−5.054 kcal/mol) in the H1/H2 double mutant. An H1/C3 mutant was constructed to assess the influence of the charged residues in the pro-region on the hydrophobic H1 mutant signal peptide function.

The cleavage site of the rat PTHrP signal peptide was predicted to be between G24 and R25 using SignalP 4.0 (*13*). A *de novo* designed signal peptide cleavage cassette was originally developed for an *E.coli* system based on the patterns of conserved amino acids that have been defined by statistical analyses and confirmed by experimental studies (*14*). It was later found to also work well in a eukaryotic *in vitro* reconstitution system composed of reticulocyte lysate, purified SRP and dog pancreas microsomes (*15*). The wild-type cleavage site sequence was replaced with the *de novo* designed cleavage cassette in a CC mutant. By virtue of this mutation, the cleavage site was moved one residue closer towards the C-terminus. CC/C3, H1/CC and H1/CC/C3 mutants were constructed to assess the influence of the charge inversion in the pro-region and/or in the hydrophobic h-region on the function of the cleavage cassette.

Effect of the mutations on cleavage of the signal peptide

Wild-type and mutant fusion proteins (ppPTHrP33-LP) were expressed in COS-1 cells, and the proportions of glycosylation and of signal peptide cleavage were assessed and calculated as described in Materials and Methods. Although the control signal peptide of the GL-LP fusion protein (*7*) was completely cleaved, the proportion of wild-type PTHrP signal peptide cleavage was limited to 84% (Fig. 2A). As expected, the signal peptide cleavage decreased to 26% in the C1 mutant signal peptide. Charge inversion of the two positively charged residues on both sides of the cleavage site (R23 and R25) in the C2 mutant had no effect on the signal peptide cleavage. However, the signal peptide cleavage slightly increased to 90% in the C3 mutant in which two tandem glutamic acid residues were substituted for arginine residues in the middle of the pro-region (R32 and R33) to invert the charge difference. The charge difference thus had some effect on cleavage of the PTHrP signal peptide.

The glycosylated prepro-form of the fusion protein was observed in the H1 mutant with a hydrophobic h-region created by replacement of the conserved region (QQWS) with tandem four leucine residues (Fig. 2B). The proportions of both the glycosylated prepro-form and the signal peptide cleavage were 43%, indicating that at least a half of the mutant signal peptide was not cleaved by the signal peptidase after the ER translocation. At least 75% of the prepro-form of the H1 mutant is likely to reside in the ER membrane as a glycosylated type II membrane protein estimated from the ratio of the proportion of the glycosylated prepro-form (PNGase −) to the proportion of the prepro-form (PNGase +). On the other hand, the increased hydrophobicity at the boundary between the h- and the c-regions in the H2 mutant had no effect on the proportion of the signal peptide cleavage. A phenotype similar to that of the H1 mutant was observed for the H1/H2 double mutant. A severe defect of the signal peptide cleavage was observed in the H1/C3 mutant where the proportion of the signal peptide cleavage decreased to 29%, and the proportion

