Characterization of parathyroid hormone/parathyroid hormone-related protein receptor and signaling in hypercalcemic Walker 256 tumor cells

P Esbrit, J Benítez-Verguizas, F de Miguel, A Valín and A García-Ocaña

Bone and Mineral Metabolism Laboratory, Research Unit, Fundación Jiménez Díaz, Madrid, Spain

(Requests for offprints should be addressed to P Esbrit, Bone and Mineral Metabolism Laboratory, Research Unit, Fundación Jiménez Díaz, Avda. Reyes Católicos, 2, 28040 Madrid, Spain)

(F de Miguel and A García-Ocaña are now at Division of Endocrinology, Department of Medicine, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, USA)

Abstract

Parathyroid hormone (PTH)-related protein (PTHrP) is the main factor responsible for humoral hypercalcemia of malignancy. Both PTH and PTHrP bind to the common type I PTH/PTHrP receptor (PTHrR), thereby activating phospholipase C and adenylate cyclase through various G proteins, in bone and renal cells. However, various normal and transformed cell types, including hypercalcemic Walker 256 (W256) tumor cells, do not produce cAMP after PTHrP stimulation. We characterized the PTHrP receptor and the signaling mechanism upon its activation in the latter cells. Scatchard analysis of PTHrP-binding data in W256 tumor cells revealed the presence of high affinity binding sites with an apparent K_d of 17 nM, and a density of 90 000 sites/cell. In addition, W256 tumor cells immunostained with an anti-PTHrR antibody, recognizing its extracellular domain. Furthermore, reverse transcription followed by PCR, using primers amplifying two different regions in the PTHrR cDNA corresponding to

the N- and C-terminal domains, yielded products from W256 tumor cell RNA which were identical to the corresponding products obtained from rat kidney RNA. Consistent with our previous findings on cAMP production, 1 μ M PTHrP(1–34), in contrast to 10 μ g/ml cholera toxin or 1 μ M isoproterenol, failed to affect protein kinase A activity in W256 tumor cells. However, in these cells we found a functional PTHrR coupling to $G_{aq/11}$, whose presence was demonstrated in these tumor cell membranes by Western blot analysis. Our findings indicate that W256 tumor cells express the PTHrR, which seems to be coupled to $G_{aq/11}$. Taken together with previous data, these results support the hypothesis that a switch from the cAMP pathway to the phospholipase C–intracellular calcium pathway, associated with PTHrR activation, occurs in malignant cells.

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Introduction

Parathyroid hormone-related protein (PTHrP), initially isolated from hypercalcemia-associated tumors, is produced by a variety of nonmalignant tissues, where it appears to act as an auto/paracrine factor (Philbrick *et al.* 1996). The N-terminal region of PTHrP exhibits structural homology with that of parathyroid hormone (PTH), which accounts for its binding to the common type I PTH/PTHrP receptor (PTHrR), cloned in bone and kidney cells, with high affinity (Schipani *et al.* 1993). In these cells, the PTHrR signals via the phospholipase C and adenylate cyclase pathways through interaction with various G proteins (Abou-Samra *et al.* 1992, Takasu *et al.* 1999). However, in HTLV-I-infected lymphocytes, in normal rat islet as well as rat insulinoma cells, and in

squamous carcinoma cells, incompletely characterized PTHrP receptors show features different from those of PTHrR, e.g. low affinity PTHrP binding and/or failure to activate the adenylate cyclase pathway (McCauley *et al.* 1992, Orloff *et al.* 1992, 1994, Gaich *et al.* 1993, Villanueva-Peñacarrillo *et al.* 1999). In addition, in the latter cells, various mRNA transcripts hybridizing with a human PTHrR cDNA have been detected (Orloff *et al.* 1995). The relationship between these transcripts and the presence of putative PTHrP receptors different from the PTHrR in squamous cells is unknown at present.

We previously found that the N-terminal region of PTHrP stimulates cell proliferation and calcium transients, but not cAMP synthesis, in the hypercalcemic cell line WS of the Walker 256 (W256) tumor (Benítez-Verguizas & Esbrit 1994), which originates in the rat mammary

gland but has hematopoietic cell features (Simpkins *et al.* 1991). The present study was carried out to characterize further the PTHrP receptor and signaling in these cells.

