The Involvement of Multiple Signaling Pathways in the Actions of Parathyroid Hormone Related Protein (PTHRP) on Osteoblastic Differentiation

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CONTRIBUTION OF AUTHORS

As first author in the manuscript included in this thesis document, I have performed all of the experiments included in the manuscript. All the figures and results are based on experiments that I have performed. Julienne Gladu provided sound advice PTHRP receptor and general G-protein signaling as well excellent technical guidance. Dr. Shafaat A Rabbani and Dr. David Goltzman provided advice throughout the analyses of the experimental data and preparation of this manuscript.

LIST OF ABBREVIATIONS

ATP	Adenosine 5'-trisphosphate
cAMP	Cyclic adenosine 3,5-monophosphate
C-terminal	Carboxyl- terminal
DAG	diacylglycerol
EGF	Epidermal Growth Factor
ERK	Extracellular regulated kinase
FBS	Fetal Bovine Serum
GDP	Guanosine diphosphate
G-protein	Guanine nucleotide-binding protein
GTP	Guanosine trisphosphate
HHM	Humoral hypercalcemia of malignancy
HM	Hypercalcemia of malignancy
IGF-1	Insulin-like growth factor
IL	Interleukin
IP3	Phosphoinositol 1,4,5-trisphosphate
JNK	c-Jun N-terminal kinase
MBP	Myelin Basic Protein
MAP kinase	Mitogen activated protein kinase
N-terminal	Amino terminal
PI-3 kinase	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
RNA	Ribonucleic acid
SH	Src homology
SOS	Son of Sevenless
TGF	Transforming Growth Factor
TNF	Tumour Necrosis factor

ABSTRACT

The study included in this thesis was aimed at elucidating the mechanisms involved in the actions of Parathyroid Hormone Related Protein (PTHRP) on osteoblast cell differentiation. PTHRP is known to mediate the complication of malignancy known as hypercalcemia. In recent years focus on PTHRP has moved away from its roles in hypercalcemia of malignancy and has since been seen to modulate the proliferation and differentiation of a number of cell types.

We have investigated the mechanisms of PTHRP action in the induction of osteoblast cell differentiation, using the osteoblast-derived osteosarcoma cell line, MG-63. Upon treatment of MG-63 cells for 8 hours with 100 nM PTHRP (1-34), maximum induction of markers of osteoblast differentiation was observed by Northern blot and histochemical analysis. Using chemical inhibitors of signal transduction targeted against adenylate cyclase, protein kinase C, PI3 kinase, Ras farnesylation, and MAPK, we have investigated the signaling pathways involved in the induction of these markers of differentiation. Transient transfection of a mutant form of Gα also served to investigate the mechanisms of signal transduction involved in our model of osteoblast differentiation.

Collectively, we have shown that multiple signaling pathways are involved in the induction of markers of osteoblast differentiation by PTHRP, acting via the classical PTH/PTHRP G protein coupled receptor.

RÉSUMÉ

Notre étude vise à élucider les mécanismes d'action du peptide apparenté à l'hormone parathyroïdienne (PTHrP) sur la différenciation cellulaire des ostéoblastes. Bien que la PTHrP fut identifiée comme le facteur causal de l'hypercalcémie humorale observée dans certains cancers malins, plusieurs études récentes se concentrent sur le rôle que la PTHrP semble jouer dans les processus de prolifération et de différenciation cellulaires.

Afin d'étudier les mécanismes d'action de la PTHrP sur l'induction de la différenciation des ostéoblastes, nous avons utilisé une lignée cellulaire ostéoblastique dérivée d'un ostéosarcome, les cellules MG-63. Les résultats obtenus suite à des analyses histochimiques et par technique de « northern » montrent que l'induction maximale des marqueurs de la différenciation ostéoblastique survient après 8 heures de traitement des cellules MG-63 par 100 nM de PTHrP (1-34). L'utilisation d'inhibiteurs chimiques d'enzymes impliquées dans la transduction des signaux cellulaires telles que l'adénylate cyclase, la protéine kinase C, la protéine kinase PI3, ras et les MAP kinases, nous ont ensuite permi d'évaluer la participation des différentes voies de signalisation cellulaire dans l'induction de ces marqueurs de différenciation. De plus, des transfections transitoires d'un mutant de la sous-unité de la protéine Gα nous ont permi d'étudier les mécanismes de transduction des signaux cellulaires dans notre modèle de différenciation ostéoblastique.

En conclusion, notre étude a démontré que de multiples voies de signalisation cellulaire sont collectivement impliquées dans l'induction des marqueurs de la

différenciation ostéoblastique engendrée par la liaison de la PTHrP sur son récepteur couplé à une protéine G hétérotrimérique.

INTRODUCTION

Discovery of PTHRP

It was long known that there were certain cancers that could cause hypercalcemia without the usual associated metastasis to bone (131). In early studies on this condition, it was believed to be parathyroid hormone (PTH) that was responsible for the elevated serum calcium in cancer patients since patients exhibited symptoms which were identical to those exhibited by patients suffering from primary hyperparathyroidism: hypercalcemia, hypophosphatemia, and an increase in urinary cAMP excretion (104). But in the early 1970s, with the advent of radioimmunoassays for PTH in the 1960s, it was discovered and reported that there was clearly a circulating factor in malignancy associated hypercalcemia that was quite immunologically distinct from PTH (88, 90, 93). Further evidence to this effect was provided by other elegant bioassays. It was discovered that tumour extracts from patients with hypercalcemia of malignancy could stimulate adenylate cyclase or glucose-6-phosphate dehydrogenase in PTH-responsive assays, but although PTH receptor antagonists were able to block the responses, anti-PTH antibodies could not (28, 105, 92). This clearly demonstrated that whatever this responsible factor was, it also interacted with the PTH receptor, but was actually immunologically distinct from PTH. Further to these studies, it was demonstrated with the use of nucleotide probes to PTH that there was no mRNA for PTH in tumours associated with hypercalcemia of malignancy (96). It was in 1987 that Moseley et al (79) were able to obtain the gene sequence, whereupon cloning (111) of PTHRP from a human squamous cell

culture was achieved. In fact, almost simultaneously, two other groups obtained identical N-terminal sequences from a breast tumour (107) and from renal carcinoma cells (109).

In little more than ten years, it has become well-established that PTHRP is the primary mediator of hypercalcemia in malignancy, and is in fact a more common tumour product than was originally believed (70, 71). Aside from its pathological roles in bone and kidney in cancer, it has been discovered that PTHRP is a far more widely distributed molecule, and which is in fact localized to normal tissues. This discovery has opened the doors to the elucidation of the roles of PTHRP in normal physiology. Its wide distribution to almost every tissue type (87), particularly in fetal development and embryogenesis, its highly conserved nature across different species, and the lethality of the condition homozygously negative for the PTHRP gene (46) points to a far more diverse and considerably more intricate role than simple mediator of hypercalcemia of malignancy.

Hypercalcemia of Malignancy

A common complication of malignancy is hypercalcemia. It is the extensive manifestations of hypercalcemia, which range from nausea, anorexia, polydipsia, polyuria, mild mental disturbances, and lethargy to eventual coma, which can lead to premature death should the condition remain untreated (80, 81, 82).

There are three general groupings of cancers that are commonly associated with hypercalcemia (80). Firstly, there are metastatic tumours, which cause hypercalcemia by metastasis to bone, whose intrinsic osteolytic activities are mediated by local release of tumour facts and enzymes that cause the dissolution of bone, and thus, release of calcium into the bloodstream. Secondly, there are the haematological cancers, such as the leukemias, adult T-cell leukemia, Burkitt's, Hodgkin's, and non-Hodgkin's lymphomas. It is believed that circulating tumour cells which reach bone mediate an increase in osteolysis via local release of cytokines which resorb bone, such as TNF-α and β, TGF-α and β, and Interleukin-1 (78). Lastly, there are the solid tumours, which are commonly squamous tumours of the lung, esophagous, and skin, renal cortical carcinoma, and tumours of the liver, breast, pancreas, bladder, and prostate. It is in this category of tumours that hypercalcemia is generally caused by PTHRP, which acts on bone and kidney in order to increase bone resorption and calcium retention, respectively. Removal of the tumour has been demonstrated to bring about a resolution of the symptoms and a decrease in circulating PTHRP (29, 56).

While the above classifications do serve as a useful guide in the recognition of the origin of hypercalcemia in cancer patients, it has since been demonstrated that the production of PTHRP by tumours is a far more common occurrence than was originally believed. A hypothesis that has yet to be proven is that PTHRP may actually act as a widely distributed cytokine (78).

Due to PTHRP's sites of action in bone and kidney, and the fact that its effects are mediated through the common, classical PTH/PTHRP receptor, it has often been a matter of some difficulty to distinguish between hypercalcemia of malignancy, and hyperparathyroidism. Biochemically, the main distinguishing aspects for hypercalcemia of malignancy are the presence of either PTHRP or PTH in the circulation and the presence of a tumour in the cancer patient. It is important to note that in hypercalcemia of malignancy, the levels of PTH are generally lowered due to the normal feedback mechanisms in response to high plasma calcium. However, both hypercalcemia of malignancy and hyperparathyroidism are characterized by high plasma calcium, low plasma phosphate, as well as increased phosphorus and cAMP excretion (57, 94). Further differences between hypercalcemia of malignancy and hyperparathyroidism are the presence of hypokalemic alkalosis and low plasma chloride in HM, as opposed to the acidosis commonly seen in hyperparathyroidism, and, a most important uncoupling of osteoclast and osteoblast activity in bone. In hyperparathyroidism there is an increase in *both* bone resorption and bone formation, but in HM, bone resorption is increased, but bone formation is decreased. The mechanism for this phenomenon of loss of equilibrium has yet to be clearly

delineated, but are thought to involve a C-terminal fragment of PTHRP (23, 24), which has been shown to inhibit bone resorption by osteoclasts, as well as the interactions of PTHRP with other tumour or bone derived cytokines. Important to note as well, are the independent actions of many of these tumour and/or bone derived cytokines, as it is likely that the overall presentation of the syndrome of hypercalcemia of malignancy is contributed to by these, along with the cancer itself, the actions of tumour factors which influence PTHRP production, as well as the interactions of PTHRP with locally produced cytokines in PTHRP target tissues.

It is known that EGF, TGFα, TGFβ, TNFα, TNFβ, AND IL-1 can influence bone turnover, and are commonly produced by tumours (78, 80, 81, 82). In fact, TGFβ is a common tumour product that has been implicated in tumour-related hypercalcemia for some time. We also know that PTHRP gene expression can be up regulated by EGF, TGFβ, and IL-1. It is obvious then that the interactions of PTHRP with other factors may well be very complex, and much has yet to be elucidated with respect to this.

PTHRP has been detected in tumours and cultured tumour cells by immunohistochemistry (21, 78), Northern or *in situ* hybridization analyses (64, 78, 111, 130), and in patient serum by radioimmunoassay (11, 47, 57). In a study of immunohistochemical localization of PTHRP in squamous tumours, the antigen was detected in 100% of the samples investigated. The fact that all tumours examined for this study were from normocalcemic patients indicates the enormous potential for PTHRP to induce hypercalcemia with progression of the disease. It is interesting to

note that PTHRP is but rarely detected in the circulation of normocalcemic patients with solid tumours commonly associated with hypercalcemia of malignancy (11, 47, 57). However, PTHRP is readily detected in those patients who are hypercalcemic, thereby suggesting that a reasonably large tumour volume must be attained prior to significant release of PTHRP into the circulation (78).

Both synthetic and recombinant PTHRP species containing the first 34 amino acids promote bone resorption *in vitro* and *in vivo* by acting on PTH-responsive osteoblasts with subsequent activation of osteoclasts (18, 33, 35, 115, 116). One of the characteristic features of hypercalcemia of malignancy is the uncoupling of bone resorption and formation, with resorption being enhanced while one formation is depressed. The contribution of bone resorption to hypercalcemia mediated by PTHRP was illustrated in studies by Rizzoli *et al* (91), who demonstrated that both resorption and hypercalcemia induced by infusion of PTHRP (1-34) into rats could be inhibited by bisphosphonates. This observation is supported by a recent study in cancer patients with PTHRP-producing tumours who were treated with bisphosphonates, which resulted in a fall in their plasma calcium levels to normal values (31).

The renal conservation of calcium is a significant factor in the development of hypercalcemia in patients with hypercalcemia of malignancy. This is apparent in patients treated with bisphosphonates (31). Complete normalization of calcium levels cannot be achieved easily in patients with high circulating PTHRP levels. In one study, the greatest fall in calcium following bisphosphonate treatment was seen in

patients with low or undetectable PTHRP levels (31). Furthermore, urinary clearance analysis in these patients indicated the persistence of increased renal calcium reabsorption. These studies serve to highlight the need for the development of strategies to overcome the renal calcium conservation by PTHRP.