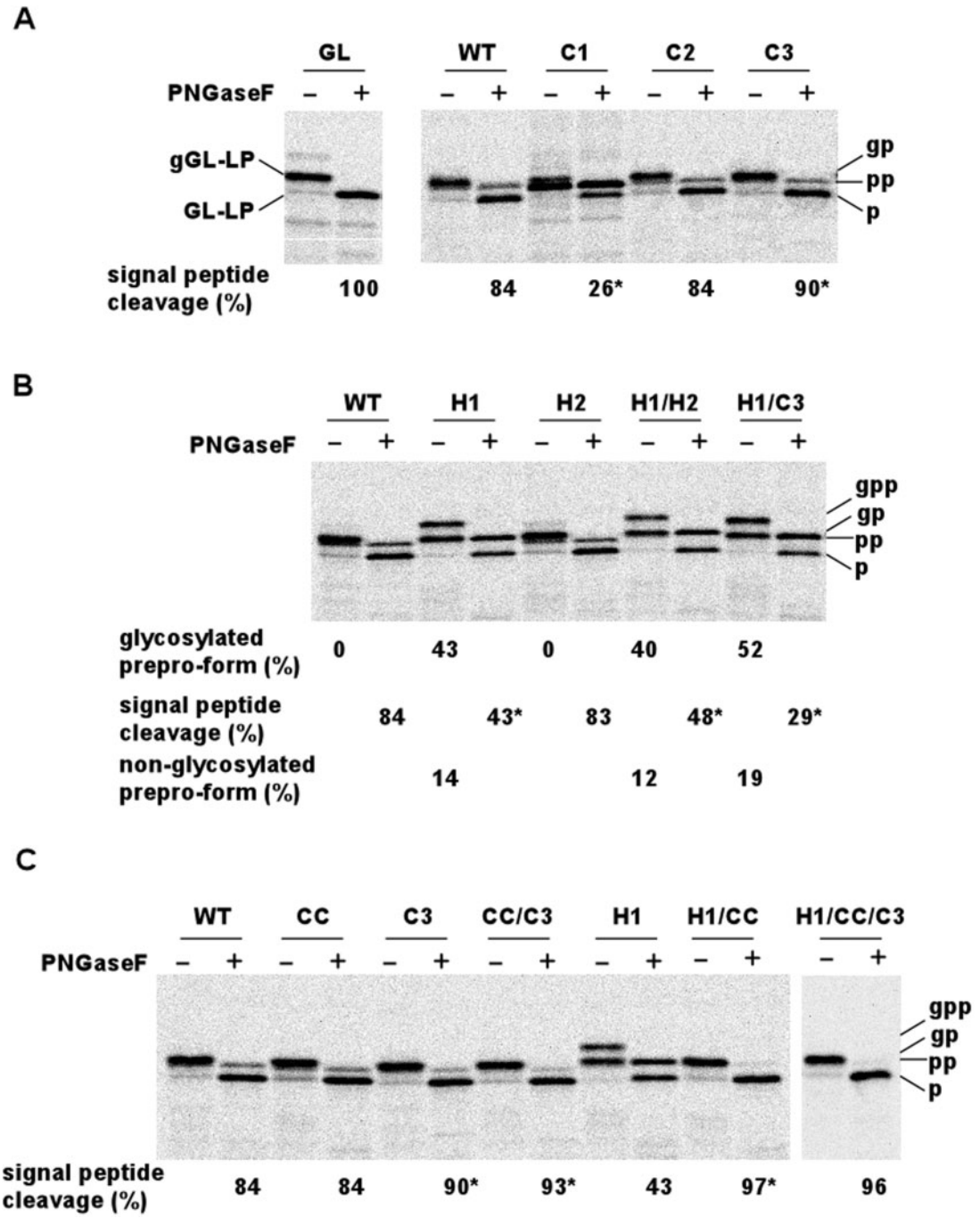

Fig. 2 Glycosylation and signal peptide cleavage of ppPTHrP33-LP fusion proteins carrying mutations in the signal peptide and/or in the pro-region. GL-LP and ppPTHrP33-LP fusion proteins were expressed in COS-1 cells, labelled with [^{35}S]-Met and [^{35}S]-Cys for 15 min, immunoprecipitated using an anti-LP antibody and separated by SDS–PAGE. The proportions of signal peptide cleavage and glycosylation were measured by BAS-5000 (Fuji film) or Typhoon FLA 7000 (GE Healthcare). An average of 2–12 experiments for each mutant is shown in the figures. The glycosylated mature form and non-glycosylated mature form of the GL-LP fusion protein are indicated by gGL-LP and GL-LP, respectively. The glycosylated prepro-form, the glycosylated pro-form, the non-glycosylated prepro-form, and the non-glycosylated pro-form of ppPTHrP33-LP fusion protein are indicated by gpp, gp, pp and p, respectively. The asterisks indicate the values showing statistically significant difference ($P<0.05$) from the value of the wild-type using the two-sample t-test. The results of the charge inversion mutants, the h-region mutants and the cleavage site mutants are shown in (**A**), (**B**) and (**C**), respectively.

of the glycosylated prepro-form concomitantly increased to 52%. Contrary to the case of the lower hydrophobic wild-type h-region, negatively charged residues of the C3 mutation in the pro-region had strong inhibitory effect on signal peptide cleavage when combined with the hydrophobic h-region of the H1 mutation in the H1/C3 mutant.

Replacement of the wild-type cleavage site with the *de novo* designed signal peptide cleavage cassette, CC, had no effect on the proportion of signal peptide cleavage (Fig. 2C). Similar to the phenotype of the C3 mutant, a little elevation of the signal peptide cleavage was observed in the CC/C3 mutant. On the other hand, almost complete cleavage (97%) of the signal peptide

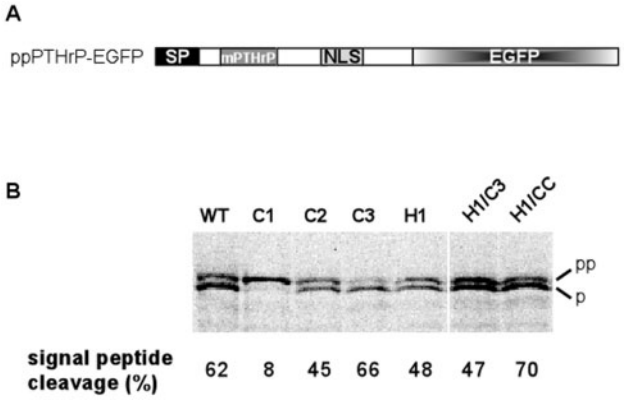

Fig. 3 Signal peptide cleavage of ppPTHrP-EGFP fusion proteins carrying mutations in the signal peptide and/or in the pro-region. The wild-type and mutant ppPTHrP-EGFP fusion proteins (**A**) were expressed in COS-1 cells, immunoprecipitated using an anti-PTHrP(122-141) antibody, and then the proportion of signal peptide cleavage was assessed as in Fig. 2 (**B**).

was observed with the H1/CC mutant. Therefore, it can be seen that the combination of the hydrophobic leucine-rich h-region and the *de novo* designed signal peptide cleavage cassette is important for efficient signal peptide cleavage. Contrary to the case of the H1 mutation, additional introduction of the C3 mutation to the H1/CC mutation (H1/CC/C3) had no inhibitory effect on cleavage of the mutant signal peptide.

Localization of the ppPTHrP-EGFP fusion protein carrying a mutation in the signal peptide and/or pro-region

To evaluate the effects of those mutations on the final localization of PTHrP, full length wild-type or mutant ppPTHrP fused to EGFP (Fig. 3A) were expressed in COS-1 cells. Similar effects of the mutations on the proportion of signal peptide cleavage to those for the ppPTHrP33-LP fusion proteins were observed for the ppPTHrP-EGFP fusion proteins, except that signal peptide cleavage of the C2 mutant was significantly inhibited (Fig. 3B, compare to Fig. 2).

Subcellular localization of the EGFP fusion proteins was detected by a fluorescence microscopy (Fig. 4). While the reporter EGFP expressed alone was found throughout the entire cell with slightly intense staining in the nucleus, the fusion protein carrying the wild-type signal peptide was almost exclusively detected in the nucleoli. Unexpectedly, such exclusive nucleolar localization was not observed with the C1 mutant in which cleavage of the mutant signal peptide was significantly suppressed. A significant proportion of the C1 mutant fusion protein was found in the nuclear matrix with faint cytoplasmic fluorescence. A similar phenotype was also observed for the C2 mutation. These results suggest that the positively charged residues in the n-region and c-region of the signal peptide are important and/or negatively charged residues in the mutants are inhibitory for the nucleolar translocation of PTHrP from the nuclear matrix. Charge inversion of the positively charged residues in the pro-region in the C3 mutant caused some effects on its final localization, whereby a significant fraction of the mutant protein was found in the perinuclear region, as well as in the nucleus.

Subcellular localization of the PTHrP-EGFP fusion proteins carrying the H1 mutant signal peptide was quite different from that of the wild-type. The substitution of tandem four leucine residues for the conserved QQWS region (H1) resulted in a significant perinuclear localization of the mutant PTHrP-EGFP fusion protein, with a concomitant reduction in the proportion of the nuclear and/or nucleolar localization. More significant perinuclear localization was observed in the H1/C3 and H1/CC mutants, in the former of which the ppPTHrP33-LP was highly glycosylated (Fig. 2B) and in the latter of which the signal peptide was efficiently cleaved both in the ppPTHrP33-LP (Fig. 2C) and ppPTHrP-EGFP fusion proteins (Fig. 3B).

Taken together, the structural features of the signal peptide and the pro-region significantly affect the nuclear and/or nucleolar localization of PTHrP. In particular, the conserved region in the h-region (QQWS) plays an important role in the regulation of ER targeting and/or membrane translocation of PTHrP nascent chain for its dual localization.

Effect of translation-inhibitory antibiotics on cleavage of the PTHrP signal peptide

COS-1 cells expressing the wild-type or either of the mutant ppPTHrP33-LP fusion proteins were treated with cycloheximide or anisomycin during the labelling reaction to assess the effects of the translation rate and ribosomal peptide elongation cycle on the proportion of the signal peptide cleavage (Fig. 5A). Both cycloheximide and anisomycin treatments had no effect on the proportion of the wild-type signal peptide cleavage even at higher concentrations of the antibiotics (Fig. 6A and B). Similarly, all the mutants in Fig. 5A, except for C1, showed no effect on the signal peptide cleavage when treated with either antibiotic.

