The parathyroid hormone-related protein receptor is expressed in breast cancer bone metastases and promotes autocrine proliferation in breast carcinoma cells

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Overproduction of parathyroid hormone-related protein (PTHRP) occurs in a high proportion of primary breast cancers (PBC) and is strongly implicated in their metastatic spread to bone. Although the PTHRPR-receptor (PTHRP-R) is often coexpressed with PTHRP in PBC, its role in regulating breast cancer cell proliferation and metastases to bone remains unclear. The aims of this study were to determine the expression of the PTHRP-R in breast cancer bone metastases (BM) and to investigate the effects of PTHRP-R overexpression on breast cancer cell proliferation. PTHRP-R expression occurred in 85% (11 out of 13) of BM compared with 58% (39 out of 67) of PBC. Median expression was higher (P<0.05) in BM compared with PBC. PTHRP increased cAMP accumulation and DNA synthesis in MCF-7 cells stably overexpressing the PTHRP-R (MCF-7WTR) but not in MCF-7VEC control cells. The increase in DNA synthesis was mimicked by the cAMP pathway activator forskolin. The receptor antagonist PTHRP7–34 reduced DNA synthesis in MCF-7WTR cells, but not MCF-7VEC cells, indicating that receptor overexpression promotes autocrine PTHRP activity. MCF-7WTR cells showed increased mitogenic responsiveness to fetal calf serum and reduced doubling times. PTHRP induced weak activation of ERK1 and ERK2 and potentiated their activation by serum growth factors. Collectively these results show that the PTHRP-R is frequently expressed in breast cancer BM and indicate that receptor overexpression drives proliferation via autocrine signals that are mediated via cAMP and ERK pathways.

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Parathyroid hormone-related protein (PTHRP) was first identified as the causative factor in the paraneoplastic syndrome humoral hypercalcaemia of malignancy (HHM) (Suva et al., 1987). The amino-terminal region of PTHRP is similar to that in parathyroid hormone (PTH) and both hormones share a common G protein-coupled receptor, known as the PTH/PTHPRP receptor (PTHRP-R). In contrast to PTH, which is produced almost exclusively by the parathyroid gland and circulates as an endocrine regulator of calcium homeostasis, PTHRP has been found in almost every tissue and acts via paracrine or autocrine mechanisms to regulate development and cell growth and differentiation (reviewed in Wysolmerski and Stewart, 1998).

Substantial evidence indicates that PTHRP is involved in breast cancer progression. In particular, a number of studies strongly implicate PTHRP as having a major role in the preferential metastatic spread of breast cancers to the skeleton. For example, compared to primary breast tumours and non-skeletal metastases, PTHRP is more frequently expressed in bone metastases (BM) (Powell et al., 1991). Moreover, expression of PTHRP in primary tumours predicts the subsequent development of BM (Bundred et al., 1992; Bouizar et al., 1993) and correlates with poor prognosis (Yoshida et al., 2000). Tumour cells secreting PTHRP may be better able to survive and proliferate in bone since localised production of PTHRP by breast cancer cells in these sites has been shown to promote osteolysis in a mouse model of skeletal metastasis (Guise et al., 1996).

In contrast to the substantial body of evidence linking PTHRP to breast cancer progression, much less is known about the role played by the PTHRP-R. Several breast tumour cell lines express the PTHRP-R and proliferate in response to PTHRP (Birch et al., 1995; Cataisson et al., 2000). Other studies have detected PTHRP-R expression in primary tumours (Carron et al., 1997; Downey et al., 1997; Iezzoni et al., 1998). Moreover, PTHRP-R and ligand are coexpressed in majority of these tumours supporting the idea that there may be paracrine or autocrine mechanisms involving PTHRP that are important for tumour progression. In support of this, we recently found that coexpression of PTHRP-R and ligand in primary breast cancers predicts poor patient prognosis (Linforth et al., 2002).

Overexpression of PTHRP and its receptor in breast tumour cells could also promote the growth of such cells in skeletal metastases by stimulating their proliferation in an autocrine fashion. To investigate this idea, we have set out to establish whether breast cancer cells maintain or upregulate PTHRP-R expression in these lesions by comparing the frequency and extent of PTHRP-R expression in BM with that in primary breast cancers. In consequence of our findings, we then characterised how overexpression of the PTHRP-R in breast cancer cells influences their proliferative responsiveness.
MATERIALS AND METHODS

Assessment of PTHRP-R expression in primary breast cancers and skeletal metastases

Following ethical approval, samples of malignant breast carcinoma tissue and BM were collected from patients undergoing surgery at the South Manchester University Hospital. After surgical excision, samples were removed from the tumour and snap frozen in liquid nitrogen. Bone metastases were collected during surgery on patients presenting with acute pathologically fractured femurs because of breast cancer, who underwent open reduction and internal fixation of the fracture.

