Rat wild-type parathyroid hormone receptor (PTH-R) and mutant PTH-R$^{P132L}$ show the different intracellular localization in vitro

Junko Shimomura-Kuroki, Sobhan Ubaidus, Paulo HL Freitas, Minqi Li, Yoko Ishida, Naoaki Saito, Kimimitsu Oda, and Norio Amizuka

1Pediatric Dentistry, The Nippon Dental University, School of Life Dentistry at Niigata, Niigata 951-8580 Japan; 2Center for Transdisciplinary Research, Niigata University, Niigata, 951-8514, Japan; and 3Division of Biochemistry, Department of Tissue Regeneration and Reconstruction, Niigata University Faculty of Dentistry, Niigata 951-8514, Japan

(Received 30 November 2007; and accepted 20 December 2007)

ABSTRACT

A replacement of proline with leucine at position 132 of the receptor for parathyroid hormone (PTH)/parathyroid hormone-related peptide (PTHrP), i.e., PTH-R, has been discovered in human Blomstrand’s lethal chondrodysplasia. As skeletal deformities in this type of chondrodysplasia appear to compromise the receptor binding to its ligands, we examined the possibility that rat PTH-R carrying P132L mutation (PTH-R$^{P132L}$) would result in abnormal intracellular localization. Osteoblastic MC3T3-E1 cells were transfected with expression vectors containing cDNAs encoding either wild-type PTH-R or mutant PTH-R$^{P132L}$. The cells expressing the wild-type PTH-R produced a receptor protein with a molecular mass of 66.3 kDa, which localized its immunoreactivity mainly on the cell surfaces. In contrast, the PTH-R$^{P132L}$ was hardly detected on the cell surfaces, but accumulated within the rough-surfaced endoplasmic reticulum. Consistent with this localization, the cells expressing the mutant receptor failed to generate cyclic AMP in response to PTH. Furthermore, a remarkably weaker intensity of the 66.3 kDa band compared with the wild-type counterpart suggests that PTH-R$^{P132L}$ is prone to degradation in the transfected cells. In summary, these findings indicate that defective transport of PTH-R$^{P132L}$ to the cell surface would be a molecular basis for Blomstrand’s chondrodysplasia.

Parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP) share homology on their N-terminal amino acid sequence, which allows both peptides to bind to a common G protein-coupled cell surface receptor: PTH-R. While PTH maintains systemic calcium and phosphate homeostasis, PTHrP acts at the local level, regulating cell proliferation and differentiation. PTHrP and PTH-R are expressed in chondrocytes of the epiphyseal cartilage, and in osteoblasts (2–4). PTH normally regulates serum calcium levels by binding and activating PTH-R in bone and kidney (5, 15), in addition to promoting trabecular bone formation (8, 20). Targeted disruption of PTHrP or PTH-R in mice results in lethal chondrodysplasia, primarily due to reduced proliferation and premature chondrocytic hypertrophy (2, 16, 19). These studies have suggested that PTH/PTHrP signaling stimulates proliferation of chondrocytes and inhibits their passage to a hypertrophic phenotype in developing cartilage.

Mutations in genomic DNA encoding PTH-R induce several types of chondrodysplasias, including the Jansen (23, 24) and Blomstrand types (9, 14, 17, 28), enchondromatosis (Ollier and Maffucci disease) (10), and Eiken and Blomstrand syndromes (7).
Among these conditions, the skeletal deformities in Blomstrand type chondrodysplasia have been linked to hindered binding of PTH or PTHrP to their receptor, and the consequent absence of cAMP or inositol phosphate production as a response to these ligands (14, 17, 28). The first report of a point mutation in the PTH-R encoding gene was in a patient with Blomstrand chondrodysplasia, who was shown to be heterozygous for a G to A substitution at nucleotide 1176 inherited from his mother (14). This mutation caused the deletion of the first 11 amino acids of exon M5 of PTH-R, for the base substitution resulted in the formation of a novel splice site. Also, a single homozygous nucleotide exchange in exon E3 of the PTH-R gene (a proline-to-leucine substitution at position 132 in the receptor’s N-terminal extracellular domain) was identified in an infant with Blomstrand chondrodysplasia born to consanguineous parents (17, 28). Consistent with the first report on Blomstrand type PTH-R, cells transiently transfected with a mutant receptor (PTH-R\(^{P132L}\)) were unable to bind the ligands, suggesting that proline 132 is critical for the binding capability of the receptor. To date, however, no research has been undertaken to elucidate the intracellular localization of Blomstrand type PTH-R.