For the C1 mutant, the proportion of signal peptide cleavage was increased more than a 2-fold with cycloheximide treatment but not with anisomycin (Fig. 5A). The proportion of C1 mutant signal peptide cleavage increased concomitantly with a decrease in the rate of translation by cycloheximide (Fig. 6A–C, left panels).

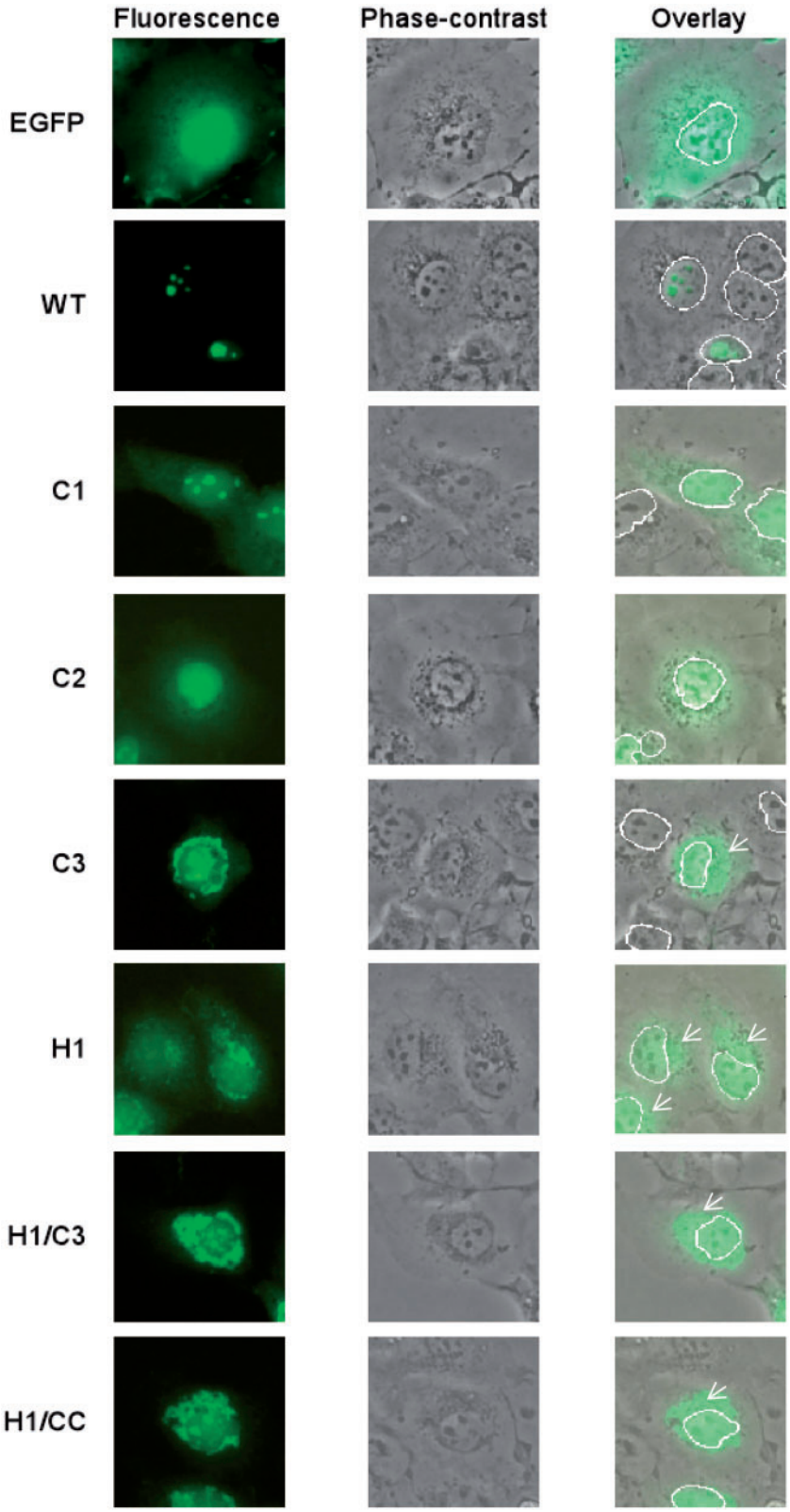

Fig. 4 Localization of the ppPTHrP-EGFP fusion protein. EGFP, the wild-type, and mutant ppPTHrP-EGFP fusion proteins (Fig. 3A) were expressed in COS-1 cells and detected by fluorescent microscopy (left panels). Phase-contrast micrographs of the corresponding fields for the fluorescence are shown in the middle panels. Overlay of both are shown in the right panels. Lines and arrows indicate nuclear envelope and perinuclear regions, respectively. The expressed proteins are indicated on the left of the panels.

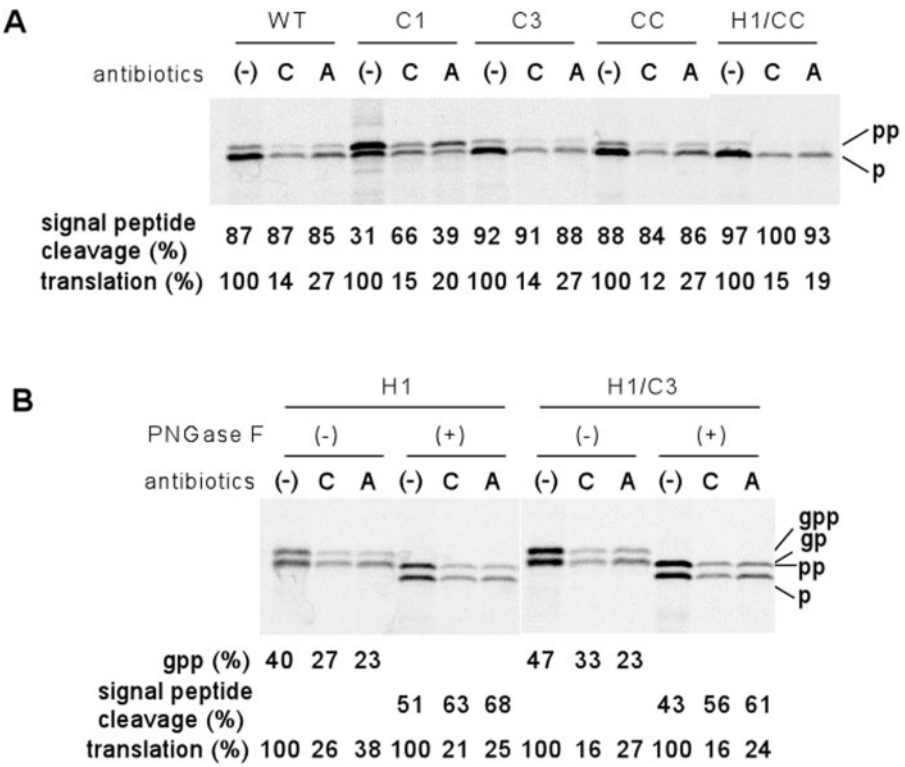

Fig. 5 Effects of cycloheximide and anisomycin on the function of the signal peptide. (**A**) The proportion of the signal peptide cleavage for the wild-type and the mutants indicated was measured as in Fig. 2. Where indicated, cycloheximide (C, 2 μg/ml) or anisomycin (A, 0.08 μg/ml) was added during labelling reaction. The immunoprecipitates were treated with PNGase and further analysed. (**B**) The proportion of the signal peptide cleavage for the conserved region mutants (H1 and H1/C3) was measured as in (A), except that the immunoprecipitates were divided and either treated (+) or untreated (−) with PNGase before being further analysed.