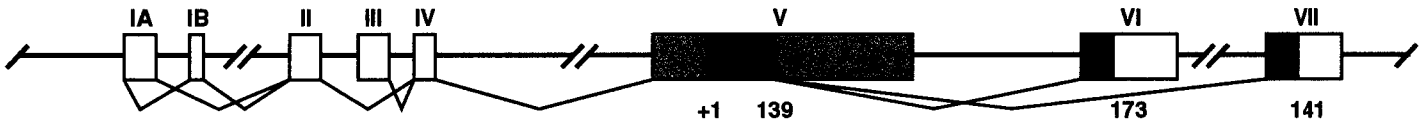
Organization of the Parathyroid Hormone Related Protein Gene

Human PTHRP is encoded on a single gene residing on the short arm of chromosome 12. It is likely that the genes for PTH and PTHRP arose through a duplication event from a common ancestor since, firstly, the two peptides share a great deal of nucleotide homology in the regions encoding amino acids 1 to 13, and secondly, the gene for PTH is found in the identical position to the PTHRP gene, but on the short arm of chromosome 11. It has in fact been demonstrated that the genes for several other closely related proteins are located on these chromosomes, consistent with the theory that chromosomes 11 and 12 arose from a common ancestral gene (20, 68).

While the gene for PTH is a relatively simple structure, the gene encoding PTHRP is a very complex transcriptional unit that predicts the potential to synthesize three isoforms of 139, 141, and 179 amino acids in length (69) (Figure 1). Of these three isoforms, the 141 amino acid peptide is the common isoform, and is in fact the only one encoded in both the rat and the mouse. These three isoforms share identical amino-terminal amino acid sequences, which in turn share strong sequence homology with parathyroid hormone. It is due to this homology that PTHRP is able to reproduce the major effects of PTH, including bone resorption, impaired calcium excretion, increased excretion of phosphate at the renal tubules, enhanced activity of adenylate cyclase in the kidney, and increased excretion of cAMP at the level of the nephrons, via binding with equal affinity to PTH to the PTH receptor (33, 50, 117).

Figure 1: Human PTHRP Gene Structure and Organization of Pre-Pro PTHRP

A: Human PTHRP Gene Structure



B: Organization of Pre-Pro PTHRP

The human PTHRP gene is approximately 15 kb long. There is some contention as to the number of exons the gene contains, but the issue seems to be one of nomenclature as no uniform nomenclature for the exonic organization of the gene has been agreed upon. While some groups have designated the first three exons as exons 1a, 1b, and 1c, others have named the same three exons I, II, and III, yielding nine exons (70, 77, 131). Alternative splicing of these nine exons yields three mature forms of the protein: 139, 141, and 173 amino acids long. Two of the nine exons are present in all PTHRP transcripts: exon V, which codes for the pre-pro region of the immature peptide, and exon VI, which codes for the majority of the mature peptide. Three prime alternative splicing yields the above mentioned three isoforms of PTHRP, 139, 173, and 141 amino acids long, depending on whether exon VI is spliced to exons VII, VIII, or X, respectively (69) (Figure 1A).

The gene is under the control of three spatially distinct promoters, two of which are TATA promoters (63, 64, 111, 115, 130) and another, which is a GC-rich promoter(120). The structural organization of the gene, with its three promoters and the potential for 3' or 5' splicing (63, 66, 67, 115, 130), suggests that the expression of PTHRP may be regulated in a tissue specific or developmental stage specific manner. Each of these promoters appear to be differentially regulated, thereby allowing for alteration of the rate of gene transcription. The PTHRP gene can also be regulated via alteration of the stability of the mRNA transcript, via the AUUUA-rich regions in the 3'-untranslated region of each of the three PTHRP mRNAs (78).

Regulation of the Parathyroid Hormone Related Protein Gene

PTHRP gene expression can be inhibited by glucocorticoids, and has been shown to do so in various cell lines, including human squamous carcinoma cells (26, 48, 49) and rat Leydig tumour cells(59). PTHRP gene expression has also been shown to be regulated by 1, 25-dihydroxyvitamin D_3. Down-regulation of PTHRP expression has been demonstrated in keratinocytes(34, 53) and rat Leydig tumour cells(59), among other cell types. The regulatory capacity of several factors on the expression and secretion of PTHRP by rat Leydig tumour cells, H-500, was examined and documented by Liu et al in 1993. It was found both fetal bovine serum (FBS) and epidermal growth factor (EGF) were stimulatory to PTHRP mRNA expression and secretion in vitro. Also investigated were the roles of Dexamethasone and 1, 25-dihydroxyvitamin D_3, which proved to be inhibitory to PTHRP gene expression and secretion. This is in keeping with the fact that a 47-bp vitamin D responsive element has been located in the rat PTHRP promoter (54). The studies by Liu et al served to indicate that growth factors and steroidal hormones may have stimulatory or inhibitory effects, respectively. The roles of these factors on either the promotion or inhibition of PTHRP production have been investigated in various cell lines, across different species. These studies have suggested, due to some of the cross-species efficacy of some of these factors, that these are important biological regulators of the PTHRP gene.

Functional Domains of Parathyroid Hormone-Related Protein

As previously mentioned, the PTHRP gene can, through alternative splicing, give rise to three different isoforms (101) (Figure 1B). Each human cell that expresses PTHRP has the ability to give rise to all three isoforms, but evidence exists for tissue-specific expression of transcripts, with tissue-specific preference for one or two alternative splicing patterns by individual tumours or tissues (69, 84). Common to all three isoforms is an identical 36 amino-acid putative "pre-pro" sequence, followed by the mature PTHRP species. Each of these isoforms is identical through amino acid 139, but then diverges to encode a unique C terminus (84). The first 13 amino acids exhibit 70% amino acid and nucleotide homology with PTH, but the remaining sequences diverge completely. Removal of the first two amino acids dramatically reduces the ability of PTHRP to stimulate adenylate cyclase (78, 84), indicating that the PTH-like bioactivity of PTHRP requires an intact N-terminus. Amino acids 14 to 34, despite sharing no primary sequence homology with PTH, have been shown to be functionally important in binding to the classical PTH/PTHRP receptor (7, 9, 10, 32, 103, 108), thereby indicating that PTH and PTHRP, despite their primary sequence divergence, share tertiary structural similarity. Due to the extraordinarily highly conserved nature of amino acids 35 to 111 across species, even more strictly conserved than peptides such as insulin and growth hormone, it is believed that this region may have a critical physiological role. After amino acid 112, PTHRP sequences diverge completely among species. Finally, the human version of the peptide, the 173 amino acid isoform, contains a unique carboxy terminal extension,

comprised of amino acids 141-173. The function of this unique carboxy terminus, the gene product of exon V of the PTHRP sequence, has yet to be elucidated. PTHRP post-translational processing at various residues serve to yield PTHRP (1 to 36) (89, 61), a mid-region fragment beginning at amino acid 38 and extending approximately 70 to 80 amino acids (15, 22, 83, 98, 129), a large N-terminal O-glycosylated form (106, 123), and a carboxy-terminal PTHRP species (11, 22, 85).

The amino terminal portion of PTHRP acts through a common PTH/PTHRP receptor in order to elevate plasma calcium via promotion of increased bone resorption and decreased calcium excretion in the kidneys. The classical PTH/PTHRP receptor contains seven membrane-spanning helixes, an intracellular carboxyl terminal tail of approximately 120 amino acids, and an amino-terminal, extracellular domain which is comprised of approximately 180 amino acids, part of which is the signal peptide (40). The receptor is a glycoprotein receptor, of 593 amino acids in length. Despite an overall structure similar to other G-protein coupled receptors, it is now recognized that the PTH/PTHRP receptor belongs to a novel receptor family (41). Both the lack of amino acid sequence homology with other previously isolated receptor proteins and the unique organisation of its gene, which is comprised of 14 coding exons and 3 non-coding exons in the 5' non-coding region of the gene (51, 75). Implicit to any discussion regarding the various proposed roles of the differentially spliced and/or post-translationally modified form of PTHRP, is the concept that each of these mature secretory and circulating forms of the protein has their own specific receptors and signal transduction pathways.

Actions of Parathyroid-Hormone Related Protein

PTHRP has been detected, in most cases at very low levels, by immunohistochemistry, *in situ* hybridization, and Northern blotting in a wide range of normal tissues, including brain, skin, endocrine tissues, kidney, lung, gut, bone and muscle, breast and breast milk (6, 13, 64, 65, 78). Because at this time there has been no convincing demonstration that PTHRP circulates in the normal, non-pregnant adult, it is likely that PTHRP fulfills an autocrine or paracrine function in most normal tissues.

Localization studies have indicated that PTHRP may be important as both a local cytokine and also as an endocrine regulator of calcium metabolism during fetal development. For example, PTHRP has even detected in tetracarcinoma cells with an embryonic stem cell phenotype, in embryonic tissues after implantation, and in many fetal tissues, including, developing epithelia, smooth muscle, skeletal and cardiac muscle, kidney, bone, and endocrine tissues, including parathyroids. Homozygous deletion of the PTHRP gene is lethal, indicating that PTHRP is an important factor essential for normal development, although the only abnormalities were observed in bone.

It has been known for some years now that circulating calcium concentrations in the mammalian fetus are raised relative to maternal levels, calcium being removed from the fetal circulation to provide for developing bones (37). An active calcium pump within the placenta maintains the maternal/fetal calcium gradient, and it has been demonstrated that this gradient is lost when fetal lambs are parathyroidectomized

during the last month of gestation. Plasma levels of immunoreactive PTH in the fetus are low in the fetus relative to the mother, but PTH-like biological activity is higher in the fetus than in the mother. The demonstration that PTHRP can stimulate placental calcium transfer in a placental perfusion model from parathyroidectomized sheep fetus and the detection of PTHRP in fetal parathyroids suggest that PTHRP is the most likely candidate to regulate the placental calcium pump. The placental transport function of PTHRP does not lie within the PTH-like region of PTHRP, and some evidence suggest that the activity may localize somewhere within the portion of the protein between residues 34 to 86 (37, 78).

The source of PTHRP for placental calcium transfer early in gestation may be the placenta itself. In keeping with this hypothesis, PTHRP immunoreactivity and biological activity have been detected in early placentas from human and sheep.

Although evidence is far from definitive, it is possible that PTHRP may be the fetal equivalent of PTH regulating calcium by its actions on placental calcium transport and renal calcium conservation. It is hypothesized that at some time close to parturition, PTH becomes the major calcium-regulating hormone, controlling calcium in the adult by its principal actions on kidney and bone. The possibility that PTHRP is the principal regulator of calcium homeostasis in the fetus leads one to speculate that PTHRP may also have a fundamental role in the control of calcium in species lower on the evolutionary tree (37).

The presence of PTHRP in the lactating breast and in maternal milk suggests one or more roles for PTHRP in lactation. Although there is, to date, substantial evidence to

point to its involvement in the processes of milk production, the nature of its actions are unknown. Breast milk contains a very high concentration of PTHRP (20 to 200nM) (2, 37), and in keeping with its potential role in calcium transport across mammary epithelia, a positive correlation between PTHRP levels in breast milk and calcium concentration has been observed in the cow (58), although this correlation is not apparent in other species. PTHRP may have an endocrine role in the mother to mobilize skeletal calcium for milk production - clinical studies that have demonstrated maternal bone loss and renal calcium retention associated with lactation support this hypothesis. PTHRP is readily detectable in lactating rat breast, and its production is enhanced following the administration of prolactin or by suckling. Several different studies suggest that PTHRP may have a role in breast cell growth and maintenance of the breast in an activated state (78).

It is known that calcium levels modulate the state of growth and differentiation of keratinocytes and epithelial cells, with high calcium levels inducing differentiation. It is therefore possible that PTHRP may have some involvement in the regulation of calcium in the microenvironment. PTHRP stimulates calcium uptake into PTH-responsive cells, and PTH/PTHRP receptors have been demonstrated in a skin cell line, in dermal fibroblasts, in mammary epithelial cells, and in lymphocytes. The differentiation of embryonic stem cells is accompanied by an increase in the expression of both PTHRP mRNA and PTH/PTHRP receptors, suggesting that PTHRP may also act as a paracrine differentiation signal in the early embryo. Further studies will need to include evaluation of the actions of different portions of the

PTHRP molecule and careful definition of the state of differentiation of the cells under investigation in order to elucidate the role of PTHRP in growth and differentiation.

PTHRP can also relax smooth muscle from blood vessels, bladder, and stomach, and PTHRP gene expression increases with stretch of the bladder and uterus. The role of PTHRP in uterine smooth muscle relaxation appears to e linked to functional regulation. Both PTHRP mRNA and protein can be detected in cultures of uterine cells, and in virgin uterus, predominantly in the endometrium, but also in the myometrium. The levels of mRNA expression are modulated by estrogen and the estrus cycle, with estrogen treatment also increasing the sensitivity and amplitude of the response to PTHRP. Presence of the fetus upregulates PTHRP mRNA expression in the uterus and the levels continue to increase during pregnancy, with its highest point in the myometrium, just prior to parturition. It is therefore hypothesized that PTHRP serves to help maintain the uterus in a relaxed state during pregnancy (37, 78).

Role of Parathyroid Hormone-Related Protein in Skeletal Development

The demonstration of PTHRP production by bone cells and fetal bones prompted the question as to the possible novel actions of PTHRP produced locally in bone. Further to this, the heterozygous elimination of the PTHRP gene resulted in extensive abnormalities in endochondral bone development. Animal death occurs just postnatally due to respiratory distress secondary to inappropriate calcification of costochondral cartilage. This lethal form of chondrodysplasia is a short-limbed dwarfism characterised by premature and inappropriate ossification of the developing skeleton. It appears that in the absence of PTHRP, chondrocyte differentiation accelerates, resulting in an overall foreshortened epiphysis due to premature closure of the epiphyseal growth plate. PTHRP is expressed in the perichondrium, while the PTH/PTHRP receptor is expressed in the lower zone of proliferating and maturing prehypertrophic chondrocytes as well as in mature osteoblasts near the growth plate. The current evidence indicates that PTHRP can act as an autocrine or paracrine growth factor in a growing number of tissues. The existence of both PTHRP and the PTH/PTHRP receptor in bone, and the ability of a variety of bone-derived cells to produce PTHRP both in culture and in vivo, strongly suggest a local role in bone. Strong evidence for such a role in the local control of bone development was seen by the effects of targeted disruption of the PTHRP gene in mice. Such observations suggested that PTHRP may modulate the maturation and differentiation of osteoblasts.