RNA was extracted from tissue samples using Trizol™ (Invitrogen, Paisley, UK) in accordance with the manufacturer’s protocol. RNA yield was quantitated by UV spectrophotometry and the integrity verified on agarose gels. PCR analysis of PTHRP-R expression was carried out following global amplification of expressed genes by poly(A) PCR (Brady and Iscove, 1993; Al-Taher et al, 2000). Direct comparison of mRNA expression levels measured using this technique gave identical results to those obtained using conventional RT–PCR or TaqMan real-time quantitative PCR (Brady, unpublished data). For gene-specific PCR, reactions, were carried out in a total volume of 22 μl and contained 1 ng of poly(A) cDNA, 0.33 μM each oligonucleotide PCR primer, 0.5 U Taq polymerase (Roche Biochemicals, Lewes, UK) and 0.25 mM dNTPs. PCR primers for the PTHRP-R were directed towards the mRNA sequence within 300 bp of the poly(A) addition site, as described previously (Al-Taher et al, 2000): forward primer (1734–1756) CCG CCT ACT GCC CAC TGC CAC CAC, reverse primer (1973–1996) TCC ATC CAG TAT GTC AGG TCC. The reactions were carried out in a programmable thermocycler (Techne PHC-3) using the following conditions: one cycle of 1 min at 94°C, then 25–35 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, and finally one cycle of 5 min at 72°C.

In order to quantify gene expression, PCR reaction products were run on a 1% agarose gel alongside a series of standards containing known amounts of PCR product DNA. These were prepared by combining the gene-specific PCR products generated from several reactions and diluting these to produce a series containing a known amount of DNA molecules from 1.5 × 10^6 to 5.0 × 10^7, as described (Al-Taher et al, 2000). Dilutions were made using a solution of sonicated carrier lambda DNA.

Following agarose gel electrophoresis, gels were denatured in 1.5 M NaCl, 0.5 M NaOH for 45 min and then neutralised with 1 M NH₄OH before blotting on to Hybond-N membrane (Amersham, Bucks, UK). The membrane was then rinsed in sodium phosphate buffer and prehybridised for 2 h at 55°C with denatured salmon sperm DNA. Membranes were then hybridised at 55°C overnight with a 32P-labelled oligonucleotide probe directed to a specific sequence within the PTHRP-R PCR product, as follows: (1852–1875) GAC GAT GGG TTC CTC AAC GGC TGC. The membrane was then washed, dried and exposed to X-ray film for 1–3 days. In addition, the hybridised bands were quantified using a phosphor-imager. The values for the DNA standards were used to construct a standard curve from which values for the samples were read. Samples falling out with the linear range of the standard curve were resubjected to PCR using a modified number of amplification cycles.

PTHRP-R cDNA constructs and generation of stable transfected cell populations

Wild-type human PTHRP-R cDNA in pcDNA1 plasmid vectors were generously provided by Dr E Schipani (Boston). The receptor cDNA was excised and subcloned into pcDNA3 (Promega, Southampton, UK) vector. Insertion was confirmed by restriction digestions using EcoR1 and XhoI, and by direct sequencing. To generate cell lines stably expressing the PTHRP-R, MCF-7 cells (obtained from the ATCC and grown in DMEM containing 10% fetal calf serum (FCS) and 2 mM glutamine) were transfected with 9 μg of the receptor cDNA or with empty pcDNA vector as a control using FuGene6 (Roche Biochemicals) according to the manufacturer’s instructions. Following transfection, cells were placed in a selection medium containing 0.5 mg ml⁻¹ G418 (Invitrogen). After approximately 2 weeks, during which the medium was changed every 2 days, resistant cells were expanded for analysis. Subcloning of transfected cells was not performed and all of the experiments reported in this study were carried out using the entire population of G418-resistant cells.