On the other hand, a moderate elevation of signal peptide cleavage was observed by anisomycin only at concentrations higher than 0.20 μg/ml, a concentration that reduces the rate of translation to as low as 10% that of the untreated control (Fig. 6A–C, right panels). These results suggest that suppression of the defective signal peptide cleavage seen with the C1 mutant depends more on the conformation of the ribosome having high affinity for cycloheximide rather than on the rate of translation.

The proportion of the glycosylated prepro-form decreased by treatment with either cycloheximide or anisomycin with the H1 and H1/C3 mutants (Fig. 5B). Cleavage of the mutant signal peptides increased concomitantly with the reduction of the glycosylated prepro-form (Fig. 7). In contrast with the C1 mutant, suppression of defective signal peptide cleavage in the H1 and H1/C3 mutants seem to be essentially dependent on the rate of translation, irrespective of which antibiotics are used (Figs 6C and 7). Taken together, it is clear that the mechanisms underlying the inefficient cleavage of wild-type, C1 and H1 mutant signal peptides are quite different from one another.

Discussion

Important roles of the conserved residues in the signal peptide and in the pro-region for the dual localization of PTHrP

The signal peptide and the pro-region of PTHrP have several conserved structural features that are predicted to be unfavourable for ER targeting and for membrane translocation, but are critical for its dual localization. Such evolutionary conservation of the primary structure of a signal peptide and a pro-region is unusual even among mammalian species, where usually their functional properties, rather than the peptide sequence *per se*, are well conserved (*16*). Among these structural features, the conserved QQWS region in the h-region of the PTHrP signal peptide seems to be the most important. In addition, the conserved positively charged residues in the pro-region significantly affect mode of insertion and/or cleavage of the signal peptide.

Like secretory proteins in general, most newly synthesized PTHrP enters the secretory pathway (Fig. 8, A→B→C→D, bold arrows). However, a proportion of PTHrP is transported to the nucleus/nucleolus (Fig. 8, G) by partitioning during ER targeting and translocation. Two possible pathways for this dual localization of PTHrP are conceivable.

The first possible pathway is that the nascent chain might fail to be targeted to the ER, due to the weak interaction between the less hydrophobic PTHrP signal peptide and the SRP. In this situation, the NLS of PTHrP could interact with importin β (*17*), and the protein could then be transported to the nucleus/nucleolus (Fig. 8, A→A′ →G, thin arrows).

Similar to the suppression of inefficient ER translocation in SRP deficient cells by antibiotics (*18*, *19*), the defect of the C1 mutant is efficiently suppressed by cycloheximide treatment, and less so by anisomycin

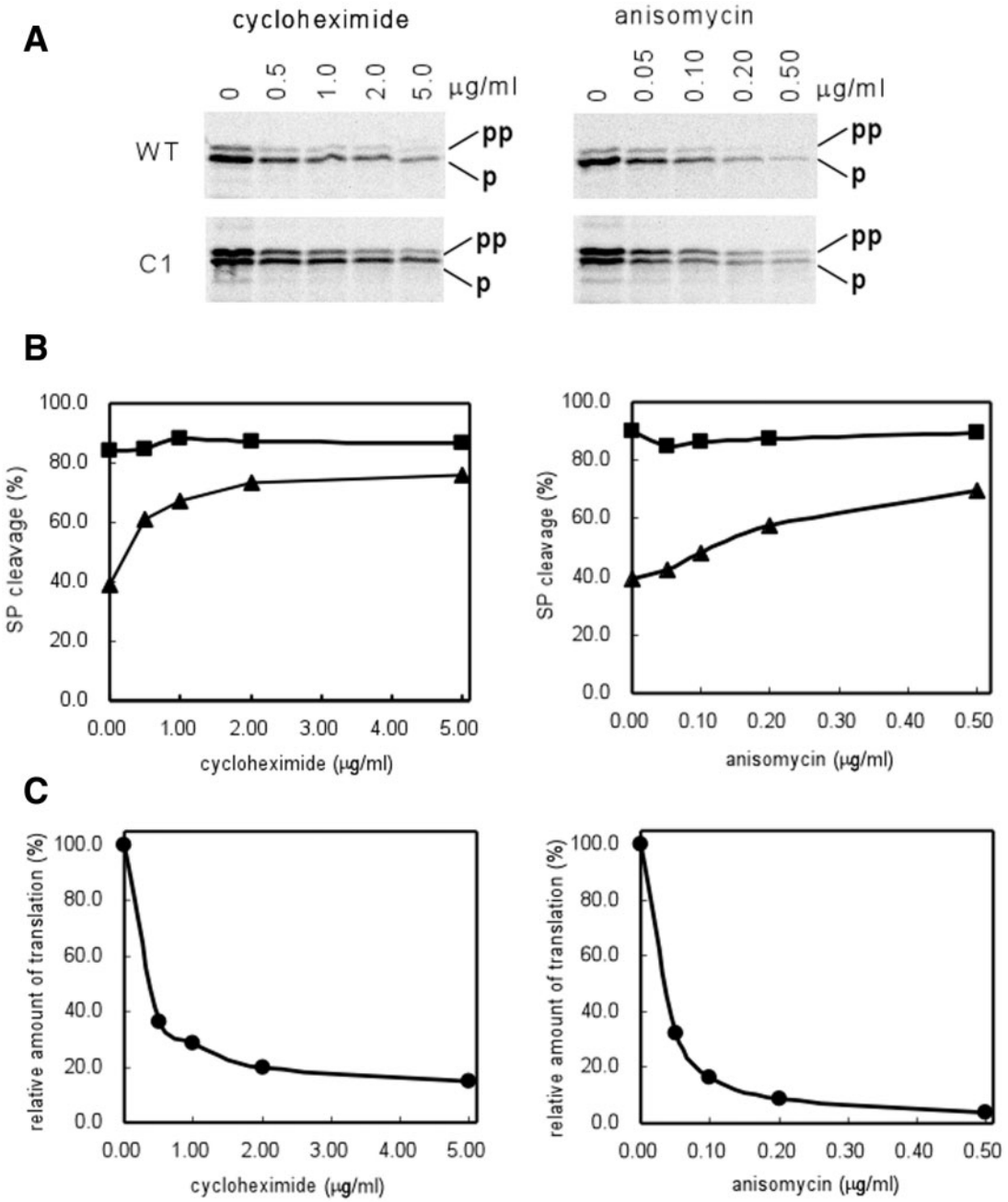

Fig. 6 Effects of antibiotic concentration on the wild-type and C1 mutant signal peptide cleavage. The effects of antibiotic concentration on the proportions of the wild-type and C1 mutant signal peptide cleavage were analysed (**A**) and quantified (**B**) as in Fig. 5A. The wild-type and C1 mutant are indicated by closed squares and closed triangles, respectively. The relative amounts of the sum of the prepro-form and pro-form, which are defined as 100% when the antibiotics are absent, are plotted (**C**).