Meanwhile, the dysfunctional mutation of PTH-R may result not only in its inability to bind the ligands, but also in its altered intracellular localization or its instability/degradation after translation. In general, most mammal cells have developed a “quality control” system when translating proteins: During the biosynthesis of secretory and membrane-bound proteins, these molecules must be correctly folded and assembled in the rough-surfaced endoplasmic reticulum (ER) before being transported to the cell surface. When protein folding is not sufficient, the malformed proteins would be retained in the ER and eventually transported to the cytosol, where they are targeted for degradation by the ubiquitin-proteasome pathway. This quality control system is played mainly by the ER, and would avoid the production of mal-conformed proteins (18, 26, 29). One may raise the feasible possibility that the mutant PTH-R may not be efficiently targeted to the cell membrane, being instead eliminated at some stage of that “quality control” process.

In this study, we have explored the possibility that the transport of mutant PTH-R\(^{P132L}\) to its final destination at the cell membrane is aborted as a consequence of this mutation.

**MATERIALS AND METHODS**

**Culture of mouse osteoblastic MC3T3-E1 cells and rat chondrocytic CFK2 cells.** Mouse calvarial osteoblastic cells (MC3T3-E1 line, purchased from Riken Cell Bank; RCB No. 1126, Tsukuba, Japan) and rat chondrocytic cells (CFK2 line, kindly provided by Dr. David Goltzman, McGill University, Calcium Research Laboratory, Royal Victoria Hospital, Canada), which has been previously confirmed to synthesize type II collagen, PTHrP and PTH-R (1, 6) were cultured in a minimal essential medium (αMEM; Flow Laboratories, Irvine, Scotland) containing 10% fetal calf serum (FCS) until 70% confluence before the transfection.

**Transfection of cDNAs encoding the wild-type and the mutant PTH-R.** For the P132L mutation, the cDNA of wild-type rat PTH-R (27) was subcloned into a pALTER-Max\(^{®}\) vector (Promega, Madison, WI). The mutation was introduced to generate PTH-R\(^{P132L}\) using Alteredsites\(^{®}\) II mammalian mutagenesis system (Promega) as previously described (12, 13). The oligonucleotide used was P132L: 5’-AAATGTAATCTAGACAAGGTACT-3’. A silent mutation was structured in the P132L mutation site (CCC → CTA) to make an Xba I restriction site for identification of mutagenesis. The mutations were verified by DNA sequencing (data not shown). MC3T3-E1 and CFK 2 cells (1.0–1.3 × 10\(^5\) cells per 35 mm dish) were transfected with 0.8–1.0 μg of plasmids containing either the wild-type PTH-R or the mutant peptide using Lipofectamine Plus (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Transfected cells were then incubated for 24 h in 5% CO\(_2/95%\) air (v/v) incubator (NAPCO 5420-2; Termo Fisher Scientific, Inc., MA) before conducting the examinations.

**Immunolocalization of the wild-type and the mutant PTH-R in transfected cells.** Transfected MC3T3-E1 and CFK2 cells were fixed with 4% paraformaldehyde diluted in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C. The specimens were pretreated with 1% bovine serum albumin (BSA) in phosphate buffered saline (1% BSA-PBS, pH 7.4) for 30 min, and then, were incubated with goat polyclonal antiserum to rat PTH-R (3, 5) at a dilution of 1 : 300 with 1% BSA-PBS for 24 h at 4°C. The specimens were rinsed with PBS for 6 h. For immunofluorescence, some specimens were incubated with FITC-conjugated anti-rabbit immunoglobulins (Kirkegaard & Perry laboratories Inc. Gaithersburg, MD) prior to observation.
Localization of mutant PTH-R