PTHRP-R expression by RT–PCR in transfected stable lines

Total RNA was extracted from approximately 5 × 10⁶ cells using the RNeasy kit (Qiagen, Crawley, UK). RT–PCR was carried out using the enhanced avian RT–PCR kit (Sigma) with the following primers designed to amplify a 71 bp fragment:

forward 5’-AGGAACAGATCTTCTCTGCTGCA-3’;
reverse 5’-TGGCATGTGGATGGATGTGGCGGT-3’.

The reactions used 100 ng RNA with primers at a final concentration of 250 nM in the presence of 3 mM MgCl₂. First strand synthesis was conducted at 42°C for 30 min, followed by denaturation at 94°C for 2 min. A total of 35 cycles of denaturation (94°C, 15 s) and annealing (68°C, 1 min) were followed by extension at 68°C for 5 min. Reaction products were run alongside 100 bp size markers on 1% agarose gels containing ethidium bromide and the resulting gel photographed under UV light.

Cyclic AMP (cAMP) assay

This was carried out using the cAMP binding protein competitive assay as described previously (Yarwood et al., 1998). In brief, cells in six-well plates were starved overnight in serum-free medium prior to stimulation with factors as described. Perchloric acid extracts were then prepared followed by neutralisation and removal of precipitated material by centrifugation. Supernatants were stored at −80°C prior to the assay. Samples were assayed in the presence of 0.1 μCi [³H]cAMP and 8 μg cAMP binding protein (Sigma) alongside a series of standard cAMP solutions. Following binding to equilibrium (2–3 h), unbound cAMP was precipitated by the addition of activated charcoal suspension. The suspension was centrifuged and an aliquot of the supernatant solution counted on a scintillation counter. Results are expressed as picomoles of cAMP formed per million cells.

Thymidine uptake assay

Cells were grown to approximately 80% confluence in 24-well plates. Cells were starved for 24 h in serum-free medium and then test agents were added in fresh serum-free medium for a further 24 h. In experiments testing the effects of PTHRP₂₋₃₄, following incubation in serum-free medium, cells were treated for a further 24 h in fresh serum-free medium or in a medium containing 0.5% serum. Cultures received additionally either drug vehicle (0.0001% acetic acid) or PTHRP₂₋₃₄ (1 μM). During the final 4 h 0.25 μCi [³H]thymidine (ICN) was added. Cells were then washed three times with PBS followed by the addition of 0.5 ml ice-cold TCA (20% w/v) for 1 h. The cell precipitates were washed twice with 20% TCA, once with 95% ethanol, then dissolved in 0.25 ml 1 M NaOH and neutralised with an equal volume of 1 M HCl. Aliquots were counted on a scintillation counter.
Measurement of doubling time

Cells were plated in quadruplicate at low density in a medium containing 2% serum. Cell numbers were counted using a haemocytometer every 24h for a total of 96h. The average time taken for each cell population to double in number was then calculated from the logarithmic phase of the growth curve.

Measurement of PTHRP secretion

PTHRP was measured using a two-site immunoradiometric assay (IDS Ltd, Tyne and Wear, UK). Cells were plated in triplicate (10 000 cells well−1) in a 48-well plate. After 72h, the medium was changed to serum-free medium and the cells were incubated for 48h. The conditioned medium was then collected and immediately assayed alongside a series of PTHRP standards (0–40 pmol). To allow for variations in growth rate (see Results), cells were removed and counted. PTHRP accumulation was expressed as picomoles produced by 106 cells over the 48h incubation period.

ERK activation

ERK activation was assessed by immunoblotting cell lysates with antiphospho-ERK antibodies as described previously (Yarwood et al, 1999). Briefly, cells were starved in serum-free medium overnight and then stimulated in fresh medium with either EGF, IGF-I or PTHRP1–34 at the concentrations and for the times detailed in the figure legends. Lysates were prepared and equivalent amounts of cellular protein (20 μg) were run on 12% SDS–PAGE gels. The samples were transferred to Hybond ECL (Amersham Bioscience, Bucks, UK), which was then blocked and probed with antiphospho-ERK antibodies (Cell Signalling Technology, Hitchin, UK) at 1:2000 dilution. Immunoreactive bands were visualised following HRP-linked secondary antibody incubation and reaction of the blots with Supersignal West Pico (Pierce, Warrington, UK). Identical samples were run in parallel and immunoblotted with anti-ERK total antibodies (Santa Cruz, CA, USA) to confirm equivalent sample loading.