Materials and Methods

Reagents

Human PTHrP(1–34) amide (PTHrP(1–34)) was from Peninsula Laboratories (Belmont, CA, USA). Human [Tyr³⁶]PTHrP(1–36) amide (PTHrP(1–36)) was a generous gift of Dr A F Stewart (University of Pittsburgh, PA, USA). Recombinant human epidermal growth factor (EGF) was kindly supplied by Serono (Madrid, Spain). Cholera toxin, 3-isobutyl-1-methylxanthine (IBMX), Arg-vasopressin, and isoproterenol were from Sigma (St Louis, MO, USA). Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was from Boehringer Mannheim (Mannheim, Germany). [γ -³²P]GTP (6000 Ci/mmol) was obtained from New England Nuclear (Brussels, Belgium). Na¹²⁵I (2000 Ci/mmol) was from Amersham International (Amersham, Bucks, UK).

Cell culture

Walker 256 tumor cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 50 U/ml penicillin, 50 µg/ml streptomycin and 10% fetal bovine serum (FBS) (culture medium), and subcultured weekly by dilution to 5×10^4 cells/ml (Benítez-Verguizas & Esbrit 1994). Cells in the exponential growth stage were used for the following experiments. Human osteoblastic (hOB) cells, supplied by Dr M E Martínez (Department of Biochemistry, Hospital La Paz, Madrid, Spain) were cultured in DMEM with 15% FBS and antibiotics, as reported previously (Martínez *et al.* 1997).

PTHrP binding assay

PTHrP(1–34) was radioiodinated by the Hunter and Greenwood method, with minor modifications (Hunter & Greenwood 1962). Briefly, 4 µg PTHrP were added to 1 mCi Na¹²⁵I in 80 µl 0.5 M sodium phosphate, pH 7.5. Iodination was allowed to proceed for 3 min following the addition of 10 µl 1.8 mM chloramine T. The reaction was stopped by adding 50 µl 0.1 M sodium metabisulfite. The iodinated hormone was separated from free iodine by using SepPak C18 cartridges (Waters, Milford, MA, USA), and was subsequently purified to maximal specific activity by HPLC on a µBondapak C-18 column with a 60-min linear gradient of 27–40% acetonitrile with 0.1% trifluoroacetic acid, at a flow rate of 1 ml/min (Orloff *et al.* 1989).

For binding studies, W256 tumor cell medium was removed, and the cells were preincubated with DMEM containing 0.2% bovine serum albumin (BSA) for 1 h at

37 °C. Then, the cells were washed and resuspended at a final density of 2×10^6 cells/ml in DMEM with 0.2% BSA, and 1 µg/ml pepstatin, 50 µg/ml bacitracin, 60 KIU/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA (protease inhibitor cocktail), $25\text{--}30 \times 10^3$ c.p.m. labeled PTHrP(1–34) and various concentrations of unlabeled peptide, in a final volume of 100 µl. Binding was carried out with gentle agitation at 20 °C for 3 h, when steady-state binding was achieved. Incubations were terminated by the addition of 1 ml cold phosphate-buffered saline (PBS) with 0.1% BSA, and centrifuging at 12 000 g for 5 min. The cell pellet was subsequently washed three times with the same buffer. Cell-bound radioactivity was recovered by the addition of 200 µl 1 M NaOH, and was γ -counted.