under a fluorescence microscope (Eclipse, Nikon, Ltd, Tokyo, Japan). For immunoelectron microscopy, some specimens were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit F(ab)2, (Chemicon International Inc., Temecula, CA). Immune complexes were visualized using diaminobenzidine-staining. Subsequently, the specimens were post-fixed with 1% OsO4 in 0.1 M cacodylate buffer (pH 7.4) for 4 h at 4°C, dehydrated with ascending concentrations of acetone and embedded in epoxy resin (Taab, Berkshire, UK). Ultra-thin sections parallel to the culture dish were obtained with a microtome (Sorvall MT-5000; Ivan Sorvall, Inc., Norwalk, CT) and stained with lead citrate for TEM examination (Hitachi H-7100; Hitachi Co. Ltd, Tokyo, Japan) at 80 kV.

Cell fractioning and Western blotting. MC3T3-E1 cells transfected with rat wild-type PTH-R or mutant PTH-R(P132L) were cultured for 24 h after the transfection, and proteins obtained from these cells were divided into membrane/organelle-associated fraction and cytosolic fraction using a Proteo-Extract™ Kit (Merck KGaA, Darmstadt, Germany) according to the manufacturer’s protocol. For Western blotting, 10 μg of protein from each sample was diluted in a sample loading buffer and boiled for 3 min before loading. The samples were then separated in a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The Western blot was probed with anti-rabbit PTH-R antibody (Sigma-Aldrich Co., Saint Louis, MI). Immunoreactive bands were visualized using an ECL detection system (GE Healthcare UK Ltd., Buckinghamshire UK).

Cyclic AMP accumulation using MC3T3-E1 cells with or without PTH-R cDNA transfection. Transfected MC3T3-E1 cells were subcultured into 24-well plates (5 x 104/well). After 2 days of culture, cells were washed with PBS and incubated with 500 μl of αMEM (FCS−/phenol red−) containing 20 mM HEPS (pH 7.5), 0.5 μg/μL aprotinin and 2 mM IBMX (a phosphodiesterase inhibitor; isobutylmethylxanthine) for 10 min. Five μL of rat PTH (1–34) (Sigma-Aldrich) ranging from 10−10 to 10−6 M were applied to the treated cells for 15 min. Subsequently, 500 μL of 20% trichloroacetic acid (TCA) was added into the incubation medium, and transferred to glass tubes. The samples were centrifuged at 3000 rpm for 10 min. The supernatants were extracted three times with 1 mL of water-saturated ether to remove the TCA. The samples were dried by lyophilization, and cyclic nucleotides were determined using a cAMP radioimmunoassay kit (GE Healthcare UK Ltd. Buckinghamshire, UK).

Detection of fragmented DNA using TUNEL and percentage of TUNEL-positive cells transfected with the wild-type and the mutant PTH-R. Transfected MC3T3-E1 cells were subjected to TdT-mediated dUTP-biotin nick end labeling (TUNEL), using TACS™ 2 TdT-Blue Label In Situ Apoptosis Detection Kit (Trevigen, Inc., Gaithersburg, MD). Control MC3T3-E1 cells were transfected with an empty pALTER-Max® vector and subjected to TUNEL examination thereafter. All processes were performed according to the manufacturer’s protocol, as reported previously (22). The number of TUNEL-positive cells were counted and divided by the whole cell numbers, providing the percentage of TUNEL-positive cells, with the statistical results expressed as mean ± SD (n = 8, each group). Significant differences were determined by the Student’s t-test.