(Figs 5A and 6). These results imply that suppression of the C1 mutant phenotype is not merely due to the reduced translation rate with antibiotics treatment, but is also dependent on the conformation of the ribosome. Here, it is possible that the cycloheximide-binding ribosome, which has a higher affinity with the SRP, compensates for the weak interaction between the C1 mutant signal peptide and the SRP. In accordance with this possibility, electrostatic interactions between the positively charged residues of the n-region of signal peptide and the phosphate backbone of SRP RNA are suggested to play a significant role in signal peptide recognition (*20*). In contrast with the C1 mutant signal peptides, incomplete cleavage of the wild-type PTHrP signal peptide was not suppressed either by cycloheximide or by anisomycin (Figs 5A and 6). The mechanism of incomplete cleavage of the wild-type PTHrP signal peptide seems not to depend on conformation of the ribosome.

The second possible pathway is release of the PTHrP nascent chain to the cytosol after the ER targeting (Fig. 8, B→E′→G, thin arrows). The charge distribution of the PTHrP signal peptide including the pro-region might affect the orientation of insertion and hairpin loop formation during the interaction between the protein conducting channel (PCC) and the signal

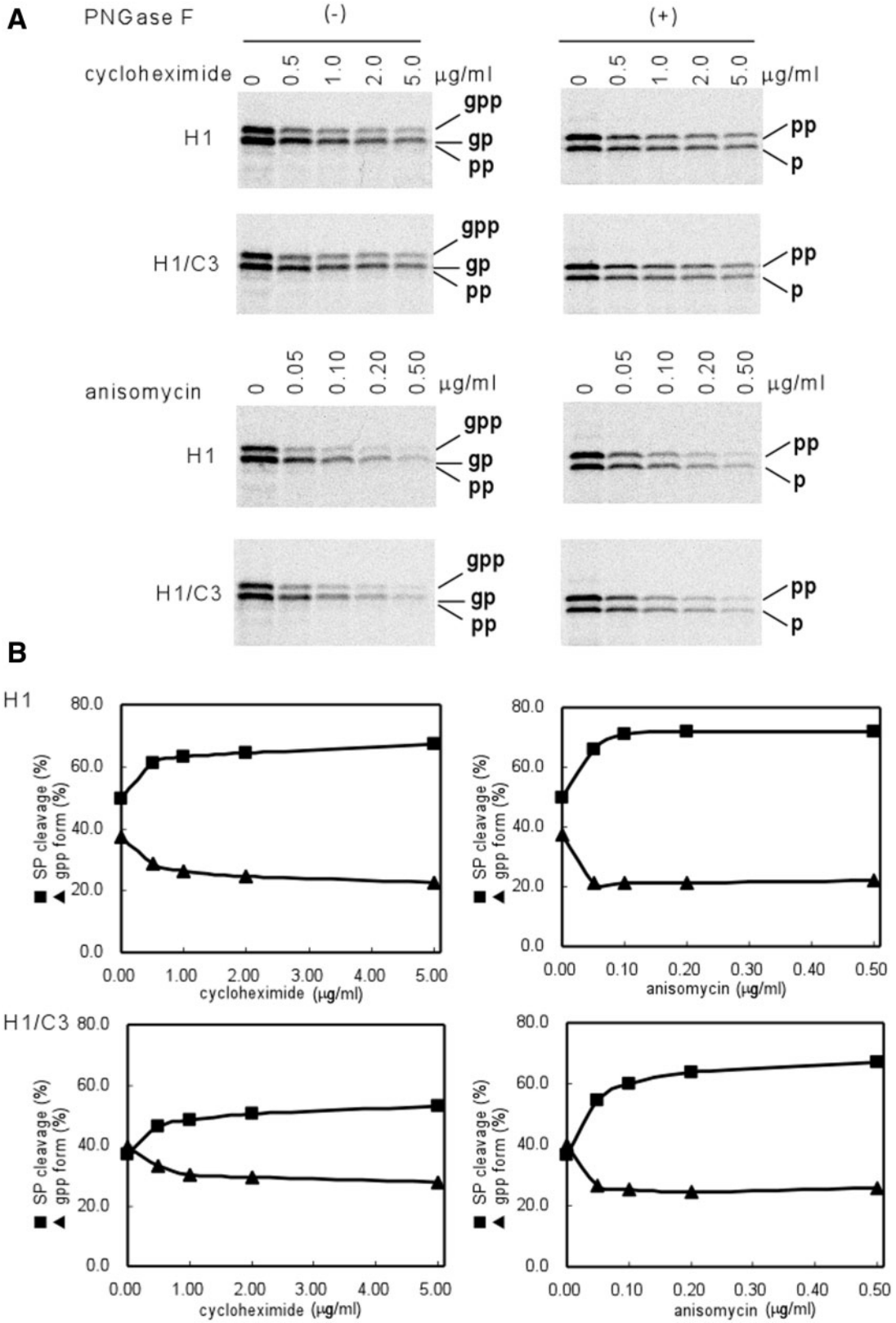

Fig. 7 Effects of antibiotic concentration on the signal peptide cleavage carrying the H1 mutation. The effects of antibiotic concentration on the proportions of the H1 and H1/C3 mutant signal peptide cleavage were analysed (**A**) and quantified (**B**) as in Fig. 5B. The closed squares indicate the proportion of signal peptide cleavage, and the closed triangles indicate the proportion of the glycosylated prepro-form.

peptide (Fig. 8, B→C). Inefficient formation of the hairpin loop facilitates exposure of the C-terminal portion of the nascent chain to the cytosol and can promote interaction of the NLS with importin β (*17*) to transport the protein into the nucleus/nucleolus (Fig. 8, E′→G).