Figure 2: Developmental Progression in the Osteoblast Lineage

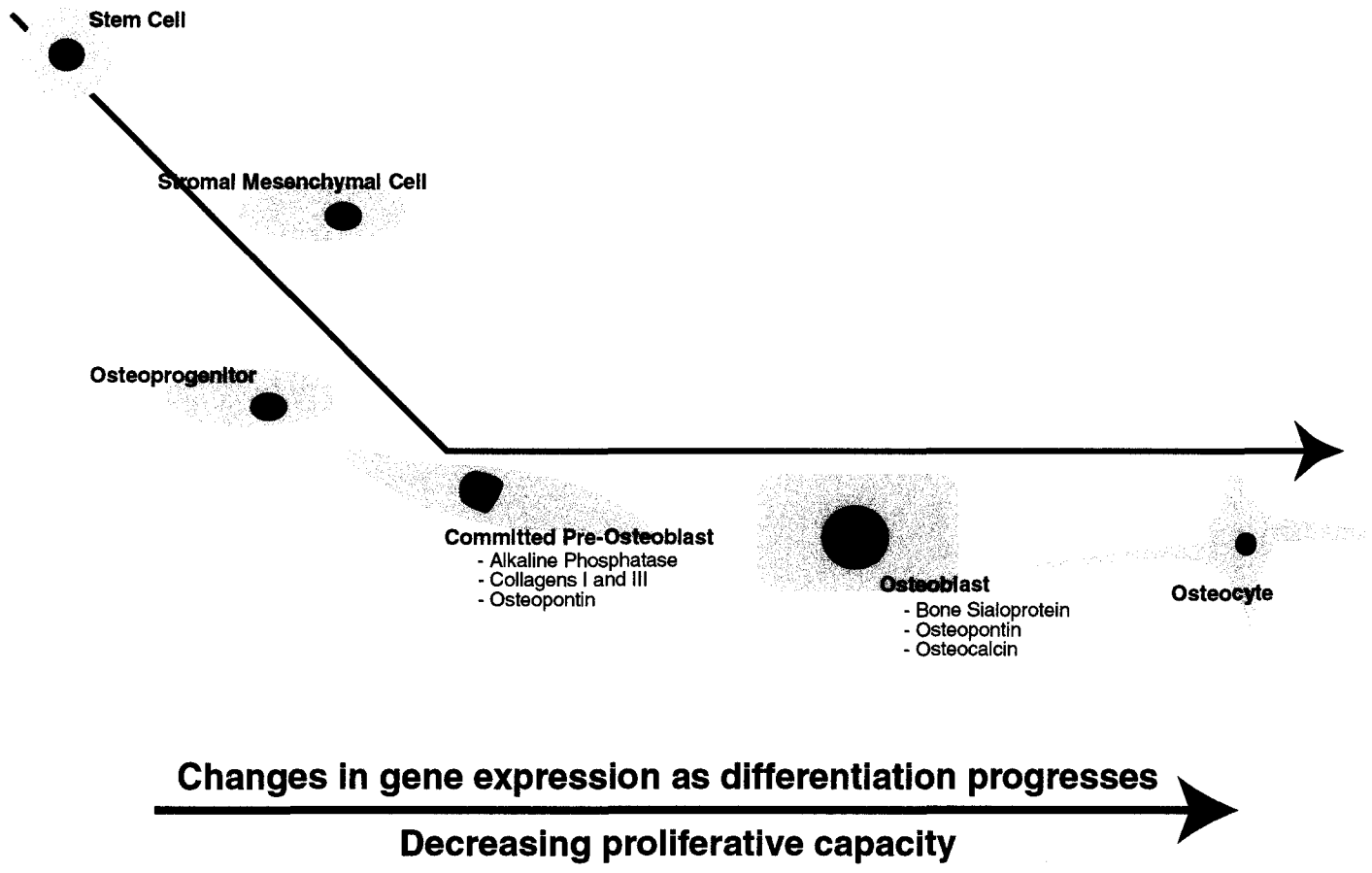


Table 1: Expression of Markers of the Osteoblast Phenotype at Progressive Stages of Differentiation

Marker	Immunocytochemistry			*In Situ* Hybridization		
	Pre-OB	OB	Osteocyte	Pre-OB	OB	Osteocyte
Type I Collagen	-	+	-	-/+	+	-
Alkaline Phosphatase	+	+	-	+	+	-
Osteopontin	+	+	+	-	+	+
Bone Sialoprotein	-	+	-	-	+	+
Osteocalcin	-/+	+	+	-	+	+

Osteoblast differentiation is fundamental to the processes of bone formation and bone maintenance. Multipotential mesenchymal cells have the capacity to differentiate into progenitor cells for mature osteoblasts, chondroblasts, myoblasts, adipocytes, and fibroblasts. Though the course of osteoblast differentiation from mesenchymal stem cell to osteoprogenitor cell is not very clearly defined with respect to discrete markers of differentiation, the course of differentiation from osteoprogenitor to osteocyte is well documented. This course of differentiation is known to be paralleled by a decrease in proliferative capacity, as well as various changes in both the profile and levels of the genes expressed (Figure 2). As differentiation progresses, one of the first proteins to be expressed is Type I collagen, followed shortly by alkaline phosphatase, and as the bone matrix is deposited and organized for mineralization, there follows osteocalcin, osteopontin, and bone sialoprotein. These markers have been detected by both immunocytochemistry and in situ hybridization (Figure 3).

An action of PTHRP on osteoclasts has been localized to the short peptide at positions 107 to 111 in the PTHRP molecule. This peptide has been shown to be a potent inhibitor of osteoclastic activity *in vitro.* Preparations of highly purified isolated osteoclasts exhibit significant bone resorbing activity on slices of devitalized cortical bone. This bone resorbing activity was completely abolished by the addition of PTHRP (107-111), at doses as low as 10^{-14} M (78). Although PTHRP (107-111) is a potent inhibitor of isolated osteoclasts, it has not been shown to inhibit bone resorption in assays using fetal long bones or mouse calvaria, and the physiological significance of this action of PTHRP *in vivo*, is currently in doubt. Recent evidence

using long-term cultures of isolated rat osteoclasts suggests that a most significant action of the peptide may be to inhibit osteoclast recruitment (18).

The Classical Parathyroid Hormone / Parathyroid Hormone-Related Protein Receptor

The amino terminal portion of PTHRP acts through a common PTH/PTHRP receptor in order to elevate plasma calcium via promotion of increased bone resorption and decreased calcium excretion in the kidneys. The classical PTH/PTHRP receptor contains seven membrane-spanning helixes, an intracellular carboxyl terminal tail of approximately 120 amino acids, and an amino-terminal, extracellular domain which is comprised of approximately 180 amino acids, part of which is the signal peptide (40). The receptor is a glycoprotein receptor, of 593 amino acids in length. Despite an overall structure similar to other G-protein coupled receptors, it is now recognized that the PTH/PTHRP receptor belongs to a novel receptor family (41). Both the lack of amino acid sequence homology with other previously isolated receptor proteins and the unique organisation of its gene, which is comprised of 14 coding exons and 3 non-coding exons in the 5' non-coding region of the gene (51, 75). Implicit to any discussion regarding the various proposed roles of the differentially spliced and/or post-translationally modified form of PTHRP, is the concept that each of these mature secretory and circulating forms of the protein has their own specific receptors and signal transduction pathways.

Classically, binding of PTHRP to the PTH/PTHRP receptor, is equal in affinity to binding of PTH, and is known to signal through at least two different pathways, either through activation of the adenylate cyclase messenger system, or through the phospholipase C pathway (1, 55). It has been well established that the determinants

of the camp/PKA signaling in both the PTH and PTHRP ligands reside within the amino-terminal residues (27, 35, 36); however the ligand determinants of PLC/PKC activation have been somewhat more difficult to define. Recent studies have shown the non-adenylyl-cyclase-mediated pathways appear to be more complex than the adenylate cyclase/PKA pathway, and may in fact involve multiple phospholipase isoforms, such as PLD and PLC. The phospholipase-mediated pathways appear to be sensitive to variations in cell type, PTH/PTHRP receptor density, and receptor species derivations (25, 112, 113).

The PTH/PTHRP receptor was originally cloned from the classical PTH target tissues of opossum kidney, rat bone, and human bone and kidney cells. The PTH/PTHRP receptor has been isolated different mammalian species, and its cDNA encodes homologous proteins that range in length from 585 to 593 amino acids. The receptor houses an extracellular amino-terminus, a seven transmembrane spanning region, and an intracellular carboxy terminal tail. While its general structure is similar to that of other G-protein coupled receptors, the PTH/PTHRP receptor has limited sequence homology with these, as well as a unique gene organization. As such, it has been classified to a new family of membrane proteins, which includes the calcitonin and secretin receptors.

Classically, the PTH/PTHRP receptor was reported to associate with the adenylyl cyclase and PLC-β second messenger systems, leading to increases in cAMP, inositol trisphosphate, and free calcium. In addition to these classical second messenger systems involving adenylyl cyclase and phospholipase C, G protein coupled receptors

have also been discovered to interact with mitogen activated protein (MAP) kinases. The activated PTH/PTHRP receptor has been shown to interact with the MAP kinase pathway. PTH/PTHRP receptor interaction with this pathway appears to be cell type specific. Verhiejen et al have shown growth factor mediated of MAP kinase activity in osteosarcoma cells, as well as activation of MAP kinases when expressed in Chinese hamster ovary and parietal yolk sac carcinoma cells.

Conclusions

The discovery of PTHRP was originally sparked by clinical features resembling hyperparathyroidism displayed by some cancer patients in whom circulating PTH was not detectable. Subsequently, PTHRP has been shown to be produced by a far broader spectrum of cancers than was appreciated on the basis of clinical studies. It is now becoming clear that PTHRP has a significant role in the etiology of hypercalcemia associated with many types of malignancy.

PTHRP has also been shown to be responsible for a number of biological actions relevant to normal physiology. Evidence indicates major roles for PTHRP in smooth muscle relaxation, fetal calcium regulation, and milk production during lactation. As a potential cytokine produced by many normal as well as malignant cells, the actions of PTHRP may be far-reaching, and an understanding of the nature of its role on cell growth and differentiation will prove to be of chief importance.

INDUCTION OF OSTEOBLAST DIFFERENTIATION INDEXES BY PARATHYROID HORMONE RELATED PEPTIDE (PTHRP) IN MG-63 CELLS INVOLVES MULTIPLE SIGNALING PATHWAYS

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ABSTRACT

Parathyroid hormone related peptide (PTHRP) can modulate the proliferation and differentiation of a number of cell types including osteoblasts. PTHRP can activate a G protein coupled PTH/PTHRP receptor, which can interface with several second messenger systems. In the current study, we have examined the signaling pathways involved in stimulated type I collagen and alkaline phosphatase expression, in the human osteoblast-derived osteosarcoma cells, MG-63. Using Northern blotting and histochemical analysis, maximum induction of these two markers of osteoblastic differentiation occurred after 8 hours of treatment with 100 nM PTHRP (1-34). Chemical inhibitors of adenylate cyclase (H-89) or protein kinase C (chelerythrine chloride), each diminished PTHRP mediated type I collagen and alkaline phosphatase stimulation in a dose dependent manner.

These effects of PTHRP could also be blocked by inhibiting the Ras-MAPK pathway with a Ras farnesylation inhibitor, B-1086, or with a MAPK inhibitor, PD-98059. Transient transfection of MG-63 cells with a mutant form of $G\alpha$, which can sequester $\beta\gamma$ subunits, showed significant down regulation of PTHRP-stimulated type I collagen expression, as did inhibition of phosphoinositol-3-kinase (PI-3-kinase) by Wortmannin. Consequently, the $\beta\gamma$-PI-3-Kinase pathway may be involved in PTHRP stimulation of Ras. Collectively, these results demonstrate that, acting via its G-protein coupled receptor, PTHRP can induce indices of osteoblastic differentiation utilizing multiple, perhaps parallel, signaling pathways.

INTRODUCTION

The search for the responsible pathogenetic factor in the development of hypercalcemia of malignancy culminated in the discovery of Parathyroid Hormone Related Protein (PTHRP) little more than a decade ago (11, 79, 109). Since then, PTHRP has been observed to be expressed by a wide variety of normal adult and fetal tissues (30, 99). Due to its wide tissue distribution, and the degree to which it is conserved across evolution, it was proposed that PTHRP may well have a significant developmental role. It was subsequently discovered that PTHRP plays a role in the proliferation and differentiation of a variety of cell types, including chondrocytes, osteoblasts (45), and keratinocytes (43, 44). Despite the evidence that PTHRP plays an important role in cellular turnover and maturation, little is currently known of the molecular mechanisms involved in PTHRP-mediated cellular differentiation. Due to the N-terminal sequence homology between PTH and PTHRP, these two peptides can interact with a common receptor PTH/PTHRP receptor (1). The presence of this receptor in bone is well established, and high receptor levels are seen in osteoblasts that are actively differentiating (74) suggesting a role for the receptor in osteoblast development. Active mineralization of bone matrix involves the production of type I collagen and alkaline phosphatase, which among others, are established markers of osteoblast differentiation (5).