Reverse transcription-polymerase chain reaction (RT-PCR) amplification of PTHrP, IHH, VEGF, RANKL, Runx-2, ALP, osteopontin and GAPDH transcripts. The expression levels of genes related to skeletal development—PTHrP, indian hedgehog (IHH), vascular endothelial growth factor (VEGF), receptor activator nuclear factor κB ligand (RANKL), runt-related transcription factor 2 (Runx-2), alkaline phosphatase (ALP) and osteopontin (OPN)—were examined by semi-quantitative RT-PCR as reported by Suda et al. (21). MC3T3-E1 and CFK2 cells transfected with cDNA encoding wild-type PTH-R were cultured in αMEM for 24 h at 37°C. Total RNAs of the transfected MC3T3-E1 and CFK2 cells were then extracted by Trizol reagent (Invitrogen). Five μg of total RNA were subjected to reverse transcription using Superscript II (Invitrogen). PCR reactions (50 μL) contained 5 μL of 10 × reaction buffer, 1 μL of 25 pmol of each primer, 1 μL of 10 mM of dNTPs mixture, 1 U of Taq polymerase (Invitrogen), 3 μL of 25 mM MgCl2 and 2 μL of RT- DNA and sterile DW. Amplification was performed with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min using TaKaRa PCR Thermal Cycler Dice® (Takara Bio Inc. Ohtsu, Japan). Primer constructions of GAPDH, PTHrP, ALP, IHH, VEGF, Runx-2, RANKL and
J. Shimomura-Kuroki et al.

and RANKL levels were increased in the transfected MC3T3-E1 cells. In CFK2 cells, however, RANKL was not detected regardless of their transfected status. PTHrP, VEGF and OPN expression levels were not different between transfected and non-transfected cells.

**Statistical analysis for TUNEL-positive MC3T3-E1 cells transfected with the wild-type or the mutant PTH-R cDNAs**

The percentage of TUNEL-positive cells per whole cell numbers was examined in MC3T3-E1 cells transfected with the wild-type or the mutant PTH-R cDNAs.

**RESULTS**

**cAMP accumulation in the transfected MC3T3-E1 cells**

After stimulating the MC3T3-E1 cells with different concentrations of rat PTH (1–34) for 15 min, we measured the intracellular cAMP accumulation (Fig. 1). The MC3T3-E1 cells transfected with wild-type PTH-R displayed a dose-dependent increase of intracellular cAMP. MC3T3-E1 cells transfected with mutant PTH-R and control cells transfected with an empty vector showed a delayed, moderate increase in cAMP levels that was similar between the two groups.

**Comparative analysis for gene expressions related to skeletal development in transfected MC3T3-E1 and CFK2 cells**

We compared the gene expression levels of PTHrP, IHH, VEGF, RANKL, Runx-2, ALP and OPN between transfected and non-transfected MC3T3-E1 or CFK2 cells, since successful transient overproduction of the wild-type PTH-R is supposed to affect these genes (Fig. 2). With GAPDH used as a standard between the transfected and non-transfected cells, the expression of ALP was shown to be reduced in both MC3T3-E1 and CFK2 cells when transfected with PTH-R cDNA. As expected, Runx-2 and RANKL levels were increased in the transfected MC3T3-E1 cells. In CFK2 cells, however, RANKL was not detected regardless of their transfected status. PTHrP, VEGF and OPN expression levels were not different between transfected and non-transfected cells.

**Table 1** Primer designs for PCR amplification

<table>
<thead>
<tr>
<th></th>
<th>MC3T3-E1 cell</th>
<th>CFK2 cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>forward 5’-CATGGAGAAGGCTGGGCTC-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td></td>
<td>backward 5’-CATGGAGAAGGCTGGGCTC-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td>ALP</td>
<td>forward 5’-CGCTATCTGCTTGGCTGTA-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td></td>
<td>backward 5’-AACGGATACATTTGGGGTAG-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td>PTHrP</td>
<td>forward 5’-CGGTTTGGGCTACAGCTG-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td></td>
<td>backward 5’-AACGGATACATTTGGGGTAG-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td>IHH</td>
<td>forward 5’-CAGCCACCTGCTTGCCAGC-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td></td>
<td>backward 5’-AACGGATACATTTGGGGTAG-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td>Runx-2</td>
<td>forward 5’-TCTTCCCACAAGGAGTAGG-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td></td>
<td>backward 5’-AACGGATACATTTGGGGTAG-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td>RANKL</td>
<td>forward 5’-GCCATATGGGAGAGGTCTG-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td></td>
<td>backward 5’-AACGGATACATTTGGGGTAG-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td>VEGF</td>
<td>forward 5’-TACATGCCTACACCACC-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td></td>
<td>backward 5’-TACATGCCTACACCACC-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td>OPN</td>
<td>forward 5’-TCTTCTACTCTCATCACC-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
<tr>
<td></td>
<td>backward 5’-TCTTCTACTCTCATCACC-3’</td>
<td>5’-AACGGATACATTTGGGGTAG-3’</td>
</tr>
</tbody>
</table>

OPN are shown in Table 1. The intensity of PCR products were examined by NIH Image J as reported previously (11).