RESULTS

In order to investigate the potential importance of PTHRP-mediated autocrine actions in metastatic breast cancer cells, we initially examined whether the PTHRP-R was expressed in skeletal metastases. Expression was measured by semiquantitative RT–PCR followed by Southern blotting and scanning densitometry. The data, presented in Figure 1, show that the PTHRP-R is detectable in around 58% (39 of 67) of the primary breast cancers compared to 85% (11 of 13) of the BM. While this difference just fails to reach statistical significance (P = 0.053, χ2-test), these results indicate that the PTHRP-R is expressed more frequently in BM. In addition, although expression levels varied considerably, especially among the primary cancer samples, the median receptor expression level was higher in the metastases samples compared to the primary cancers (2818 (1189–7483) vs 299 (10–3000); median and interquartile range (P < 0.05; Mann–Whitney U-test)) (Figure 1).

Expression of the PTHRP-R in metastatic breast cancers supports the idea that tumour cells within these lesions are responsive to PTHRP. Based on previous reports, showing that PTHRP can increase proliferation in some breast cancer cell lines, we reasoned that overexpression of the receptor would increase mitogenic responses of cells to PTHRP. To examine this, MCF-7 breast carcinoma cells were transfected with a cDNA encoding the human PTHRP-R. Following transfection and selection, we confirmed stable receptor expression by RT–PCR and by measuring the response of these cells to PTHRP in terms of cAMP generation. Stable populations of cells transfected with PTHRP-R cDNA (MCF-7TR) expressed the receptor, whereas vector-transfected cells (MCF-7TEC) did not (Figure 2A). MCF-7TR cells exhibited a ≈ five-fold increase in cAMP accumulation in response to PTHRP1–34 whereas MCF-7TEC cells showed little or no responsiveness to PTHRP1–34 (Figure 2B). The response of both cell types to forskolin, which directly activates adenyl cyclase, was virtually identical indicating that receptor transfection did not affect the basic functioning of the cAMP generation system.

To investigate the effects of PTHRP-R overexpression on mitogenesis, we first examined the effect of exogenously added PTHRP on thymidine uptake, as a measure of cell cycle progression. MCF-7TEC cells were unresponsive to PTHRP1–34, whereas MCF-7TR cells showed a significant increase in thymidine uptake in response to PTHRP1–34 treatment. Exposure of both cell types to 100 nm forskolin resulted in quantitatively similar increases in thymidine incorporation indicating that cAMP signals a mitogenic response in these cells.

We next sought evidence for autocrine-mediated effects of PTHRP on proliferation. Previous studies have shown that MCF-7 cells synthesize PTHRP mRNA (Birch et al, 1995) and secrete PTHRP into the medium (Rong et al, 1999). To confirm these findings, we measured PTHRP secretion in each cell type using a sensitive two-site immunoradiometric assay. These assays revealed that PTHRP was present in the conditioned medium and that the degree of PTHRP production was similar in each cell type.

**Figure 1** Expression of the PTHRP-R in primary breast cancers and BM. PTHRP-R expression was measured in 67 primary breast cancers and 13 bone metastases by RT–PCR as described in Materials and Methods. Relative expression was quantified by Southern blotting of the agarose gels using a specific 32P-labelled probe followed by phosphorimaging, and related to a series of standards of known amounts of DNA corresponding to the PCR product. Owing to the wide variation in receptor expression within the primary cancer samples data were log10 transformed for clarity.
Figure 2 Comparison of PTH-R expression and responsiveness to PTHRP in vector- and receptor-transfected MCF-7 cells. (A) Total RNA was isolated from MCF-7VEC and MCF-7WTR cells, and PTH-R expression was measured by RT-PCR as described in Materials and Methods. The specific receptor fragment of 571 bp is indicated. M = 100 bp size markers, B = water blank reaction without RNA, C = control reaction containing irrelevant RNA and primers. (B) Cells in six-well culture plates were treated for 10 min with IBMX (0.5 mM) followed by treatment for a further 10 min with vehicle (ethanol, 0.01%; open bars), PTHRP (125 nM; grey bars) or forskolin (100 μM; black bars). Extracts were then prepared and assayed for cAMP content as described in Materials and Methods. The data represent means ± s.e.m. from three independent experiments for each cell population. Individual assays were performed in triplicate. *Values significantly different from the corresponding control value (P < 0.05; ANOVA).