The common PTH/PTHRP receptor is a member of the family of seven transmembrane receptors that are coupled to heterotrimeric G-proteins. PTH and PTHRP are known to activate several second messenger pathways which are linked

by distinct mediators to the PTH/PTHRP receptor (86). For example, ligand binding is known to stimulate both intracellular cAMP and inositol trisphosphate through Gαs and Gαq, respectively (1, 42). G protein coupled receptors are also known to activate mitogen-activated protein kinase (MAPK) activity in a manner that is dependent upon the profile of the involved G protein, the receptor to which it is coupled, and the cell type in which they are found (110). Previous studies have reported differential activation of PKA or PKC pathways and this may be cell type specific, and vary according to the differentiation stage and exposure time to the ligand. Activation of MAPK has been shown to be essential in the differentiation of several cell types, and it has been shown that PKA and/or PKC activation can influence MAPK activity. G protein activation will also involve the release of a βγ dimer subunit (8, 60), and this subunit may regulate the phosphorylation of the protein Shc, which can then lead to the formation of a protein complex involving Shc-Grb2-SOS, and the subsequent activation of the proto-oncogene Ras (19). Ras activation is known to result in activation of Raf, and then in activation of the enzymes MEK and MAPK (62). Receptors coupled to trimeric G-proteins may therefore activate one or more of these possible pathways. While it has been definitively established that PTH/PTHRP receptor signaling may occur via $G\alpha_s$ and/or $G\alpha_q$, the question of βγ mediated signaling by this receptor is not yet as clearly proven.

The present study was undertaken to further examine the possible role of multiple signal transduction pathways in the stimulation of osteoblastic differentiation by PTHRP. Through the use of chemical inhibitors of signal transduction and transient

transfection of βγ-sequestering mutant form of $G\alpha_s$, and employing a human osteoblast-like osteosarcoma model, MG-63, we show the importance of activation of protein kinase A, protein kinase C, as well as Ras by PTHRP in inducing osteoblastic differentiation. Our results also demonstrate the involvement of MAPK, which may be a point of convergence of these activated signaling pathways in this system. These results therefore emphasize the involvement of multiple pathways in PTHRP induced indices of osteoblastic differentiation.

MATERIALS AND METHODS

Cell Culture

MG-63 osteosarcoma cells were maintained *in vitro* in MEM (with Earle's Salts) supplemented with 10% FBS, 100 units/ml of penicillin-streptomycin sulphate (BRL/GIBCO). For transient transfection assays, cells were plated at $1x10^5$ cells per 60-mm dish 24 hours before transfection and growth in 5% CO_2 in MEM (ES). Cells were incubated with lipofectin, 10 μg/ml (BRL/GIBCO) and cultured overnight in serum free MEM (ES) culture media with 0.1, 1, or 10 μg of plasmid DNA. After overnight incubation with lipofectin, fresh culture media containing 10% FBS was added. PTHRP treatment assays were performed within 48 hours post transfection.

PD98059 (Biomol), Wortmannin (Sigma Canada) and B1086 (Eisai Research Institute, Andover MA), H-89 (Biomol), and Chelerythrine Chloride (Biomol) were dissolved in dimethyl sulfoxide and stored at appropriate stock concentrations and diluted to the desired concentrations immediately prior to use.

The plasmid encoding the G alpha triple mutant was obtained from the laboratory of Dr. H.R. Bourne and has been previously described (38).

Northern Blot Analysis

Total cellular RNA was extracted by Trizol extraction from control and experimental cells following treatment with vehicle alone, PTHRP (1-34) alone, or graded concentrations of chemical inhibitors. 10 μg of total cellular RNA was electrophoresed on a 1.1% agarose-formaldehyde gel, transferred to a nylon membrane (Nytran, s&S,

Keene, NH) by capillary blotting, and then fixed by drying and UV cross-linking for 10 minutes. The integrity of the RNA was assessed by ethidium bromide staining. Hybridization was carried out with a ^{32}P-labelled type I collagen cDNA and with an 18S RNA probe using a ^{32}P dCTP as previously described (Thomas 1980). After a 24 hour incubation at 65°C, filters were washed twice under low stringency conditions (1xSSC and 1% SDS; at 60°C for 2x20 minutes) and under high stringency conditions (0.1xSSC, 0.1% SDS; at 60°C for 2x20 minutes). Autoradiography of filters was carried out at -70°C using XAR film (Eastman Kodak Co., Rochester, NY). The levels of type I collagen expression were quantified by densitometric scanning using the MAC BAS V1.01 alias program.

Alkaline Phosphatase Detection

Alkaline phosphatase activity in control and PTHRP treated MG-63 cells was detected by a histochemical reaction. Cells were fixed in citrate buffered acetone. Slides were then immersed in alkaline-dye solution containing diazonium salts and incubated for 30 minutes. Cells were stained with Mayer's hematoxylin solution for 10 minutes in order to detect the insoluble pigments formed as a result of alkaline phosphate activity. Slides were then evaluated as integrated densities of staining using Scion Image, where total staining intensity was measured (17).

Immune Complex Protein Kinase Assay

Cells were washed twice with ice-cold PBS. Ice-cold RIPA lysis buffer was added to cell monolayers and incubated on ice for 10 minutes. Cells were scraped and transferred to eppendorf tubes and further disrupted by vortex. Cellular debris was pelletted and

supernatant retained. Upon Bio-Rad Protein Assay quantitation of total protein levels, 200-500 μg total protein was co-incubated with 0.2-2 μg Erk-1 antibody (Santa Cruz Biotechnology, Inc.) for 1 hour at 4°C. 25 μl resuspended volume of Protein-G agarose (Santa Cruz Biotechnology, Inc.) was added and incubated at 4°C, rotating for 2-16 hours. Immunoprecipitates were collected and rinsed with RIPA buffer.

Pellets were resuspended in 30 μl of appropriate kinase assay buffer containing 10-1000 ng peptide substrate MBP and (γ^{32}P)-ATP (10mCi/ml) and incubated at 30°C for 30 minutes. Kinase reaction was terminated by addition of equal volume of 2x electrophoresis sample buffer and boiling for 2-3 minutes. Samples were analyzed by SDS-PAGE and autoradiography.

Statistical Analysis

All data are shown as mean values ± SEM. Statistical analysis of results was by Student's T test or by analysis of variance. Significant values were taken at $p<0.05$. The mean, SEM and P values were performed using Excel software (Microsoft Corporation, Port Redmond, WA).

RESULTS

Effect of PTHRP on Indices of Osteoblastic Differentiation

MG-63 osteoblast-like osteosarcoma cells, which exhibit characteristics of early, immature osteoblasts, were incubated in the absence or presence of 100 nM PTHRP (1-34) for 0, 1,3, 6, 8, 12, and 16 hours. As shown in Figure 3A, treatment with PTHRP (1-34) increased type I collagen mRNA transcript levels within 6 hours and peak levels were reached after 8 hours of treatment. Type I collagen levels were augmented greater than two-fold at this time point, as compared to control, untreated cells. Type I collagen levels decreased to basal by approximately 16 hours. A second marker of osteoblast differentiation, alkaline phosphatase, was detected by histochemical reaction. After treatment of cells with 100 nM PTHRP (1-34) for 6, 12 and 24 hours, cells were fixed and alkaline phosphatase activity was detected by a histochemical reaction. As seen in Figure 3B and 3C, PTHRP induced alkaline phosphatase activity, with highest levels occurring at 24 hours. While staining density increased slightly in untreated cells, the overall increase in staining in treated cells was considerably greater, reaching levels as high as 7-fold after 12 hours of treatment. These results indicate that PTHRP can induce indices of differentiation in MG-63 osteoblastic cells.

Effects of Inhibiting Protein Kinase C (PKC) on Indices of Osteoblastic Differentiation

In order to determine the involvement of PKC in PTHRP mediated MG-63 cell differentiation, cells were pretreated with chelerythrine chloride (4) (12), a specific inhibitor of PKC, followed by co-incubation with 100 nM PTHRP (1-34) for 8 hours, the time of maximal induction of type I collagen transcript levels by PTHRP treatment.

As seen in Figure 4, inhibition of PKC by chelerythrine chloride treatment resulted in a decrease in PTHRP-stimulated type I collagen levels in a dose dependent manner. Cell morphology (Figure 5), viability as determined by trypan blue dye exclusion (>95% viable), and basal levels of type I collagen mRNA were unaffected by treatment of MG-63 cells with chelerythrine chloride alone. These results were paralleled by a reduction in alkaline phosphatase levels. Pretreatment with 5.0 μM chelerythrine chloride, followed by co-incubation with 100 nM PTHRP (1-34) for 24 hours resulted in alkaline phosphatase levels which were comparable to levels in untreated cells cultured for the same time period (Figure 5). The ability of this inhibitor to curtail the effects of PTHRP on MG-63 cell differentiation suggests that PKC activation is involved in this phenomenon.

Effects of Inhibiting Protein Kinase A (PKA) on Indices of Osteoblastic Differentiation

In a number of cell types, PTHRP is known to be a strong activator of adenylate cyclase via its stimulation of Gαs. The cAMP generated then leads to activation of PKA. In order to determine whether activation of PKA is involved in PTHRP induced MG-63 cell differentiation, cells were pretreated with the PKA inhibitor H-89. After incubation in serum-free media for approximately 16 hours, cells were pretreated with H-89 at 15 and 30 μM concentrations for 1 hr, followed by co-incubation with 100 nM PTHRP (1-34). Inhibition of PKA by H-89 treatment resulted in a dose dependent decrease in PTHRP-stimulated type I collagen mRNA levels (Figure 6). The specificity of this response was confirmed by the treatment of MG-63 cells with H-89 alone, which showed little to no effect on basal type I collagen mRNA levels. Cell viability by

Trypan blue dye exclusion (> 94% viable) and morphology (Figure 5) were unaffected by H-89 at the doses indicated. These results were paralleled by a reduction in alkaline phosphatase levels. Pretreatment with 30 μM H-89 for 1 hr, followed by co-incubation with 100 nM PTHRP (1-34) for 24 hours resulted in levels of alkaline phosphatase which were comparable to those in untreated cells (Figure 5). The ability of this inhibitor to curtail the effects of PTHRP on MG-63 cell differentiation suggests that PKA activation is involved in this phenomenon.

Effects of a Gαs Mutant on Indices of Osteoblastic Differentiation

In order to confirm results obtained regarding PTHRP signaling via Gαs in PTHRP mediated osteoblast differentiation, MG-63 cells were transiently transfected with a Gαs mutant. The Gαs mutant used in our studies is designed to stabilize a receptor-Gαs-βγ complex, effectively blocking signaling from both αs and βγ. Following transient transfection, MG-63 cells were treated with 100 nM PTHRP (1-34) for 8 hours. As seen in Figure 7, in cells transfected with the Gαs mutant, PTHRP stimulation of type I collagen mRNA levels was significantly reduced, confirming the involvement of Gαs in PTHRP-stimulated osteoblastic differentiation and suggesting the possible involvement of βγ subunits.

Effects of Inhibiting Phosphatidyl Inositol-3-Kinase (PI3 Kinase) on Indices of Osteoblastic Differentiation

In order to explore signal transduction components related to the βγ complex which might be implicated in PTHRP induced osteoblastic differentiation, we examined the

involvement of PI3 kinase by using the chemical inhibitor Wortmannin. In preliminary experiments, Wortmannin was confirmed to have no effects on cellular viability by trypan blue dye exclusion (> 97%) or morphology (Figure 5) at the doses indicated. Cells were pre-treated with Wortmannin for 3 hours, followed by co-incubation with 100 nM PTHRP (1-34). Type I collagen mRNA levels were minimally affected in cells treated with Wortmannin alone. The ability of Wortmannin to cause a dose dependent decrease in PTHRP-stimulated type I collagen levels (Figure 8) suggests that PI3 kinase is a component of the PTHRP induced signaling involved in MG-63 cell differentiation. This was confirmed by the ability of Wortmannin to abrogate the PTHRP mediated increase in alkaline phosphatase activity after 24 hours of co-incubation (Figure 5). Alkaline phosphatase activity in the Wortmannin treated cells is comparable to basal, untreated cells (Figure 5). These results, taken together, suggest the involvement of PI3 kinase in PTHRP mediated osteoblast differentiation.

Effects of Ras Inhibition on Indices of Osteoblastic Differentiation

Ras proteins are a major point of convergence of numerous signal transduction pathways. Ras is required to be anchored to the plasma membrane in order to function and must undergo the post-translational addition of a farnesyl group which facilitates Ras insertion into the plasma membrane (95, 119). We therefore examined the effects of B1086 which is an inhibitor of the enzyme farnesyl transferase and which therefore inhibits Ras activity. In preliminary experiments, B1086 was confirmed to have no effects of cellular viability as assessed by trypan blue dye exclusion (> 97%) or morphology (Figure 5) at the doses indicated. Overnight pre-treatment of MG-63 cells

with B1086 was followed by 8 hours of co-incubation with 100 nM PTHRP (1-34). Total RNA was collected and subjected to Northern blot analysis. Type I collagen mRNA levels were significantly decreased in B1086 treated cells (Figure 9), while type I collagen mRNA levels were little affected in cells treated with B1086 alone. Histochemical examination of alkaline phosphatase activity following identical treatment conditions also revealed a significant decrease in this parameter in B1086 treated cells (Figure 5).