Immunocytochemistry

W256 tumor cells were washed with PBS, and deposited on glass slides by cytocentrifugation at 700 g for 10 min. Human OB cells were plated at 10 000 cells/cm² on multiwell chambers (Labtek; Nunc, Naperville, IL, USA) and cultured until preconfluency. The cells were subsequently fixed in 95% ethanol, washed with PBS, treated with 3% H₂O₂ for 5 min to abolish endogenous peroxidase activity, and then incubated with 1.5% nonimmune goat serum for 10 min. Immunostaining was conducted with 5 µg/ml of the polyclonal anti-rat PTHR antibody VII (Babco, Richmond, CA, USA), recognizing the N-terminal extracellular domain. This antibody detected the PTHR in rat kidney cortex (Largo *et al.* 1999). Subsequent incubations, 2 h with the primary antibody, 30 min with biotinylated anti-rabbit IgG, and 30 min with avidin-biotin-peroxidase complex (Vectastain Elite; Vector, Burlingame, CA, USA), were performed at room temperature. Positive staining was developed with 3,3'-diaminobenzidine. As negative controls, some cell preparations were incubated with saline buffer instead of the primary antibody.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Rat kidneys were harvested from adult (300 g) Sprague-Dawley rats and immediately frozen in liquid nitrogen. Total RNA was prepared from W256 cells and rat kidneys using the Trizol method (Gibco-BRL, Grand Island, NY, USA). Polyadenylated (poly A⁺) RNA was isolated from W256 cells by oligo(deoxythymidine)-cellulose chromatography (Gibco-BRL).

Complementary DNA was synthesized by reverse transcription of 2–5 µg total RNA or 1 µg poly A⁺ RNA using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA) with oligo(dT)₁₈ as primer, in a final volume of 40 µl. For PCR reactions, 10 µl of 2- to 20-fold diluted cDNA were added to a reaction mixture (50 µl)

containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl₂, 0.1 mM each deoxynucleotide, 1.25 U Taq DNA polymerase (Promega, Madison, WI, USA), and 0.4 µM of the specific primers: 5'-GATGCGGACGATGTCTTTACC-3' (sense), and 5'-GGCGGTCA AATACCTCC-3' (antisense), yielding a 481-bp product, corresponding to nucleotides 151-631 (exons E1-M1) of the rat PTHR cDNA sequence (Abou-Samra *et al.* 1992), encoding the N-terminal extracellular domain, or 5'-CATTGTGGCAGATCCAGATGC-3' (sense), and 5'-AGTCTAGCCCCCTAGTGCC-3' (antisense), yielding a 495-bp product, corresponding to nucleotides 1376-1870 of this cDNA (exons M6-7/T), encoding a region including the whole C-terminal intracellular tail of the rat PTHR (Abou-Samra *et al.* 1992). The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was coamplified using specific primers for the rat sequence (García-Ocaña *et al.* 1998). PCR products were visualized by ethidium bromide staining following electrophoresis on 1.6% agarose gels. The bands were excised, purified with Quantum Prep Freeze 'N Squeeze Spin columns (Bio-Rad, Hercules, CA, USA) and cloned into the pCRII-TOPO plasmid using the TOPO TA cloning kit as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). Miniprep plasmid DNA was prepared from PCR-product-containing colonies using QIAprep Spin columns (QIAGEN, Valencia, CA, USA). Inserted PCR products were sequenced in both directions using M13 primers and the dideoxy terminators method in an automated ABI PRISM 377 sequencer (Perkin Elmer, Norwalk, CT, USA).

Measurement of cAMP-dependent protein kinase A (PKA)

W256 tumor cells were maintained in FBS-depleted medium for 24 h. Fifteen minutes before stimulating with the different agonists, this medium was changed to the same fresh FBS-free medium with 10 µM IBMX. The cells were then stimulated in this medium with the agonists, 1 µM PTHrP(1-34), 1 µM isoproterenol for 5 min, or 10 µg/ml cholera toxin, for 2 h. After microcentrifugation for 1 min, followed by two washes with PBS, an ice-cold extraction buffer (50 mM potassium phosphate, pH 7.2, 10 mM EGTA, 10 mM EDTA, 2 mM IBMX, 2% Triton X-100, 0.2 mM dithiothreitol, 20 mM NaF) was added to the cell pellet. The cells were gently vortexed and then microcentrifuged for 1 min. PKA activity in the resulting supernatant was determined as previously described (Valín *et al.* 1997). PKA activity was expressed as the ratio of activity measured in the absence of added cAMP to that in the presence of 6.25 µM cAMP.