Figure 3 Effect of PTHRP7–34 on DNA synthesis in vector and PTH-R-transfected MCF-7 cells. Cells in 24-well culture dishes were incubated in serum-free medium for 24 h prior to treatment for a further 24 h with PTHRP7–34 (25 nM; open bars or 125 nM; grey bars) or forskolin (1 μM; black bars). [3H]thymidine (0.25 μCi well−1) was added for the final 4 h, then cellular uptake of thymidine was measured as described in Materials and Methods. The data represent means ± s.e.m. from three independent experiments for each line. Individual measurements were performed in triplicate. *Values significantly different from the respective control (P < 0.05, ANOVA).

(MCF-7VEC: 1.67 ± 0.05 pmol 10⁴ cells⁻¹ h⁻¹; MCF-7WTR cells: 1.58 ± 0.06). Since MCF-7 cells produce PTHRP, overexpression of the PTH-R may sensitize such cells to autocrine activity. To test this further, we examined the effects of PTHRP7–34, which acts as an antagonist of the PTH-R and blocks ligand-induced receptor activation (Nagasaki et al, 1989). Figure 4 shows that incubation of MCF-7VEC cells, in either serum-free medium or medium containing 0.5% serum, with PTHRP7–34 significantly reduced thymidine incorporation by 18 and 36%, respectively. In contrast, MCF-7VEC cells were unaffected by treatment with PTHRP7–34. These experiments demonstrate that overexpression of the wild-type PTH-R alone, in the absence of any alterations in PTH ligand production, is sufficient to confer autocrine proliferative responsiveness to PTHRP.

We next examined whether overexpression of the PTH-R affected the general mitogenic responsiveness of MCF-7 cells. Exposure of previously starved cells to normal growth medium containing 10% FCS led to increases in thymidine uptake that were similar in MCF-7VEC and MCF-7WTR cells (data not shown). However, when the cells were treated instead with a medium containing 2% serum, significant differences in responsiveness were revealed. Stimulation of MCF-7VEC cells resulted in a 122 ± 9% increase in thymidine uptake compared with MCF-7WTR cells where the increase was 78 ± 9% (Table 1). Consistent with their apparent increased mitogenic responsiveness to serum, the MCF-7WTR cells also exhibited a doubling time that was significantly lower than that measured for MCF-7VEC cells (Table 1). These results suggest that overexpression of the PTH-R increases the capacity of MCF-7 cells to respond to mitogenic factors present in FCS.

The preceding data indicate that overexpression of the PTH-R increases the mitogenic responsiveness of breast cancer cells not only to endogenous and exogenous PTH-R but also to heterologous serum-derived growth factors. To begin to explore the intracellular mechanisms that might mediate these effects, we elected to characterise the signalling pathways that mediate the proliferative actions of PTHRP in MCF-7 cells. The PTH-R can couple to multiple signalling systems, including Ca²⁺/protein kinase C, cAMP and Ras/ERK pathways (Carpio et al, 2001; Miao et al, 2001). PTH-R did not induce Ca²⁺ accumulation in MCF-7 cells (data not shown) in agreement with a previous report (Birch et al, 1995), making it unlikely that this pathway contributes to the mitogenic actions of PTHRP. We attempted to address directly whether cAMP signals were required for the proliferative actions of PTHRP using H89, a selective inhibitor of cAMP-dependent protein kinase. However, at concentrations required to block PKA (5–10 μM) this agent caused extensive cell detachment and apoptosis within a few hours, thus precluding its use in the mitogenesis assay.
Cells were plated in serum-free medium for 24 h and then stimulated with a medium containing 2% serum for a further 24 h. ([3H]thymidine uptake was measured as described in Materials and Methods. To measure doubling times, cells were plated in quadruplicate at low density in a medium containing 2% serum. Cell numbers were counted every 24 h for a total of 96 h. The average time taken for each cell population to double in number was then calculated from the logarithmic phase of the growth curve. The data are means ± s.d. and are derived from three independent experiments. *A significant difference between MCF-7 WT and MCF-7 WTR cells (P < 0.05, ANOVA and Student’s t-test).