**Fig. 1** Measurement of intracellular cAMP accumulation in MC3T3-E1 cells transiently expressing PTH-R after 15 min of stimulation with increasing concentrations of rat PTH (1–34). MC3T3-E1 cells transfected with wild-type PTH-R revealed a dose-dependent increase in intracellular cAMP levels. Cells transfected with the mutant PTH-R and the control MC3T3-E1 cells showed a moderate increase.
Localization of mutant PTH-R

The PTH-R protein carrying the Blomstrand type mutation was barely detected on the cell surface; patchy and focal immunoreactivity was found in the cytoplasm of transfected MC3T3-E1 and CFK2 cells (Figs. 5A, B). Some cells showed faint but widespread immunoreaction of the mutant PTH-R throughout their cytoplasmic region. Under transmission electron microscopy, some mutant PTH-R

Transfected with cDNAs encoding either the wild-type PTH-R or the mutant receptor. This percentage was significantly higher in cells transfected with the mutant receptor than in those transfected with the wild-type receptor (2.02 ± 1.01 vs 0.45 ± 0.24, p < 0.005). There was no significant difference between control MC3T3-E1 cells transfected with the empty vector and the cells carrying the wild-type PTH-R (0.23 ± 0.16 vs 0.45 ± 0.24, NS).

**Western blotting analysis of the cytosolic and membrane-associated cell fractions**

PTH-R protein harvested from transfected MC3T3-E1 cells was divided into cytosol and membrane/cell organelle fractions, and the PTH-R molecule of each fraction was examined by Western blotting (Fig. 3). Wild-type PTH-R with 66.3 kDa was clearly detected in the membrane/organelle-associated fraction, while there was a smear labeling of that protein in the cytosolic fraction. On the other hand, a weaker intensity of the 66.3 kDa band for mutant PTH-R<sup>P132L</sup> was detected in the membrane/organelle-associated fraction (b-2). The cytosolic fraction of mutant PTH-R has a very faint labeling (b-1). w-1; wild-type cytosolic fraction, w-2; wild-type membrane/organelle associated fraction, b-1; Blomstrand type cytosolic fraction, b-2; Blomstrand type membrane/organelle associated fraction.

**Immunolocalization of the wild-type and mutant PTH-R in MC3T3-E1 and CFK2 cells**

MC3T3-E1 cells transfected with the wild-type PTH-R cDNA displayed immunofluorescence for the receptor mainly on the cell surface (Fig. 4A). Like MC3T3-E1 cells, the transfected CFK 2 cells displayed evenly distributed receptor immunoreactivity on the cell surface (Fig. 4B). Immunochemistry microscopic observation unveiled the presence of the wild-type PTH-R on the cell membranes of MC3T3-E1 cells, and in addition, some small vesicles close to the cell membranes, Golgi apparatus and ER showed the PTH-R immunoreactivity (Figs. 4C, D). There was virtually no reaction in the cytoplasm. In contrast, MC3T3-E1 cells in which PTH-R was not overexpressed did not show visible immunoreaction on their cell membrane (data not shown).

The PTH-R protein carrying the Blomstrand type mutation was barely detected on the cell surface; patchy and focal immunoreactivity was found in the cytoplasm of transfected MC3T3-E1 and CFK2 cells (Figs. 5A, B). Some cells showed faint but widespread immunoreaction of the mutant PTH-R throughout their cytoplasmic region. Under transmission electron microscopy, some mutant PTH-R

---

**Fig. 2** Expression of genes related to skeletal development in transfected MC3T3-E1 (A) and CFK2 (B) cells. ALP expression is decreased in both transfected MC3T3-E1 and CFK2 cells. The expression levels of Runx-2 and RANKL are increased in the transfected MC3T3-E1 cells. In CFK2 cells, RANKL is not found in either the transfected or in the non-transfected status. PTHrP, VEGF and OPN expression levels did not differ between transfected and non-transfected cells. Con: control cells transfected with empty vector, WT: MC3T3-E1 or CFK2 cells transfected with wild-type PTH-R cDNA.