It is likely that the positively charged residues in the c-region of the signal peptide or the pro-region of

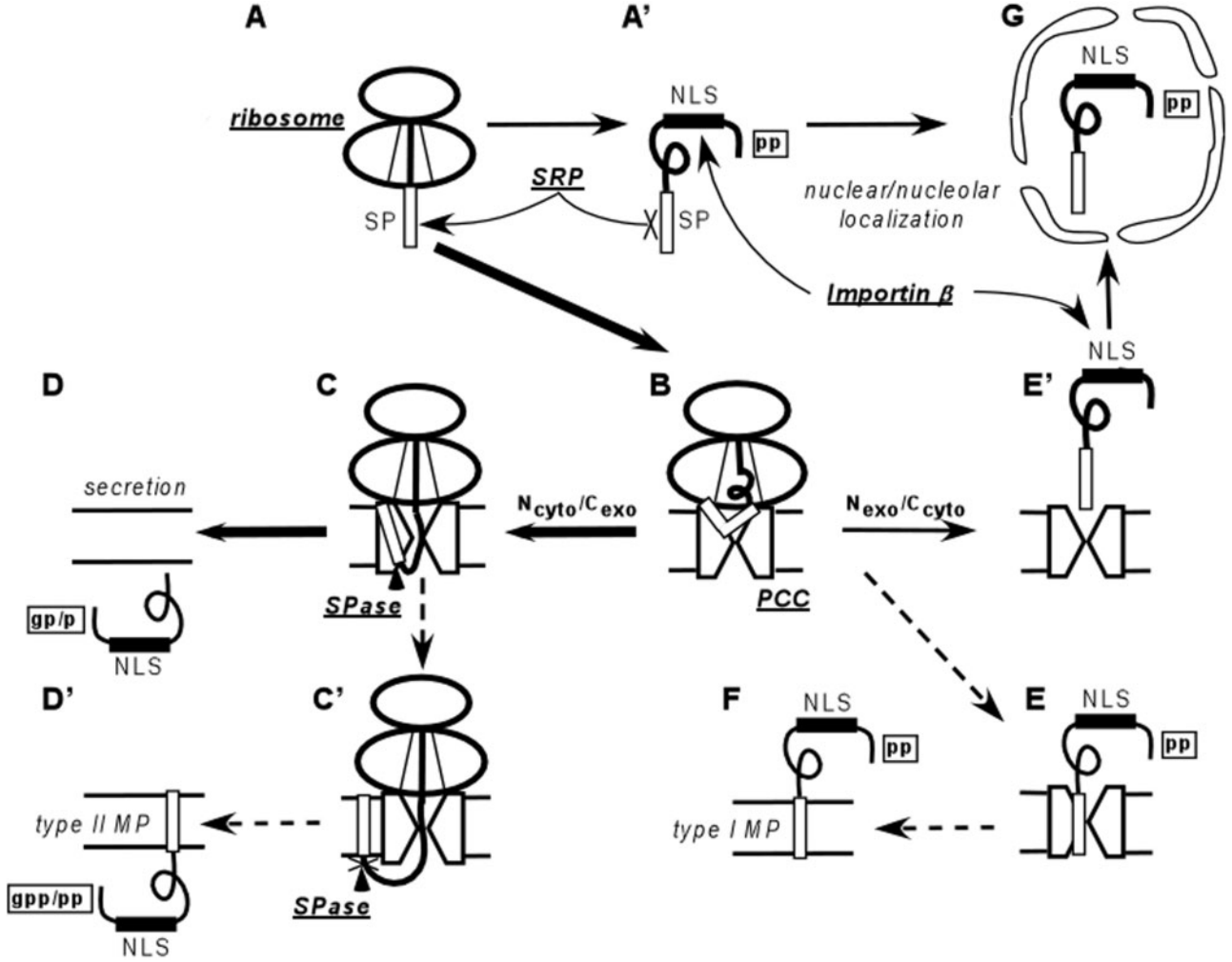

Fig. 8 Possible pathways for the nuclear/nucleolar localization of PTHrP. Most of the newly synthesized PTHrP enters the secretory pathway (A→B→C→D, bold arrows), however, a part of the PTHrP is transported to the nucleus/nucleolus by escaping ER-targeting due to a weak interaction of PTHrP signal peptide (SP) with the SRP (A →A′ →G, thin arrows) or by release of the PTHrP nascent chain from the PCC (B→E′→G, thin arrows). In the H1 and H1/C3 mutant, significant proportion of the signal peptide was not cleaved (gpp and pp) by the signal peptidase (SPase) and the mutant proteins are integrated into the ER membrane as type II membrane proteins (type II MP, C→C′→D′, dotted arrows) or as type I membrane proteins (type I MP, B→E→F, dotted arrows). For further details, see the text.

PTHrP might interfere with the hairpin loop formation (Fig. 8, B→C) of the signal peptide (*10*, *21*). At least in some cases, hairpin loop formation occurs by a reorientation of the signal peptide after its head-on insertion (*22*, *23*). Although charge inversion in the c-region (C2) had no effect on the signal peptide cleavage, that in the pro-region (C3) slightly enhanced signal peptide cleavage (Fig. 2A). In addition, the ppPTHrP-EGFP fusion protein carrying the C3 mutation was detected in perinuclear region (Fig.4). These results suggest that the conserved positively charged residues in the pro-region facilitate the release of the PTHrP nascent chain from the ER membrane by inefficient formation of the hairpin loop. In addition to the signal peptide itself, positively charged residues in the pro-region also had some effect on the function of the PTHrP signal peptide for the dual localization. Evolutionary conservation suggests the importance of these positively charged residues (Fig. 1A) in the functional regulation of PTHrP signal peptide. As reported in some cases, the downstream region from the cleavage site of a secretory protein can affect the gating of the PCC, which is one of the critical steps for translocation of the signal peptide (*16*).

Hydrophobic interaction between the signal peptide and the PCC is important for the opening of the lateral gate (Fig. 8, B→C or B→E) through which the signal peptide gains access to the lipid bilayer (*12*, *24*). It is possible that the low hydrophobicity of the h-region of the PTHrP signal peptide causes a cytosolic release of a significant fraction of the nascent chain through its inefficient lateral gate opening activity (Fig. 8, E′). The relatively high predicted ΔG_{app} (1.973 kcal/mol, Fig. 1B) of the h-region of the wild-type PTHrP signal peptide seems to be energetically unfavourable for the opening of the lateral gate, but favourable for release of the PTHrP nascent chain from the PCC into the cytosol where it would then be available for nuclear/nucleolar localization (Fig. 8, B→E′→G, thin arrows). Indeed, nuclear/nucleolar localization was significantly precluded when the conserved region was replaced with a tandem of leucine residues in the H1 mutation, for which the predicted ΔG_{app} was −3.382 kcal/mol, (Fig. 4). In the H1 and H1/C3

mutants, a significant proportion of the signal peptides were not cleaved by the signal peptidase (SPase), resulting in the formation of the glycosylated prepro-form (gpp) and non-glycosylated prepro-form (pp). A major proportion of the uncleaved mutant proteins is integrated into the ER membrane as type II membrane proteins, in which sequestration of the NLS into the ER would prevent it from interacting with cytosolic importin β (Fig. 8, C→C′→D′, dotted arrows). It is also possible that the non-glycosylated prepro-form (pp) of the mutant proteins are integrated into the ER membrane as type I membrane proteins (Fig. 8, B→E→F, dotted arrows). Although the NLS can interact with importin β in this case, tethering of the mutant proteins in the ER membrane by the non-cleavable hydrophobic mutant signal peptides may prevent its nuclear/nucleolar transport.