To examine whether the ERK pathway might play a role in the mitogenic actions of PTHRP, MCF-7 WTR cells were stimulated with PTHRP1–34 and, for comparison, EGF and IGF-1, two growth factors known to act as potent mitogens for MCF-7 cells (Pollak et al., 1988; Godden et al., 1992). ERK activation was assessed by immunoblotting with antibodies recognising the dually phosphorylated forms of ERK1 and ERK2, which accurately reflects their activation status (Anderson et al., 1990). As shown in Figure 5A, PTHRP1–34 induced a small increase in the phosphorylation of both ERK1 and ERK2. The size of this increase was negligible compared to that induced by either EGF or IGF-1. However, when PTHRP was administered prior to EGF or IGF-1, there was a potentiation of the resultant ERK phosphorylation (Figure 5A). The degree of potentiation was dependent upon the dose of EGF administered. Use of suboptimal EGF concentrations revealed the potentiation action of PTHRP on EGF-induced ERK activation more clearly (Figure 5B). Conversely, potentiation of EGF-induced activation of ERKs was also dependent on the concentration of PTHRP administered (Figure 5C). These results indicate that PTHRP by itself is a weak activator of the ERK pathway in MCF-7 cells, but that it can boost ERK activation by other growth factors. We finally tested whether the ERK pathway was required for the proliferation of MCF-7 cells. Cells were incubated with PD098059, which blocks the activation of MEK, the protein kinase required for activation of ERKs (Alessi et al., 1995). As shown in Figure 5D, although blockade of the ERK pathway almost completely prevented the increase in [3H]thymidine induced by 2% serum, it had no effect on the ability of PTHRP to increase thymidine uptake. Thus, although PTHRP-induced mitogenesis may occur through an ERK-independent mechanism, the ability of PTHRP to potentiate the activation of ERK by EGF and IGF-1, together with the ability of cells to respond to autocrine PTHRP, could provide one explanation for the increased mitogenic responsiveness and reduced doubling times of MCF-7 cells overexpressing the PTHRP-R.

**DISCUSSION**

PTHRP is now well recognised as an important factor in the development of osteolytic BM from breast cancer. The mechanisms involved remain to be fully resolved but studies by Guise and colleagues provide evidence that production of PTHRP forms part of a vicious cycle involving osteoclast activation followed by the release of mitogenic growth factors such as IGF-1 from the bone matrix (Guise, 2000). In this model, PTHRP exerts its actions by binding to receptors present on osteoblasts, which in response secrete factors leading to osteoclast activation. The presence or absence of the PTHRP-R on the tumour cells themselves is unlikely to exert a major influence in such a process. On the other hand, PTHRP-R expression may give metastatic breast cancer cells a selective advantage that allows them to colonise and expand in secondary sites by permitting autocrine PTHRP signalling. In this study, we demonstrate that PTHRP-R expression is indeed maintained in breast cancer BM. Indeed our data suggest that
PTHRP-R expression occurs more frequently and at a higher level in BM compared to the primary cancer. This suggests that tumour cells overexpressing the PTHRPR have a selective survival or growth advantage in BM. Alternatively, tumour cells may upregulate PTHRPR expression as a result of their exposure to the bone microenvironment. Distinguishing between these possibilities is a goal for future studies.

The presence of the PTHRPR in metastatic breast tumour cells clearly indicated that these cells could respond to PTHRP in a paracrine or autocrine manner. The results of our analyses of MCF-7 breast cancer cells engineered to express the receptor provide evidence that PTHRPR overexpression may be sufficient to give cells a selective advantage in terms of their proliferative capability. We show that this advantage may not only an increased capacity to respond to both exogenous and endogenous PTHRPR, but also to heterologous growth factors.

A previous report (Birch et al, 1995) showed that parental MCF-7 cells responded mitogenically to exogenously applied PTHRPR. The reason why we failed to observe any such effect of PTHRPR in our vector-transfected cells may be because of differences in the culture conditions used. Nevertheless, our work shows that mitogenic responsiveness to exogenous PTHRPR increases when the receptor is overexpressed.

Our results also show that the proliferation of MCF-7 cells overexpressing the PTHRPR is sustained in part by PTHRPR acting in an autocrine manner. The antiproliferative effects of PTHRPR antagonism were greater in cells growing in the presence of serum. Since PTHRPR is not present at detectable levels in normal FCS (CS and NGA, unpublished data), this suggests that the proliferation of MCF-7 cells in serum is supported to a greater extent by autocrine PTHRPR. This may be because growth factors present in serum stimulate the production of endogenous PTHRPR. Interestingly, PTHRPR gene expression is reported to be promoted via the Ras-ERK pathway (Aklilu et al, 2000), and many of the growth factors present in serum are well known to activate this pathway.