Western blotting of G_{aq/11} proteins

W256 cell membranes were prepared from subconfluent W256 cell cultures. The cells (20 × 10⁶ cells/ml) in PBS

with the protease inhibitor cocktail described above, were homogenized by sonication three times for 15 s in a Branson (Danbury, CT, USA) B-12 sonifier, at maximal setting. The resulting suspension was centrifuged at 100 000 g for 1 h at 4 °C. The pellet containing cell membrane fragments was resuspended in the same buffer and stored at -70 °C. Membrane proteins (30 µg) were electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel under reducing conditions, according to the method of Laemmli (1970). The separated proteins were transferred to a nitrocellulose membrane filter (Amersham International) by electroblotting, and then it was blocked with PBS containing 0.1% Tween (PBT), with 5% blocking agent (Amersham International) and 1% BSA, for 2 h at room temperature. After washing twice with PBT, the membrane was incubated for 1.5 h, at room temperature, with 4 µg of a rabbit polyclonal anti-G_{aq/11} antiserum (C-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA), which had been pre-incubated with or without 4 µg G_{aq/11} protein for 2 h at 4 °C. After two washes with PBT, the membrane was incubated with horseradish peroxidase-labeled donkey anti-rabbit IgG for 1 h, and then developed with an enhanced chemiluminescence method (ECL; Amersham International).

GTPase assay

Measurement of GTPase activity was performed as described by Thibonnier *et al.* (1993), with minor modifications. W256 tumor cell membranes (5 µg protein) were incubated with either different concentrations of the aforementioned specific anti-G_{aq/11} rabbit antiserum or 500 ng/ml nonimmunogenic rabbit IgG for 2 h at 4 °C. W256 cell membranes were then incubated with or without 1 nM PTHrP(1-36), in 100 µl containing 50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EGTA, 10 mM MgCl₂, 0.2 mM ATP, 5 mM creatine phosphate, 100 U/ml creatine kinase, 0.2 mM ouabain, 0.1% BSA, 500 000 c.p.m./tube [γ -³²P]GTP, for 10 min at 30 °C. The reaction was terminated with 750 µl cold 5% activated charcoal in 50 mM potassium phosphate, pH 7.5. After centrifugation at 1200 g for 30 min at 4 °C, the supernatant was counted in a liquid scintillation counter. GTPase activity was calculated after subtracting the c.p.m. obtained in this assay in the absence of cell membranes (nonspecific activity), and expressed as fmol × min⁻¹ × mg protein⁻¹.

Statistics

Results are expressed as means ± s.d. throughout the text. Statistical significance ($P < 0.05$ or lower) was evaluated by either unpaired *t*-test or one-way analysis of variance, when appropriate.

Figure 1 Competition binding of ^{125}I -PTHrP(1–34) to W256 tumor cells with various amounts of PTHrP(1–34). Values are means $\pm$ s.d. of 3–5 experiments in duplicate. Inset: Scatchard plot of ^{125}I -PTHrP(1–34) binding data.

Results

PTHrP binding

Specific binding of ^{125}I -PTHrP(1–34) ranged from 4% to 6% of total radioactivity added to W256 tumor cells. Meanwhile, nonspecific binding was between 2% and 3% in these cells. During binding incubation, ^{125}I -PTHrP(1–34) was not significantly degraded (<5%), as assessed by trichloroacetic acid precipitation. PTHrP(1–34) dose dependently inhibited ^{125}I -PTHrP(1–34) binding to W256 tumor cells (Fig. 1). Scatchard analysis of the saturation binding curves demonstrated the presence of a single class of high affinity binding sites with an apparent K_d of 17 nM, and a density of 90 000 sites/cell (Fig. 1, inset). Addition of either EGF or Arg-vasopressin, at 1 μM ,

failed to displace ^{125}I -PTHrP(1–34) bound (100%) to W256 tumor cells, which was $92 \pm 10\%$ and $102 \pm 5\%$ respectively ($n=3$).