Functional interrelation between the h-region and the cleavage site of the signal peptide

Cleavage of the H1 mutant signal peptide was significantly inhibited, while its targeting efficiency was apparently unaffected (Fig. 2B). In a study of the model signal peptides consisting of various lengths of leucine residues in the h-region followed by the cleavage cassette (AQAA), signal peptide cleavage was observed in the leucine length from 8 (ΔG_{app} = 0.833 kcal/mol) to 17 (ΔG_{app} = −6.189 kcal/mol) residues, but not in the length from 20 (ΔG_{app} = −8.341 kcal/mol) to 26 (ΔG_{app} = −12.51 kcal/mol) residues (*15*). The predicted ΔG_{app} values of the H1 mutant (−3.382 kcal/mol, Fig. 1B) is within the cleavable hydrophobicity of that study. In spite of this, cleavage of the H1 mutant signal peptide was significantly reduced (Fig. 2B). A similar result was observed with the H1/H2 mutant, for which the predicted ΔG_{app} value was −5.054 kcal/mol. These results suggest that the combinations of the hydrophobic h-regions and the wild-type cleavage site sequence are unfavourable for cleavage. In contrast, almost complete signal peptide cleavage was observed when combining the hydrophobic h-region and the *de novo* cleavage cassette (H1/CC), although the combination of the wild-type h-region and the CC mutation (CC) had no effect on signal peptide cleavage (Fig. 2C). This all suggests that both the hydrophobic h-region and the well-adapted cleavage site are simultaneously required for the mutant PTHrP signal peptide to be translocated and cleaved as efficiently as typical signal peptides.

Surprisingly, the combination of the hydrophobic h-region in the H1 mutation and the negatively charged residues in the pro-region of the C3 mutation (H1/C3) caused a more severe inhibition of the signal peptide cleavage (Fig. 2B), even though the C3 mutant slightly enhanced the cleavage in combination with the wild-type h-region (Fig. 2A). Efficient hairpin loop formation and the rapid transfer of the signal peptide to the lipid bilayer, induced by the combination of the negative charge in the pro-region and the hydrophobic h-region may reduce the time window for binding of the wild-type PTHrP cleavage site with the signal peptidase (Fig. 8 C→C′). Alternatively, a conformational change around the cleavage site of the H1/C3 mutant induced by the negatively charged residues in the mutant pro-region may affect its interaction with the active site of the signal peptidase. In the case of the H1/CC/C3 mutant, the cleavage inhibitory effect of the C3 mutation was not observed (Fig. 2C). Here, it is possible that efficient cleavage (Fig. 8, B→C→D, bold lines) by a combination of the H1 and CC mutations could overcome the inhibitory effect of the C3 mutation (Fig. 8, C′).

When the H1 mutation is present, the effects of the two antibiotics on the suppression of the defect of cleavage are similar and mainly dependent on the rate of translation (Figs 5B and 7). This implies that due to the slow translation rate caused by the antibiotic treatment, a sufficient time window might be available for binding the cleavage site with the active site of the signal peptidase (Fig. 8, C).

Unlike these mutant signal peptides, incomplete cleavage of the wild-type PTHrP signal peptide was not suppressed either by cycloheximide or by anisomycin (Figs 5A and 6). The mechanism for the incomplete cleavage of the PTHrP signal peptide seems to depend on neither conformation of the ribosome nor the rate of translation. The different effects of the two antibiotic treatments observed on the inefficient cleavage of the wild-type, C1, and H1 mutant signal peptides suggest that their underlying mechanism are also different.

To the best of our knowledge, this study provides the first evidence that the unique structural features of the signal peptide of PTHrP play a significant role in its dual localization through targeting and/or translocational regulation at the ER membrane. The function of the nuclear/nucleolar PTHrP has been suggested using mutants lacking the NLS (*25*, *26*). It must be noted that some of the poorly characterized peptides such as osteostatin, generated during secretion (*1*, *27–29*), are deficient in these mutants. The possibility cannot be excluded that at least some aspect of these phenotypes are caused by deficiencies of these peptides. Also, it must be noted that C-terminal truncation of prepro-PTHrP increases the secretion of mature PTHrP (*7*, *30*). To avoid these ambiguities, the signal peptide mutants shown in this report, in which the nuclear/nucleolar translocation is severely impaired, can be useful alternative tools for the functional analysis of nuclear/nucleolar localized PTHrP. Moreover, these mutant signal peptides may be used for probing interactions of the nascent chain with the components of the ribosome, the PCC, and the lipid bilayer during ER translocation to reveal their regulatory functions.

Acknowledgements

We thank Yoshiaki Takahashi (Niigata University) for encouragement during the course of this work, Haruko Suzuki (Yokohama City University) for antigen peptide synthesis, Hui Lin Chai (Yokohama City University) for antibody preparation, Shigeru Hirayama (Niigata University) for the cell culture experiments, and Takuji Izumikawa (Niigata University) and Jun Goto (Niigata University) for the radio isotope labelling experiments.

Funding
This work was supported in part by a grant-in-aid for scientific research (13671934) from the Ministry of Education, Science, Sports and Culture of Japan.

Conflict of Interest
None declared.