Effects of Mitogen-Activated Protein Kinase (MAPK) Inhibition in Indices of Osteoblastic Differentiation

The MAPK family of serine/ threonine kinases includes extracellular signal regulated kinases (ERKs). Activation of the ERK group of MAP kinases may occur via Ras and may involve stimulation of the enzyme MEK, or MAPK kinase. MEK activates ERK MAPK directly, and thus serves as a point of control in that selective inhibition by the chemical inhibitor of MEK, PD-98059 (3, 73), and results in inhibition of MAPK. In order to determine the involvement of MAPK in the PTHRP mediated induction of type I collagen expression, MG-63 cells were incubated overnight with PD-98059. PD98059 was confirmed to have no effects of cellular viability by trypan blue dye exclusion (> 97%) or morphology (Figure 5) at the doses indicated. Following pretreatment, cells were co-incubated with 100 nM PTHRP for 8 hours, type I collagen levels were determined by Northern blot analysis. A dose dependent decrease (Figure 10) in PTHRP-stimulated type I collagen gene expression was observed, implicating MAPK in PTHRP induced MG-63 differentiation. Cells treated with PD98059 alone showed

negligible effects on type I collagen mRNA levels, confirming the specificity of the response to PTHRP induced increase of type I collagen mRNA. These results were confirmed by measurement of alkaline phosphatase activity in PD-98059 treated MG-63 cells (Figure 5). Direct measurement of the effects of PTHRP on MAPK activity in MG-63 cells was also performed. PTHRP (1-34) at 100 nM concentration induced an increase in MAPK activity as measured by *in vitro* kinase activity assay, as seen in Figure 11. Peak levels of MBP phosphorylation by MAPK were observed at 15 minutes, with activity remaining significantly elevated at 20 and 25 minutes. By 30 minutes, levels of MBP phosphorylation had returned to basal, untreated levels. We next investigated effects of the various inhibitors of upstream signaling on the ability of PTHRP to induce peak MAPK activity after 15 minutes of treatment (Figure 12). All five inhibitors inhibited the ability of PTHRP to induce MAPK activity, thereby confirming the role of MAPK as a downstream target of these signaling pathways.

Figure 3. Effect of PTHRP on MG-63 cell Differentiation.

Human osteoblast-like osteosarcoma cells MG-63 were grown in 10% serum to 80% confluence and then incubated in serum free conditions. Cells were then treated with vehicle or with 100 nM PTHRP (1-34) for timed intervals.

A Total cellular RNA was extracted from MG-63 cells. 15 μg of total cellular RNA for each time point (1–16 hr) was electrophoresed on a 1.1% agarose/formaldehyde gel. Filters were probed with type αI collagen and 18s cDNA in order to determine the ratio of αI collagen/18s mRNA expression.

B Alkaline phosphatase activity in control and PTHRP treated MG-63 cells was detected by a histochemical reaction. Cells were fixed in citrate buffered acetone. Slides were then immersed in alkaline-dye solution containing diazonium salts and incubated for 30 minutes. Cells were stained with Mayer's hematoxylin solution for 10 minutes in order to detect the insoluble pigments formed as a result of alkaline phosphate activity. Slides were then evaluated microscopically.

C Alkaline Phosphatase activity was quantified using the NIH Image based Scion Image Analysis program. Quantification is presented as Staining Density per Field. Results represent the mean ± SEM of three different experiments. Significant differences from control are represented by an asterisk * ($p<0.05$).

Figure 3A: Effect of PTHRP on MG-63 cell Differentiation

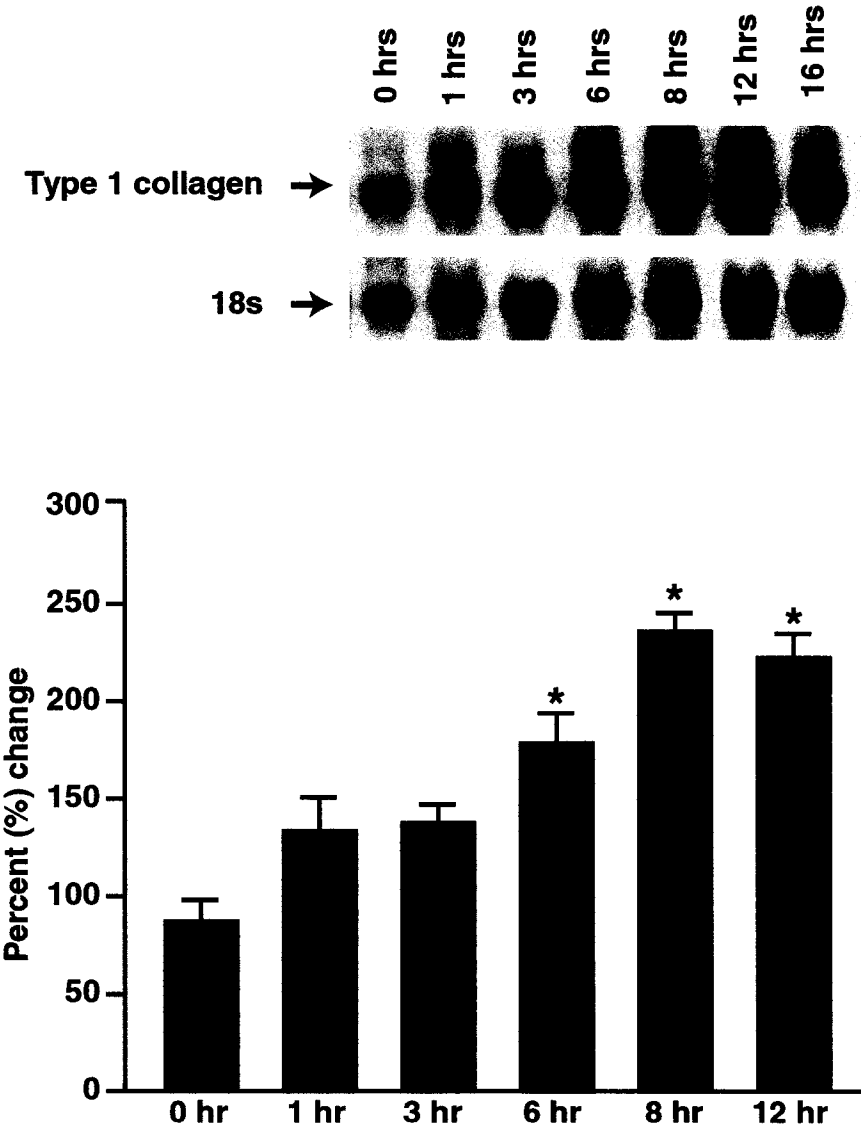


Figure 3B: Effect of PTHRP on MG-63 cell Differentiation

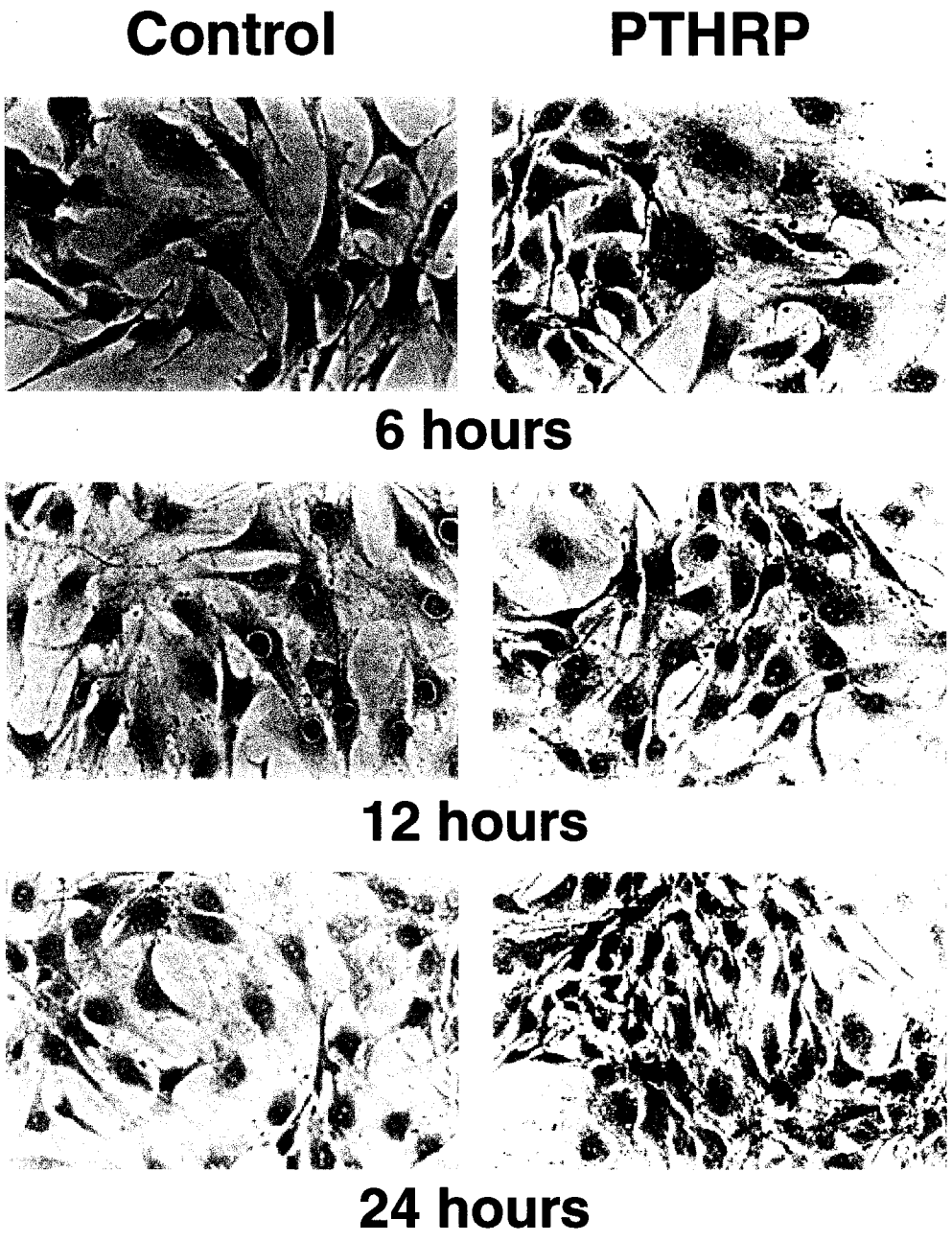


Figure 3C: Effect of PTHRP on MG-63 cell Differentiation

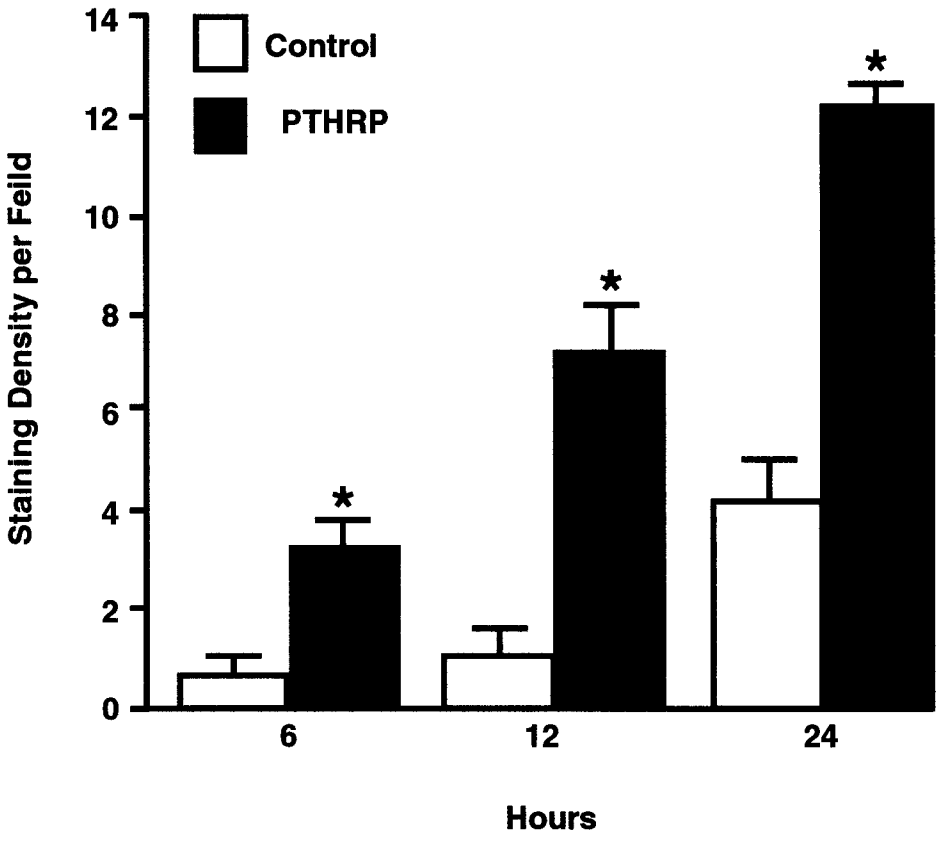


Figure 4. Effect of the PKC Inhibitor, chelerythrine chloride, on type I collagen gene expression in MG-63 cells.