Immunocytochemistry

Immunocytochemistry was also carried out to examine the presence of the PTHR in W256 tumor cells. Human OB cells, as positive control (Fig. 2B), and rat renal tissue (Largo *et al.* 1999), showed positive staining with an antibody recognizing the extracellular domain of this receptor. Using this antibody, distinct clusters of immunostaining were observed in W256 tumor cells (Fig. 2A). In contrast, these cells displayed no immunostaining upon incubation without the antibody (Fig. 2C).

Figure 2 (A) and (B).

Figure 2 (C).

Figure 2 Immunocytochemical staining for the PTHR in W256 tumor cells (A), and hOB cells (B). Negative control without primary antibody in W256 tumor cells is shown in (C). (Magnification, $\times 214$ (B); $\times 340$ (A,C).)

PTHrP mRNA expression by RT-PCR

Total and poly A⁺ RNA isolated from W256 tumor cells, and whole rat kidney total RNA as a positive control, were analyzed by RT-PCR, using primers amplifying two rat PTHR fragments. PCR analysis demonstrated the predicted products in either kidney or W256 tumor cells (Fig. 3). The nucleotide sequence of both PCR fragments obtained from W256 tumor cells RNA showed 100% homology with those corresponding from kidney, and were identical to the rat cDNA gene sequence domain reported previously (Abou-Samra *et al.* 1992).

cAMP-dependent PKA

We previously found a lack of cAMP response to the N-terminal region of PTHrP, in contrast to the response triggered by forskolin, in W256 tumor cells (Benítez-Verguizas & Esbrit 1994). Since PKA activity appears to be a more sensitive indicator of PTHrP bioactivity than cAMP production (Martin *et al.* 1992), we assessed this activity in W256 tumor cells. We found that PKA activity in control cells was 0.41 ± 0.08 ; meanwhile, it was 0.42 ± 0.05 , 0.60 ± 0.09 ($P < 0.01$), or 0.76 ± 0.13

($P < 0.01$) after treating these cells with $1 \mu\text{M}$ PTHrP(1–34), $1 \mu\text{M}$ isoproterenol, or $10 \mu\text{g/ml}$ cholera toxin respectively ($n=5$).

Western blot analysis of $G_{aq/11}$ protein

Our previous results showed an intracellular calcium response, which is likely to occur by phospholipase C activation, to N-terminal PTHrP in W256 tumor cells (Benítez-Verguizas & Esbrit 1994). In the present study, we specifically examined whether a $G_{aq/11}$ type of G protein was present in these cells (Simon *et al.* 1991). Using a specific anti- $G_{aq/11}$ antibody, we detected a protein with an apparent molecular mass of 42 kDa in W256 tumor cell membranes (Fig. 4), consistent with that of $G_{aq/11}$ protein in other cells (Allgeier *et al.* 1994).

GTPase assay

Activation of G protein-coupled receptors causes dissociation of the G protein $\beta\gamma$ heterodimer from the α subunit-GTP complex displaying intrinsic GTPase activity (Simon *et al.* 1991, Thibonnier *et al.* 1993).

Figure 3 RT-PCR analysis of W256 tumor cell (WS) total or poly A⁺ RNA, and of rat kidney tissue total RNA, using specific primers yielding two fragments corresponding to the N-terminal extracellular loop (exons E1/M1) or the C-terminal tail (exons M6–7/T) of the PTHR gene. The PCR product of a rat PTHR cDNA probe (R15B), negative control (H₂O) without total RNA, and coamplified GAPDH product are also shown. PCR products were electrophoresed on 1.6% agarose gels. DNA markers are shown on the left.

Therefore, we used this approach to determine whether the G_{aq/11} protein was coupled to the PTHrP receptor in W256 tumor cell membranes. Current evidence suggests that PTHrP(1–34) and PTHrP(1–36) were equivalent in binding to PTHR and the subsequent activation of phospholipase C (Abu-Samra *et al.* 1992, Jüppner *et al.* 1992, Schipani *et al.* 1993, Takasu & Bringham 1998, Takasu *et al.* 1999). We found that PTHrP(1–36), between 1 nM (Fig. 5) and 100 nM (not shown), maximally increased GTPase activity in these cell membranes. This activity was dose-dependently inhibited by a specific anti-G_{aq/11} antibody, but not by nonimmune IgG (Fig. 5).