Apart from acting through the PTHRPR, recent studies have uncovered an alternative mechanism through which PTHRPR can exert cellular actions. This involves an intracrine pathway through which newly synthesised PTHRPR is directed to the nucleus. The precise role that this novel pathway plays in controlling cellular function is still unclear, but it is interesting to note that the overexpression of PTHRPR in MCF-7 cells results in increased proliferation via an intracrine mechanism (Falzon and Du, 2000). Intracrine PTHRPR signalling required overexpression of PTHRPR indicating that this pathway is unlikely to operate with the endogenous levels of PTHRPR present in our cell system. Nevertheless, it would be interesting to test whether intracrine and autocrine PTHRPR activity coexist in cells with overexpression of both PTHRPR and the receptor.

In addition to conferring increased responsiveness to PTHRPR, our results also show that overexpression of the PTHRPR sensitises cells to the mitogenic actions of heterologous growth factors present in serum. One potential explanation for these results could involve, as suggested above, an increase in the synthesis of PTHRPR in cells cultured in a serum-containing medium. Cells expressing the PTHRPR-R would show increased autocrine responsiveness to PTHRPR. Our results, showing that PTHRPR-R antagonism is more effective in the presence of serum, support this conclusion.

An additional explanation for the apparent increased mitogenic responsiveness of cells expressing the PTHRPR-R derives from the results of our analysis of ERK signalling. We found that PTHRPR indirectly influences signalling through the ERK pathway; while PTHRPR had little effect on its own, it increased the resulting activation of ERK when cells were stimulated by EGF and IGF-1. The mechanisms involved remain to be established, although it is interesting to note that ATP, which also signals via a GPCR, potentiates EGF signalling in MCF-7 cells (Wagstaff et al, 2000). Since the ERK pathway appears critical for optimal proliferation in MCF-7 cells (Hermanto et al, 2000), the potentiation of its activation by PTHRPR may be significant and could provide a further explanation for the enhanced mitogenesis in cells overexpressing the PTHRPR.

Finally, our studies provide some insight into the mechanisms by which PTHRPR induces mitogenesis in MCF-7 cells. Although the ERK pathway is required for optimal proliferation in MCF-7 cells, blockade of its activation using the chemical inhibitor PD098059 failed to affect PTHRPR-induced mitogenesis, ruling out any involvement of this pathway in the direct mitogenic effects of PTHRPR.

As shown in many other systems, the PTHRPR-R couples via Gs to adenyl cyclase and the cAMP signalling pathway (Gardella and Juppner, 2001). Although we were unable to test directly whether this pathway mediates the mitogenic effects of PTHRPR in MCF-7 cells, the fact that forskolin, a direct activator of adenyl cyclase, also promoted mitogenesis indicates that cAMP is mitogenic in these cells. This appears to contradict previous reports suggesting that the cAMP signalling system inhibits proliferation in MCF-7 cells (Chen et al, 1998). We believe that this apparent difference may be explained by the relative strength of the cAMP signal generated. We have shown previously that fibroblasts can respond differentially to low/transient vs high/sustained levels of cAMP (Yarwood et al, 1998). In the present study, low concentrations of forskolin promoted mitogenesis whereas high concentrations were inhibitory (RP and NGA, unpublished data). The antiproliferative effects reported in the study by Chen et al resulted from the introduction of dominantly active version of Gs into the cells, and although they did not report the levels of cAMP generated by this procedure, it is likely that such a manipulation would lead to sustained cAMP signalling. We therefore contend that PTHRPR promotes mitogenesis in MCF-7 cells by generating transient increases in cAMP.

In conclusion, we have shown that the PTHRPR-R is expressed in breast cancer BM, and thus may mediate autocrine PTHRPR signalling in these lesions. Overexpression of the PTHRPR-R in a breast cancer cell line increases the mitogenic responsiveness of these cells not only to PTHRPR but also to heterologous growth factors. PTHRPR can promote mitogenesis in an autocrine manner via receptor-mediated increases in cAMP and by sensitising the ERK signalling pathway to stimulation by other growth factors. Strategies aimed at blocking PTHRPR-R activation may therefore slow the progression of breast cancer cells in BM.

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