MG-63 cells were grown in 10% serum to 80% confluence and then incubated overnight in serum free conditions (Ctl). Cells were then pretreated with vehicle or Chelerythrine Chloride for 1 hr. Cells were then treated with 100 nM PTHRP (1-34) for 8 hrs. Ctl represents results of treatment with vehicle only for both the pretreatment and treatment periods. Levels of type I collagen and ratios of type I collagen to 18s mRNA were determined by Northern blot analysis as described in "Materials and Methods". Results in the lower panel depict the ratios of type I collagen to 18s mRNA and are expressed as a % change relative to Ctl, which was assigned a value of 100%. Each bar represent the mean ± SEM of three different experiments. Significant differences in the ratios from Ctl cells are represented by a single asterisk * ($p<0.05$). Significant differences in the ratios from PTHRP-only treated cells are represented by two asterisks ** ($p<0.05$).

Figure 4: Effect of the PKC inhibitor, chelerythrine chloride, on type I collagen gene expression in MG-63 cells

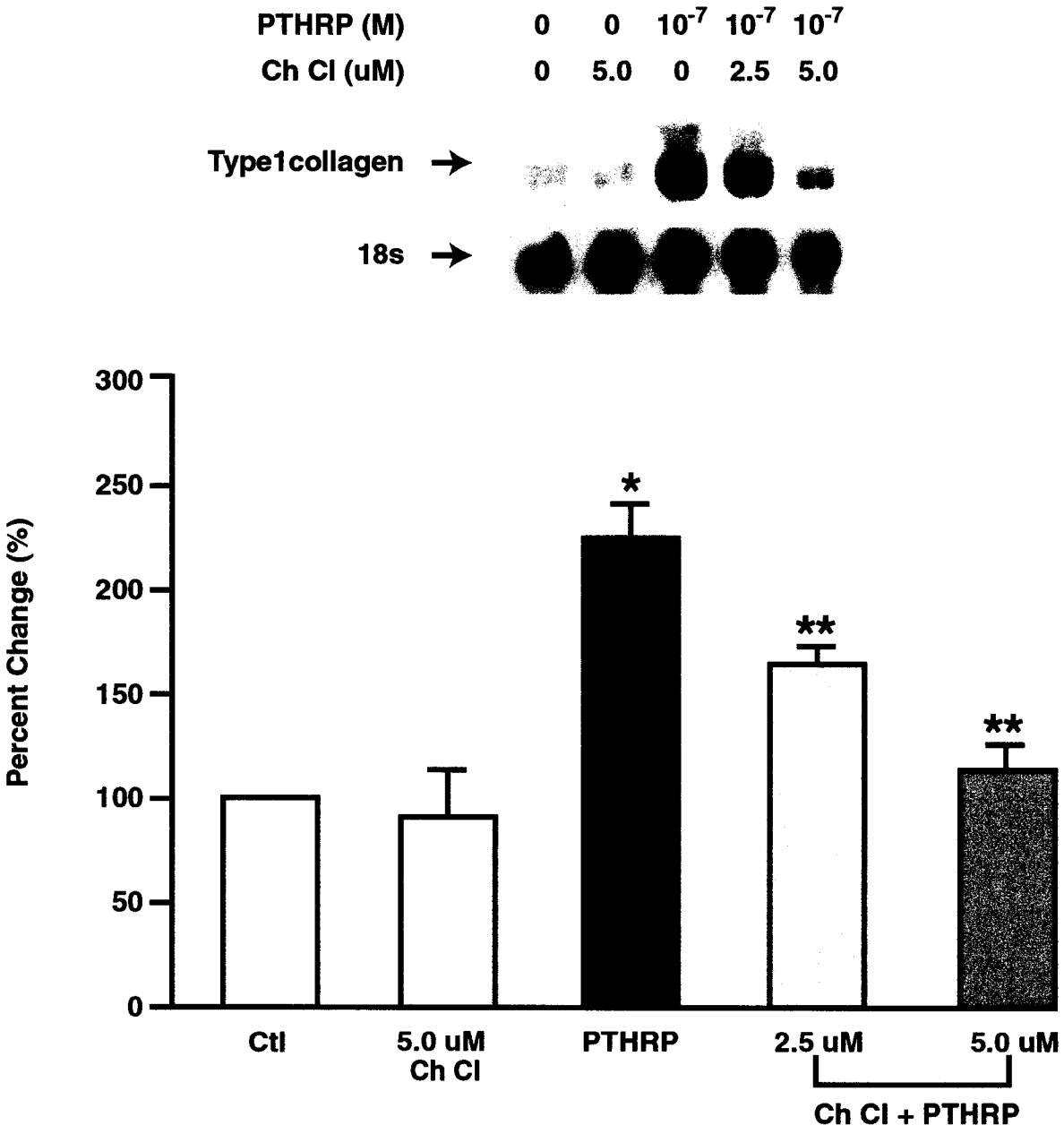
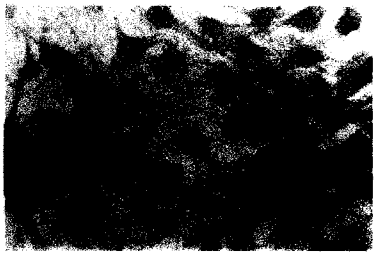


Figure 5. Effect of inhibitors of signal transduction on alkaline phosphatase levels.

A MG-63 cells were grown in 10% serum to 80% confluence on double chamber slides and then incubated overnight in serum free conditions. Cells were pretreated with vehicle or PKA inhibitor, H89 (30 μM) and PKC inhibitor, Chelerythrine Chloride (5.0 μM) for 1 hr, PI3K inhibitor, Wortmannin (100 nM) for 3 hrs, and Ras inhibitor, B1086 (5.0 μM) and MAPK inhibitor, PD98059 (100 μM) overnight. The cells were then treated with 100 nM PTHRP for 24 hrs.

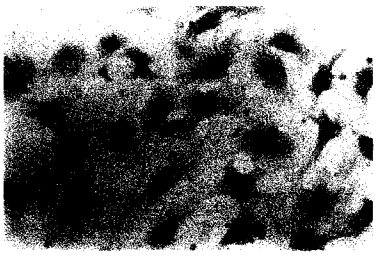
B Alkaline Phosphatase activity was quantified using the NIH Image based Scion Image Analysis program. Quantification is presented as Staining Density per Field. Significant differences in the ratios from Ctl cells are represented by a single asterisk * ($p<0.05$). Significant differences in the ratios from PTHRP-only treated cells are represented by two asterisks ** ($p<0.05$).

Figure 5A: Effect of inhibitors of signal transduction on alkaline phosphatase levels



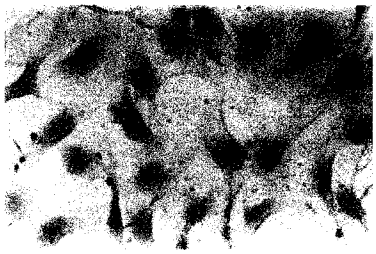
PTHRP

Control

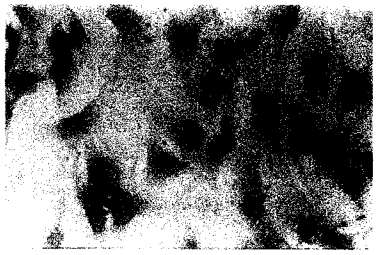


30 uM H89

5.0 uM Chelerythrine Chloride



100 nM Wortmannin



10 uM B1086

100 uM PD98059

Figure 5B: Effect of inhibitors of signal transduction on alkaline phosphatase levels

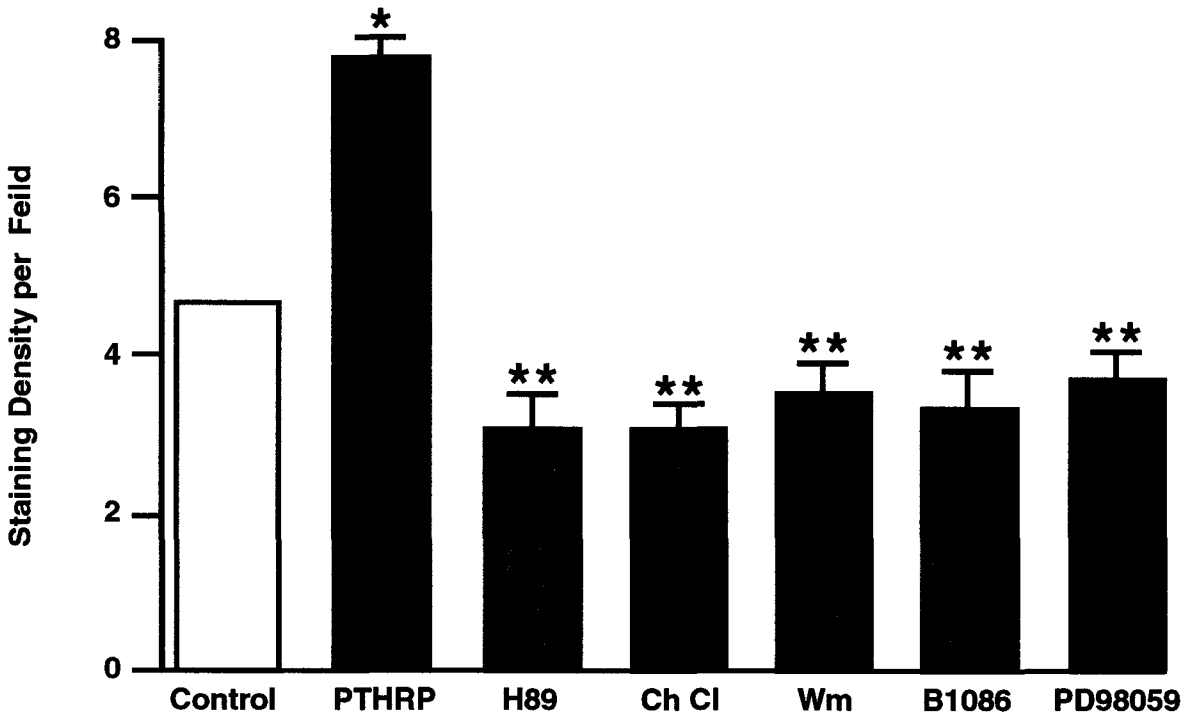
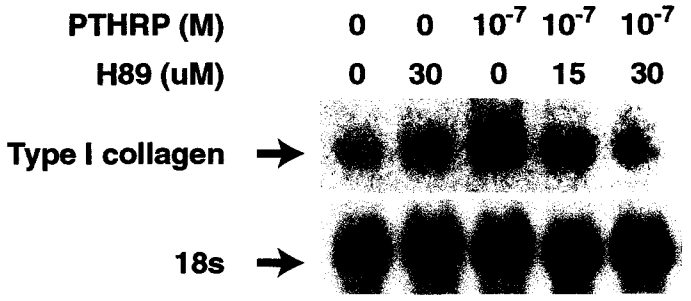


Figure 6. Effect of the PKA inhibitor, H89, on type I collagen gene expression in MG-63 cells.

MG-63 cells were grown in 10% serum to 80% confluence and incubated overnight in serum free conditions. Cells were then pretreated with vehicle or H89 for 1 hr and then incubated with 100 nM PTHRP (1-34) for 8 hrs. Ctl represents results of treatment with vehicle only for both the pretreatment and treatment periods. Levels of type I collagen and ratios of type I collagen to 18s mRNA were determined by Northern blot analysis as described in "Materials and Methods". Results in the lower panel depict the ratios of type I collagen to 18s mRNA and are expressed as a % change relative to Ctl, which was assigned a value of 100%. Each bar represent the mean ± SEM of three different experiments. Significant differences in the ratios from Ctl cells are represented by a single asterisk * ($p<0.05$). Significant differences in the ratios from PTHRP-only treated cells are represented by two asterisks ** ($p<0.05$).

Figure 6: Effect of the PKA inhibitor, H89, on type I collagen gene expression in MG-63 cells



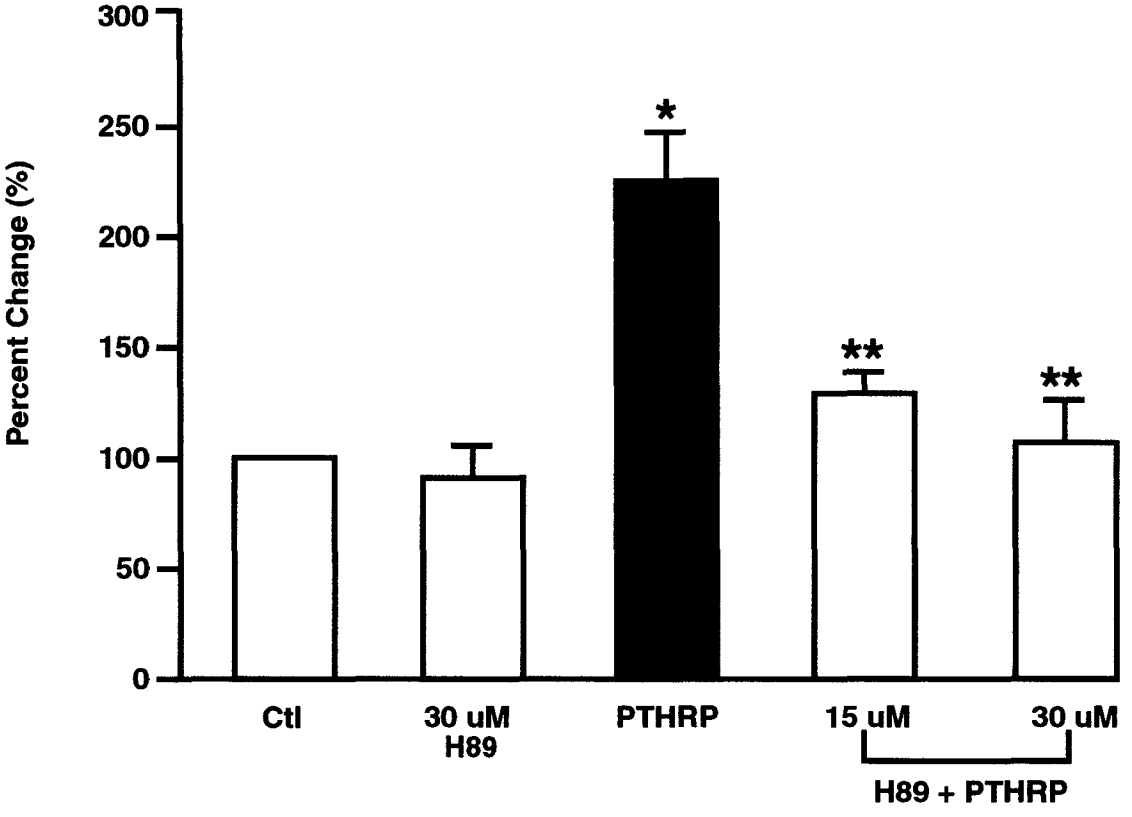


Figure 7. Effect of expressing a dominant negative Gαs mutant on PTHRP induced type I collagen gene expression in MG-63 cells.