Discussion

In the present study, analysis of the kinetics of PTHrP(1–34) binding to W256 tumor cells shows a single class of high affinity binding sites, with an apparent K_d of 17 nM, which is within the range of that found by some investigators (8–15 nM) for the PTHR (Abou-Samra *et al.* 1992, Schipani *et al.* 1993, Blind *et al.* 1995), although somewhat higher than that reported by others (within 0.3–6 nM) for this receptor (Jüppner *et al.* 1992, Iida-Klein *et al.* 1995, Takasu & Bringham 1998, Takasu *et al.* 1999). This

Figure 4 Western immunoblot analysis of W256 tumor cell membranes, using the anti-G_{aq/11} antibody (4 µg). The membrane was incubated with the antibody, preincubated with (lane 2) or without (lane 1) 4 µg G_{aq/11}. The molecular masses ($\times 10^{-3}$) of protein markers are indicated.

moderately higher apparent K_d value compared with the latter range for PTHrP binding to W256 tumor cell membranes is unlikely to be accounted for by a high concentration of PTHrP receptors, but it might reflect an inefficient G protein coupling (discussed below), as suggested occurs in some PTHR-transfected cells (Jüppner *et al.* 1992, Schipani *et al.* 1993, Blind *et al.* 1995, Iida-Klein *et al.* 1995, Takasu *et al.* 1999). In addition, we obtained a similar band by RT-PCR of either poly A⁺ or total RNA from these cells and from rat kidney, using primers amplifying two fragments corresponding to either the extracellular domain or the C-terminal tail coding regions in the rat PTHR gene (exons E1–M1 and M6–7/T respectively). Thus, both the extreme N-terminal region and the juxtamembrane end of the extracellular domain of the PTHR, necessary for promoting its surface expression and/or for ligand binding (Jobert *et al.* 1996, Carter *et al.* 1999), are likely to be homologous with the corresponding regions in the PTHrP receptor in W256 tumor cells. Moreover, these cells stain with an antibody recognizing an epitope in the extracellular domain of the rat PTHR,

Figure 5 Effect of PTHrP(1–36) in the absence or presence of anti- $G_{\alpha q/11}$ antibody (Ab) at 25, 50, and 500 ng/ml, or nonimmune IgG at 500 ng/ml, on GTPase activity in W256 tumor cell membranes. PTHrP(1–36) was added at 1 nM. Values are means $\pm$ S.D. of 3–5 experiments in duplicate. * $P < 0.01$ compared with nonstimulated control ($100 \pm 14\%$ or $7 \pm 1 \text{ fmol} \times \text{min}^{-1} \times \text{mg}^{-1}$); ^a $P < 0.01$ compared with value corresponding to stimulation with PTHrP(1–36) alone.

encoded by exon E1 in this receptor gene—an exon apparently involved in its surface expression (Lee *et al.* 1994). Taken together, these findings suggest that both N- and C-terminal domains of the PTHrP receptor in W256 tumor cells are either identical to, or share high homology with, the corresponding domains in the PTHR. In this regard, the 2.3-kb transcript encoding the PTHR has recently been detected by Northern blot analysis in a W256 tumor strain different from that studied herein (Yaghoobian *et al.* 1998).

The PTHR cloned in bone and kidney cells activates both adenylate cyclase and phospholipase C pathways (Abou-Samra *et al.* 1992, Orloff *et al.* 1994, Takasu & Bringham 1998, Takasu *et al.* 1999). However, in vascular smooth muscle cells, this receptor activates the cAMP-dependent pathway but does not stimulate phospholipase C, apparently due to a deficient PTHR coupling to the G_q protein necessary for phospholipase C activation (Maeda *et al.* 1996). On the other hand, similar to findings in the