References

1. Wysolmerski, J.J. and Stewart, A.F. (1998) The physiology of parathyroid hormone-related protein: an emerging roles as a developmental factor. *Annu. Rev. Physiol.* **60**, 431–460
2. Henderson, J.E., Amizuka, N., Warshawsky, H., Biasotto, D., Lanske, B.M.K., Goltzman, D., and Karaplis, A.C. (1995) Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apototic cell death. *Mol. Cell Biol.* **15**, 4064–4075
3. Fiaschi-Taesch, N.M. and Stewart, A.F. (2003) Parathyroid hormone-related protein as an intracrine factor—trafficking mechanisms and functional consequences. *Endocrinology* **144**, 407–411
4. Aarts, M.M., Rix, A., Guo, J., Bringhurst, R., and Henderson, J.E. (1999) The nucleolar targeting signal (NTS) of parathyroid hormone related protein mediates endocytosis and nucleolar translocation. *J. Bone Miner. Res.* **14**, 1493–1503
5. Nguyen, M.T.A., He, B., and Karaplis, A.C. (2001) Nuclear forms of parathyroid hormone-related peptide are translated from non-AUG start sites downstream from the initiator methionine. *Endocrynology* **142**, 694–703
6. Amizuka, N., Fukushi-Irie, M., Sasaki, T., Oda, K., and Ozawa, H. (2000) Inefficient function of the signal sequence of PTHrP for targeting into the secretory pathway. *Biochem. Biophys. Res. Commun.* **273**, 621–629
7. Amaya, Y., Nakai, T., Komaru, K., Tsuneki, M., and Miura S. (2008) 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. *J. Biochem.* **143**, 569–579
8. Meerovitch, K., Wing, S., and Goltzman, D. (1998) Proparathyroid hormone-related protein is associated with the chaperone protein BiP and undergoes proteasome-mediated degradation. *J. Biol. Chem.* **273**, 21025–21030
9. Rane, N.S., Kang, S.-W., Chakrabarti, O., Feigenbaum, L., and Hegde, R.S. (2008) Reduced translocation of nascent prion protein during ER stress contributes to neurodegradation. *Dev. Cell* **15**, 359–370
10. Hartmann, E., Rapoport, T., and Lodish, H.F. (1989) Predicting the orientation of eukaryotic membrane spanning proteins. *Proc. Natl Acad. Sci. USA.* **86**, 5786–5790
11. Orloff, J.J., Reddy, D., de Papp, A.E., Yang, K.H., Soifer, N.E., and Stewart, A.F. (1994) Parathyroid hormone-related protein as a prohormone: posttranslational processing and receptor interactions. *Endocr. Rev.* **15**, 40–60
12. Hessa, T., Meindl-Beinker, N.M., Bernsel, A., Kim, H., Sato, Y., Lerch-Bader, M., Nilsson, I., White, S.H., and von Heijne, G. (2007) Molecular code for transmembrane-helix recognition by the Sec61 translocon. *Nature* **450**, 1026–1030
13. Petersen, T.N., Brunak, S., von Heijne, G., and Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* **8**, 785–786
14. Nilsson, I. and Heijne, G. (1991) A *de novo* designed signal peptide cleavage cassette functions *in vivo*. *J. Biol. Chem.* **266**, 3408–3410
15. Nilsson, I., Whifley, P., and von Heijne, G. (1994) The COOH-terminal ends of internal signal and signal-anchor sequences are positioned differently in the ER translocase. *J. Cell Biol.* **126**, 1127–1132
16. Kim, S.J., Mitra, D., Salerno, J.R., and Hegde, R.S. (2002) Signal sequences control gating of the protein conducting channel in a substrate-specific manner. *Dev. Cell* **2**, 207–217
17. Cingoiani, G., Bednenko, J., Gillespie, M.T., and Gerace, L. (2002) Molecular basis for the recognition of a nonclassical nuclear localization signal by importinβ. *Mol. Cell* **10**, 1345–1353
18. Ogg, S.C. and Walter, P. (1995) SRP samples nascent chains for the presence of signal sequences by interacting with ribosomes at a discrete step during translation elongation. *Cell* **81**, 1075–1084
19. Lakkaraju, A.K.K., Mary, C., Scherrer, A., Johnson, A.E., and Strub, K. (2008) SRP keeps polypeptides translocation-competent by slowing translation to match limiting ER-targeting sites. *Cell* **133**, 440–451
20. Peterson, J.H., Woolhead, C.A., and Bernstein, H.D. (2003) Basic amino acids in a distinct subset of signal peptides promote interaction with the signal recognition particle. *J. Biol. Chem.* **278**, 46155–16162
21. Shaw, A.S., Rottier, P.J.M., and Rose, J.K. (1988) Evidence for the loop model of signal-sequence insertion into the endoplasmic reticulum. *Proc. Natl Acad. Sci. USA.* **85**, 7592–7596
22. Goder, V. and Spiess, M. (2003) Molecular mechanism of signal sequence orientation in the endoplasmic reticulum. *EMBO J.* **22**, 3645–3653
23. Devaraneni, P.K., Conti, B., Matsumura, Y., Yang, Z., Johonson, A.E., and Skach, W.R. (2011) Stepwise insertion and inversion of a type II signal anchor sequence in the ribosome-Sec61 translocon complex. *Cell* **146**, 134–147
24. Trueman, S.F., Mandon, E.C., and Gilmore, R. (2012) A gating motif in the translocation channel sets the hydrophobicity threshold for signal sequence function. *J. Cell Biol.* **199**, 907–918
25. Miao, D., Su, H., He, B., Gao, J., Xia, Q., Zhu, M., Gu, Z., Goltzman, D., and Karaplis, A.C. (2008) Severe growth retardation and early lethality in mice lacking the nuclear localization sequence and C-terminus of PTH-related protein. *Proc. Natl Acad. Sci. USA.* **105**, 20309–20314
26. Fiaschi-Taesch, N., Sicari, B., Ubriani, K., Cozar-Castellano, I., Takane K.K., and Stewart, A.F. (2009) Mutant parathyroid hormone-related protein, devoid of the nuclear localization signal, markedly inhibits arterial smooth muscle cell cycle and neointima formation by coordinate up-regulation of $p15^{Ink4b}$ and $p27^{kip1}$. *Endocrinology* **150**, 1429–1439
27. Wu, T.L., Vasavada, R.C., Yang, K., Massfelder, T., Ganz, M., Abbas, S.K., Care, A.D., and Stewart, A.F. (1996) Structural and physiologic characterization of the mid-region secretory species of parathyroid hormone-related protein. *J. Biol. Chem.* **271**, 24371–24381
28. Toribio, R.E., Brown, H.A., Novince, C.M., Marlow, B., Hernon, K., Lanigan, L.G., Hildreth, B.E. III, Werbeck, J.L., Shu, S.T., Lorch, G., Carlton, M., Foley, J., Boyaka, P., McCauley, L.K., and Rosol, T.J. (2010) The mid-region, nuclear localization sequence, and C

terminus of PTHrP regulate skeletal development, hematopoiesis, and survival in mice. *FASEB J.* **24**, 1947–1957

29. Garcia-Martin, A., Acitores, A., Maycas, M., Villanueva-Penacarrillo, M.L., and Esbrit, P. (2013) Src kinases mediate VEGFR2 transactivation by the osteostatin domain of PTHrP to modulate osteoblastic function. *J. Cell. Biochem.* **114**, 1404–1413
30. Ditmer, L.S., Burton, D.W., and Deftos, L.J. (1996) Elimination of the carboxy-terminal sequence of parathyroid hormone-related protein 1-173 increases production and secretion of the truncated forms. *Endocrinology* **137**, 1608–1617
31. Crooks, G.E., Hon, G., Chandonia, J.-M., and Brenner, S.E. (2004) WebLogo: a sequence logo generator. *Genome Res.* **14**, 1188–1190