**Fig. 3** Proteins obtained from MC3T3-E1 cells transfected with cDNAs carrying either the wild-type or the Blomstrand type PTH-R were divided into cytosolic and membrane-associated cell fractions (See M&M section). Wild-type PTH-R from the membrane/organelle-associated fraction (w-2) is clearly detected at 66.3kDa molecular weight, while the cytosolic fraction (w-1) shows a smear labeling of this protein. Only a small amount of mutant PTH-R<sup>P132L</sup> with 66.3 kDa was detected in the membrane/organelle-associated fraction (b-2). The cytosolic fraction of mutant PTH-R has a very faint labeling (b-1). w-1; wild-type cytosolic fraction, w-2; wild-type membrane/organelle associated fraction, b-1; Blomstrand type cytosolic fraction, b-2; Blomstrand type membrane/organelle associated fraction.
Fig. 4 Immunolocalization of wild-type PTH-R in MC3T3-E1 (A, C, D) and CFK2 (B) cells. MC3T3-E1 (A) and CFK2 (B) cells transfected with the wild-type PTH-R cDNA show immunofluorescence on the cell surface. Nuclei were faintly counterstained with DAPI (blue color). Immunoelectron microscopic observation demonstrates the wild-type PTH-R (darker areas) on the cell membranes (C, D, arrows), in some small vesicles close to the cell membranes (inset in C, arrowheads), in the Golgi apparatus (D) and in the ER (D) of the MC3T3-E1 cells. Original magnification, A, B: ×800, C: ×3000, an inset in C: ×6000, D: ×9000
Localization of mutant PTH-R

As expected, our study has provided evidences that the mutant PTH-R is not linked to signal transduction pathways activating cAMP production in response to PTH. After exogenous PTH administration, cAMP accumulation in cells transfected with PTH-R\textsuperscript{P132L} displayed a curve similar to that seen in control MC3T3-E1 cells transfected with an empty vector. Since the Blomstrand type PTH-R\textsuperscript{P132L} cannot bind the ligands (17, 28), it seems likely that only endogenous PTH-R may be linked to signal transduction in cells transfected with the mutant receptor. Consequently, MC3T3-E1 cells with PTH-R\textsuperscript{P132L} appeared to be concentrated in somehow enlarged ERs, and the other was present in the cytoplasm surrounding the ER in the transfected cells.

DISCUSSION

Although it has not been clarified whether PTH-R carrying the mutation found in human Blomstrand type chondrodysplasia would localize on the cell membrane, our study clearly demonstrated the abnormal intracellular localization of PTH-R\textsuperscript{P132L} in the transfected cells. Therefore, in addition to the inability of binding its ligand, PTH-R\textsuperscript{P132L} may not be transported to the cell membranes.

As expected, our study has provided evidences that the mutant PTH-R is not linked to signal transduction pathways activating cAMP production in response to PTH. After exogenous PTH administration, cAMP accumulation in cells transfected with PTH-R\textsuperscript{P132L} displayed a curve similar to that seen in control MC3T3-E1 cells transfected with an empty vector. Since the Blomstrand type PTH-R\textsuperscript{P132L} cannot bind the ligands (17, 28), it seems likely that only endogenous PTH-R may be linked to signal transduction in cells transfected with the mutant receptor. Consequently, MC3T3-E1 cells with PTH-R\textsuperscript{P132L}