W256 tumor cells (Benítez-Verguizas & Esbrit 1994, and present results), the N-terminal region of PTHrP does not increase cAMP in HTLV-I-infected lymphocytes (McCauley *et al.* 1992), in squamous carcinoma cells (Orloff *et al.* 1992), and in both normal and transformed rat islet cells (Gaich *et al.* 1993, Villanueva-Peñacarrillo *et al.* 1999). However, in contrast with the present findings in W256 tumor cells, low affinity binding sites (apparent K_d 100 nM) have been found in both rat insulinoma cells and malignant keratinocytes (Orloff *et al.* 1992, Gaich *et al.* 1993). The latter cells express multiple mRNA transcripts of different size to the 2.3-kb transcript encoding the PTHR (Orloff *et al.* 1995). Moreover, both PTH and PTHrP activate adenylate cyclase in various squamous carcinoma cell lines after transfection with the PTHR, supporting the hypothesis that a receptor different from the PTHR, and not a defective G_s protein, mediates the altered cAMP response to PTHrP(1–34) in these cells (Orloff *et al.* 1995). On the other hand, but consistent

with our findings in W256 tumor cells, HTLV-I-infected lymphocytes display high affinity binding sites for PTHrP(1–36), associated with increases in cytosolic calcium and unaltered cAMP (McCauley *et al.* 1992). To our knowledge, further characterization of this PTHrP receptor has not yet been carried out.

In the present report, in agreement with our previous findings (Benítez-Verguizas & Esbrit 1994), we found that PTHrP(1–34) failed to induce a significant activation of PKA in W256 tumor cells. However, other G_s -dependent adenylate cyclase stimulatory agonists, such as cholera toxin and isoproterenol, produced a similar increase in PKA activity in these cells. On the other hand, we demonstrated the existence of a PTHrP receptor coupling to $G_{aq/11}$, which was detected in W256 membranes by Western blot analysis, in these cells. Previous studies with the full-length and truncated forms of the PTHR stably transfected in 293 cells showed that a region of the C-terminus beyond residue 483 of this receptor modulates G_s activation (Schneider *et al.* 1994). Moreover, using COS-7 cells transiently expressing various PTHR constructs, the C-terminal region of this receptor has been shown to lower the apparent receptor affinity, and to decrease the efficacy of cAMP responsiveness without altering phospholipase C activation (Iida-Klein *et al.* 1995). However, the PCR product from RNA from W256 tumor cells, using primers amplifying a region encoding the whole C-terminal intracellular tail of the PTHR gene, was identical to the corresponding region in this gene. Thus, an alteration in this domain of the PTHrP receptor is unlikely to contribute to the lack of cAMP response to PTHrP in W256 tumor cells.

Malignant transformation of human keratinocytes with an activated H-ras oncogene was shown to be associated with a reduced cAMP response to some agonists, including PTHrP(1–34) (Henderson *et al.* 1992). Moreover, PTHrP(1–34) stimulates cAMP but not intracellular calcium in human mammary cells, Hs578Bst, although Hs578T cells, originating from a mammary carcinoma in the vicinity of the normal breast tissue which was the source for the cell line Hs578Bst, do not respond to this peptide with increased cAMP production (Birch *et al.* 1995). However, in contrast to findings herein in W256 tumor cells, H-ras-transformed keratinocytes showed no binding to PTHrP(1–34) (Henderson *et al.* 1992), and Hs578T cells had no PTHR mRNA, using PCR with primers annealing to a domain encoding the N-terminal extracellular loop of this receptor (Seitz *et al.* 1993, Birch *et al.* 1995). Interestingly, a preliminary study in human mammary epithelial cell lines has shown that their increasing malignancy is associated with a switch from the cAMP pathway to the phospholipase C–intracellular calcium pathway after PTHR activation (Cataisson *et al.* 1998). Therefore, taken together, the aforementioned and present findings suggest that such a switch associated with PTHR activation might be a common event in malignant cells.

In summary, our data indicate that W256 tumor cells express the PTHR, which appears to be coupled to a $G_{aq/11}$ protein in these cells. Since several W256 tumor variants with different tumor features are available, this tumor could be a useful model to clarify the possible role of malignant transformation on the mechanism(s) involved in the PTHR uncoupling to adenylate cyclase.

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