MG-63 cells were grown in 10% serum to semi-confluence. Cells were then cultured in serum free medium or transiently transfected for 12 hrs with the empty vector (pcDNA1) or with vector containing GαTM. Cells were then cultured for 24 hrs in serum free medium. Untransfected and transfected cells were then treated with 100 nM PTHRP(1-34) or vehicle for 8 hrs. After stimulation with 100 nM PTHRP (1-34), total cellular RNA was extracted from untreated control and experimental cells. 20 μg total cellular RNA was electrophoresed on a 1.1% agarose formaldehyde gel. After transfer of RNA, filters were probed with a (32P])type I collagen cDNA or with a (32P)-labeled 18 S RNA probe as described in "Materials and Methods". Results in the lower panel depict the ratios of type I collagen to 18s mRNA and are expressed as a % change relative to Ctl, which was assigned a value of 100%. Each bar represent the mean ± SEM of three different experiments. Significant differences in ratios from vehicle-treated cells (Ctl) are represented by a single asterisk * ($p<0.05$). Significant differences in ratios from PTHRP treated cells are represented by two asterisks ** ($p<0.05$).

Figure 7: Effect of expressing a dominant negative Gαs mutant on PTHRP induced type I collagen gene expression in MG-63 cells

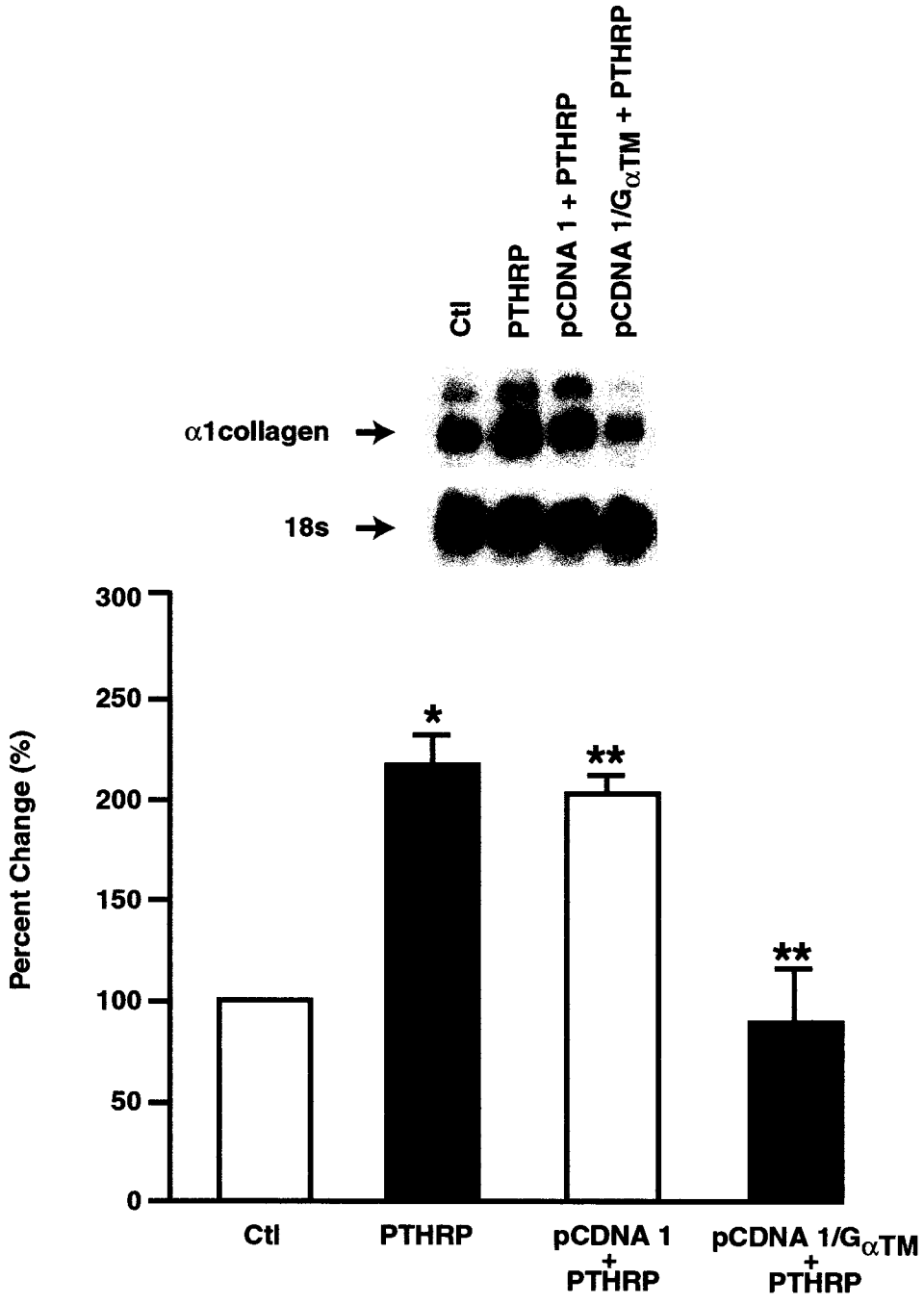


Figure 8. Effect of the PI3 kinase inhibitor, Wortmannin, on type I collagen gene expression in MG-63 cells.

MG-63 cells were grown in 10% serum to 80% confluence and incubated overnight in serum free conditions. Cells were then pretreated with vehicle or Wortmannin (50 nM, 100 nM) for 3 hrs. Cells were then treated with 100 nM PTHRP (1-34) for 8 hrs. Ctl represents results of treatment with vehicle only for both the pretreatment and treatment periods. Levels of type I collagen and ratios of type I collagen to 18s mRNA were determined by Northern blot analysis as described in "Materials and Methods". Results in the lower panel depict the ratios of type I collagen to 18s mRNA and are expressed as a % change relative to Ctl, which was assigned a value of 100%. Each bar represents the mean ± SEM of three different experiments. Significant differences in the ratios from Ctl cells are represented by a single asterisk * ($p<0.05$). Significant differences in the ratios from PTHRP-only treated cells are represented by two asterisks ** ($p<0.05$).

Figure 8: Effect of the PI3 Kinase inhibitor, Wortmannin, on type I collagen gene expression in MG-63 cells

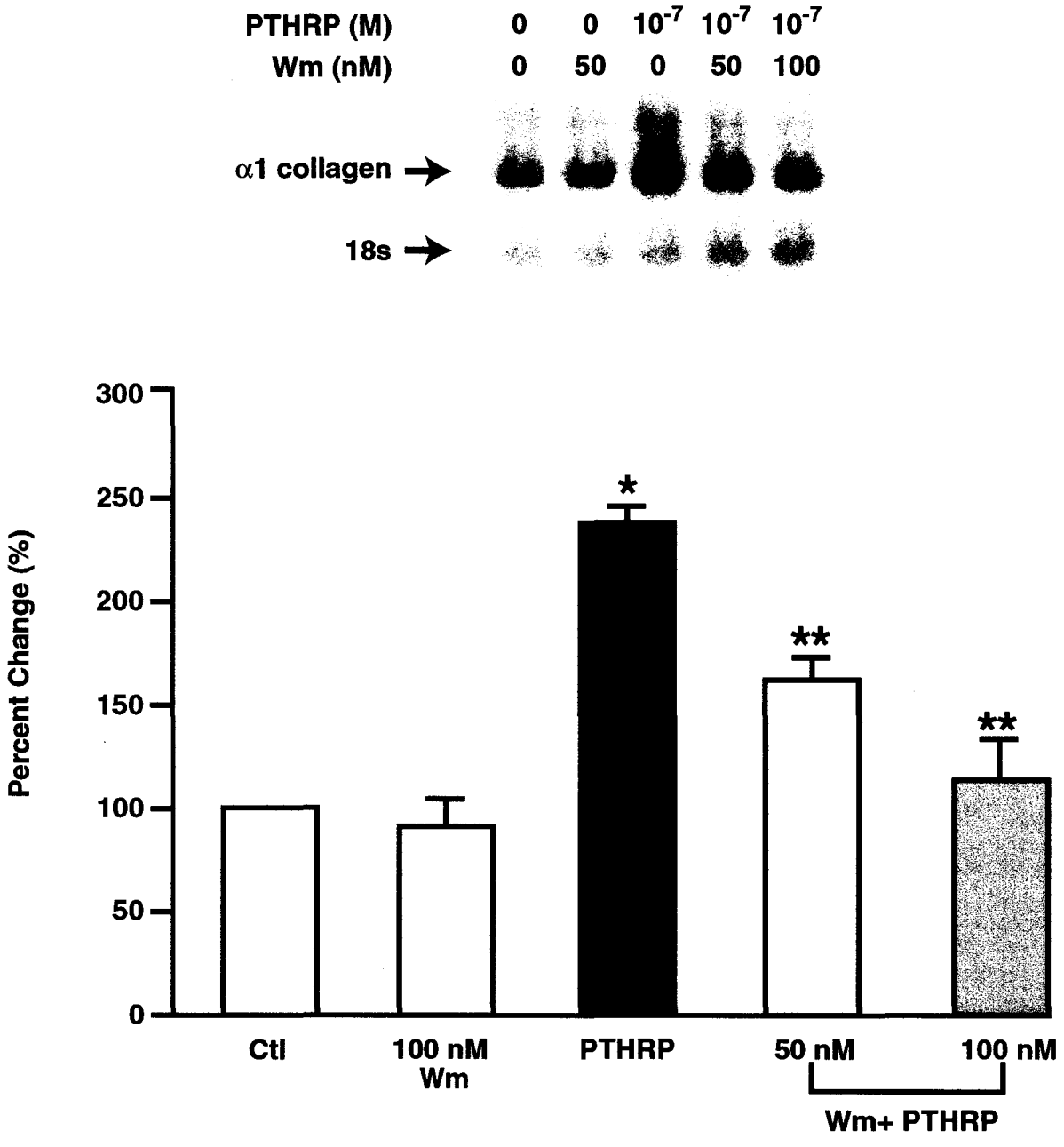
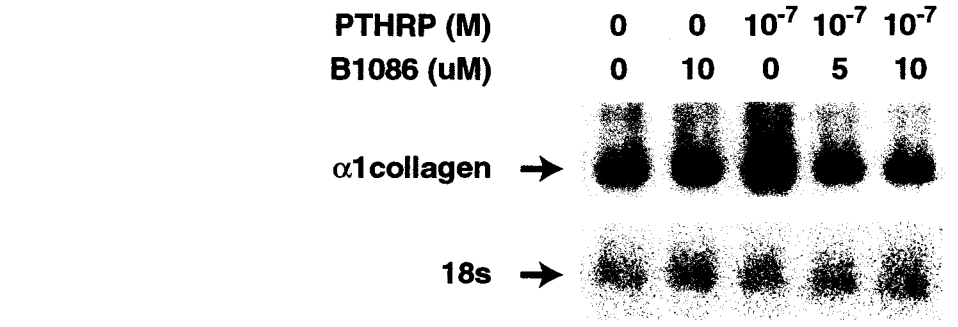


Figure 9. Effect of the Ras inhibitor, B1086, on type I collagen gene expression MG-63 cells.

MG-63 cells were grown in 10% serum to 80% confluence and then incubated overnight in serum free conditions. Cells were pretreated with vehicle or Ras inhibitor B1086 (5 μM, 10 μM) for 16 hrs. The cells were then treated with 100 nM PTHRP for 8 hrs. Ctl represents results of treatment with vehicle only for both the pretreatment and treatment periods. Levels of type I collagen and ratios of type I collagen to 18s mRNA were determined by Northern blot analysis as described in "Materials and Methods". Results in the lower panel depict the ratios of type I collagen to 18s mRNA and are expressed as a % change relative to Ctl, which was assigned a value of 100%. Each bar represent the mean ± SEM of three different experiments. Significant differences in the ratios from Ctl cells are represented by a single asterisk * ($p<0.05$). Significant differences in the ratios from PTHRP-only treated cells are represented by two asterisks ** ($p<0.05$).