Fig. 5 Immunolocalization of the Blomstrand type PTH-R in MC3T3-E1 (A, C, D) and CFK2 (B) cells. Patchy and focal immunoreactivity of PTH-R carrying a P132L mutation can be seen in transfected MC3T3-E1 (A) and CFK2 (B) cells. Note the absence of immunoreaction on the cell membrane. Under immunoelectron microscopy, the mutant PTH-R is found to have accumulated in somehow enlarged ERs (C), and in the cytoplasm surrounding the ER (D). Original magnification, A, B: x800, C, D: x8000
would produce the same amount of cAMP as did control MC3T3-E1 cells. MC3T3-E1 cells transfected with the wild-type PTH-R cDNA, on the other hand, showed an early and acute elevation of cAMP accumulation. The acute accumulation of cAMP may influence differentiation and function of these cells, and therefore, we have examined the gene expression related to bone and cartilage development. It is noteworthy that transfected, mouse calvaria-derived MC3T3-E1 and rat chondrocytic CFK2 cells showed different patterns in the expression of some genes. While both Runx-2 and RANKL expression levels were increased in the former cells, the latter showed only a slight increase in Runx-2 levels and no detectable rise in RANKL. This might correspond to the different characteristics of authentic osteoblasts and chondrocytes. Reduced ALP in transfected MC3T3-E1 and CFK2 cells seems consistent with the idea that PTH/PTHrP signaling stimulates proliferative activities and inhibits differentiation of chondrocytes and osteoblasts (2–4, 20). Thus, our successful transfection with cDNA encoding the wild-type PTH-R promoted cAMP accumulation, and affected the expression of genes related to bone and cartilage development.

As shown in Figure 3, wild-type PTH-R was abundantly found in the membrane/organelle-associated fraction, and had an assumed molecular weight of 66.3 kDa, while the presence of PTH-R

\textsuperscript{P132L} in this fraction was insignificant. In addition, immunofluorescent technique revealed the presence of wild-type PTH-R on the cell membrane, while PTH-R

\textsuperscript{P132L} showed a patchy accumulation in the cytoplasm of transfected cells (Compare Figs. 4 and 5). Taken together, it seems that the intracellular “quality control” carried out by the ER might coordinate proper protein folding and assembling of PTH-R, so as to maintain the full biological activity of the receptor (26). Generally, this control system would play a pivotal role in excluding abnormal proteins after translation, only permitting the transport of suitable proteins towards the Golgi apparatus and the cell membranes. When folding and assembly promotion is faulty, proteins would be retained in the ER and eventually translocated to the cytosol for degradation by the ubiquitin-proteasome pathway (18, 26, 29). It seems that intact PTH-R can be transported to cell membranes, whereas the mutant PTH-R

\textsuperscript{P132L} is excluded by means of the quality control system in the ER. Even if PTH-R

\textsuperscript{P132L} passes through the quality control system, only a smaller amount of the mutant receptor is able to reach the cell membranes when compared with the wild-type counterpart.

The immuno-electron microscopy provides further evidence to support our postulation, showing the presence of wild-type PTH-R in the ER, the Golgi apparatus, as well as in the cell membrane. In contrast, very few mutant receptors were found on the cell membrane but, instead, were seen in somehow enlarged ERs and in the cytoplasmic region surrounding the ER. If our postulation is correct, the immunoreactivity for the receptor in the enlarged ER may implicate its accumulation in this organelle, while immunoreactivity in the cytoplasm surrounding the ER may indicate the leakage of the mutant protein into the cytoplasm. This dual pattern of immunolocalization of the mutant receptor, in the enlarged ER or in the cytoplasmic region surrounding the ER, may be a consequence of when and how much of the mutant protein leaked into the cytoplasmic region. Accumulation of the mutant receptor protein may lead to a reaction to unfolded non-native proteins, such as the unfolded protein response or the ER stress response which is linked to signaling for cell differentiation, function and apoptosis (18, 29). If so, apoptosis in cells carrying the mutant PTH-R may be a consequence of these responses. Yet, whether mutant PTH-R is indeed degraded by proteasomes needs further examinations.

In summary, this study has provided a clue for the intracellular localization of mutant PTH-R

\textsuperscript{P132L}, a mutant PTH receptor that has been associated with the human Blomstrand type chondrodysplasia. Although the main mechanism that leads to the condition in humans has been reported to be the receptor’s inability to bind its ligands, here we raise the hypothesis that the mutant peptide is recognized by an intracellular quality control system, expelled to the cytoplasm and probably targeted into the ubiquitin-proteasome pathway.

Acknowledgements

This work was supported by from the Japanese Society for the Promotion of Science (N. Amizuka, J. Shimomura-Kuroki, K. Oda), and Naito Memorial Foundation (N. Amizuka).

REFERENCES