Figure 9: Effect of the Ras inhibitor, B1086, on type I collagen gene expression in MG-63 cells



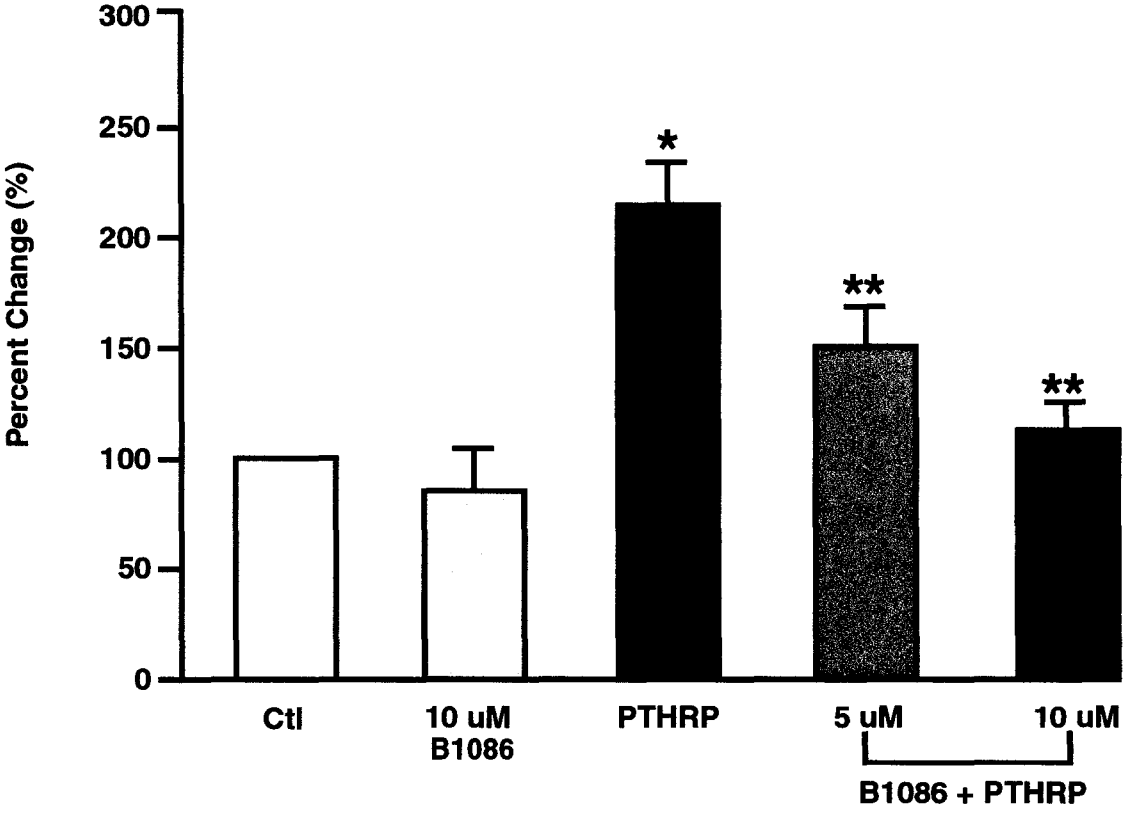


Figure 10. Effect of the MEK inhibitor, PD98059, on type I collagen gene expression in MG-63 cells.

MG-63 cells were grown in 10% serum to 80% confluence and then incubated overnight in serum free conditions. Cells were pretreated with vehicle or PD98059 (50 μM, 100 μM) for 16 hrs, followed by PTHRP treatment for 8 hrs. Ctl represents results of treatment with vehicle only for both the pretreatment and treatment periods. Levels of type I collagen and ratios of type I collagen to 18s mRNA were determined by Northern blot analysis as described in "Materials and Methods". Results in the lower panel depict the ratios of type I collagen to 18s mRNA and are expressed as a % change relative to Ctl, which was assigned a value of 100%. Each bar represent the mean ± SEM of three different experiments. Significant differences in the ratios from Ctl cells are represented by a single asterisk * ($p<0.05$). Significant differences in the ratios from PTHRP-only treated cells are represented by two asterisks ** ($p<0.05$).

Figure 10: Effect of the MEK inhibitor, PD98059, on type I collagen gene expression in MG-63 cells

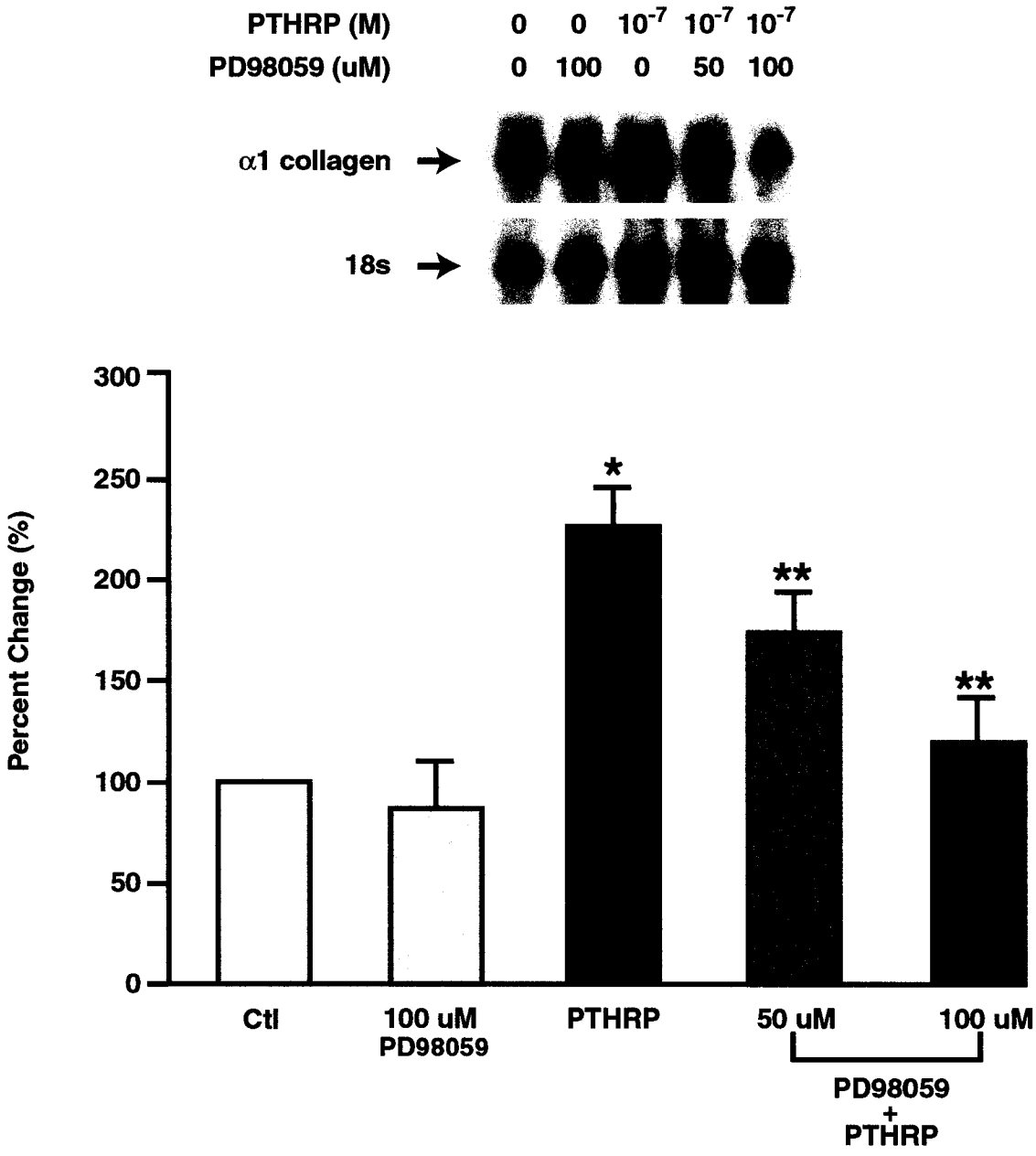


Figure 11. Effect of the PTHRP (1-34) on MAPK Activity in MG-63 cells.

MG-63 cells were grown in 10% serum to 80% confluence and then incubated overnight in serum free conditions. Cells were then treated with 100 nM PTHRP (1-34) for timed intervals. After stimulation with PTHRP (1-34), total cell lysates were collected from untreated control and from experimental cells. 200-500 μg total cellular protein was immunoprecipitated with ERK1 antibody. Immunoprecipitates were incubated in reconstituted kinase reaction buffer containing MBP and (32P)-ATP as described in "Materials and Methods". Results represent the mean ± SEM of three different experiments. Significant differences from time 0 are represented by a single asterisk * ($p<0.05$).

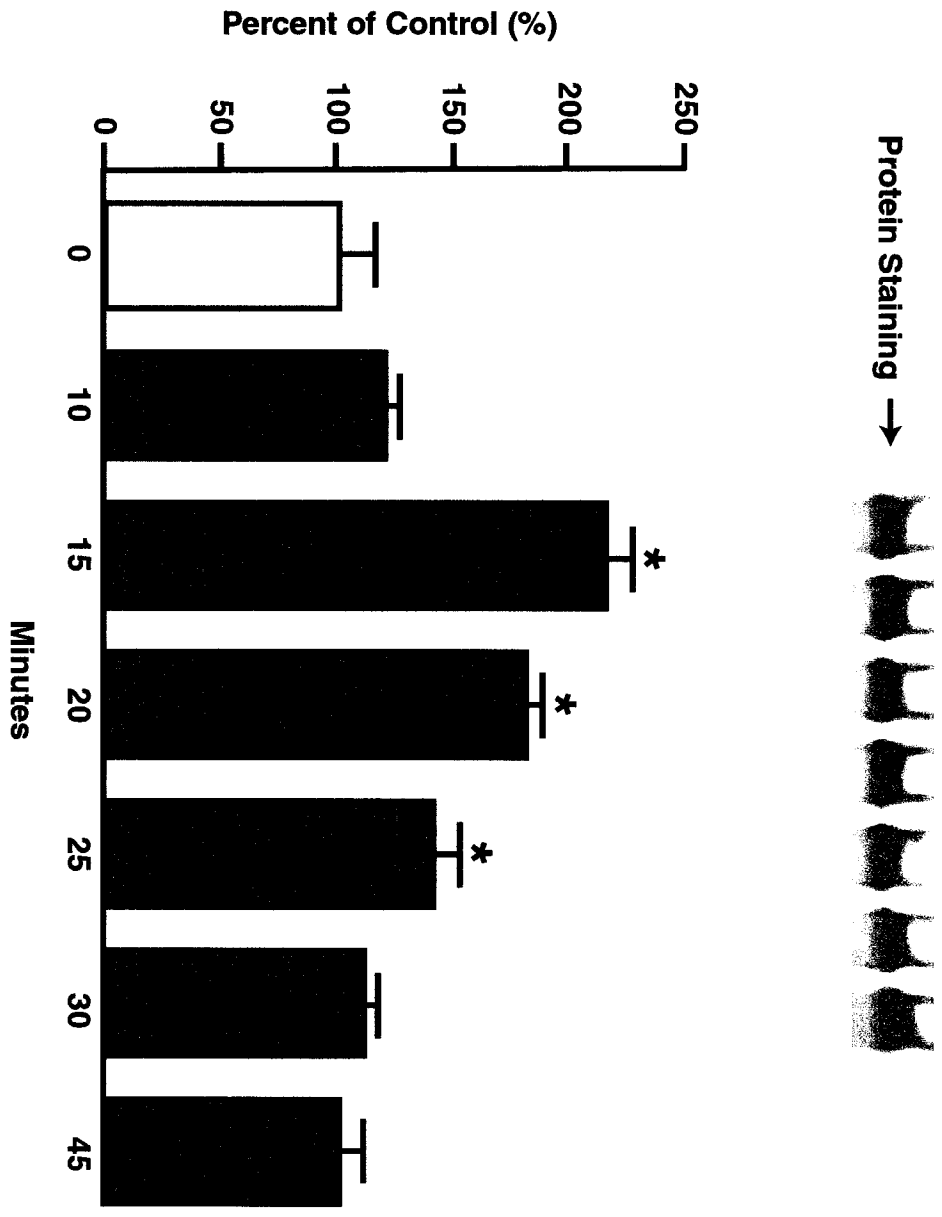


Figure 11: Effect of PTHRP (1-34) on MAPK Activity in MG-63 cells

Figure 12. Effect of inhibitors of signal transduction on MAPK activity levels.

MG-63 cells were grown in 10% serum to 80% confluence and then incubated overnight in serum free conditions. Cells pretreated with vehicle or PKA inhibitor, H89 (30 μM) and PKC inhibitor, Chelerythrine Chloride (5.0 μM) for 1 hr, PI3K inhibitor, Wortmannin (100 nM) for 3 hrs, and Ras inhibitor, B1086 (5.0 μM), and MAPK inhibitor, PD98059 (100 μM), overnight. The cells were then treated with 100 nM PTHRP for 15 minutes. MAPK activity was then determined as described in "Materials and Methods". Results are expressed as a % of vehicle-only treated cells (Ctl) and represent the mean ± SEM of three different experiments. Significant differences from control are represented by a single asterisk ($p<0.05$) and significant differences from PTHRP-only treated cells are represented by two asterisks ($p<0.05$).

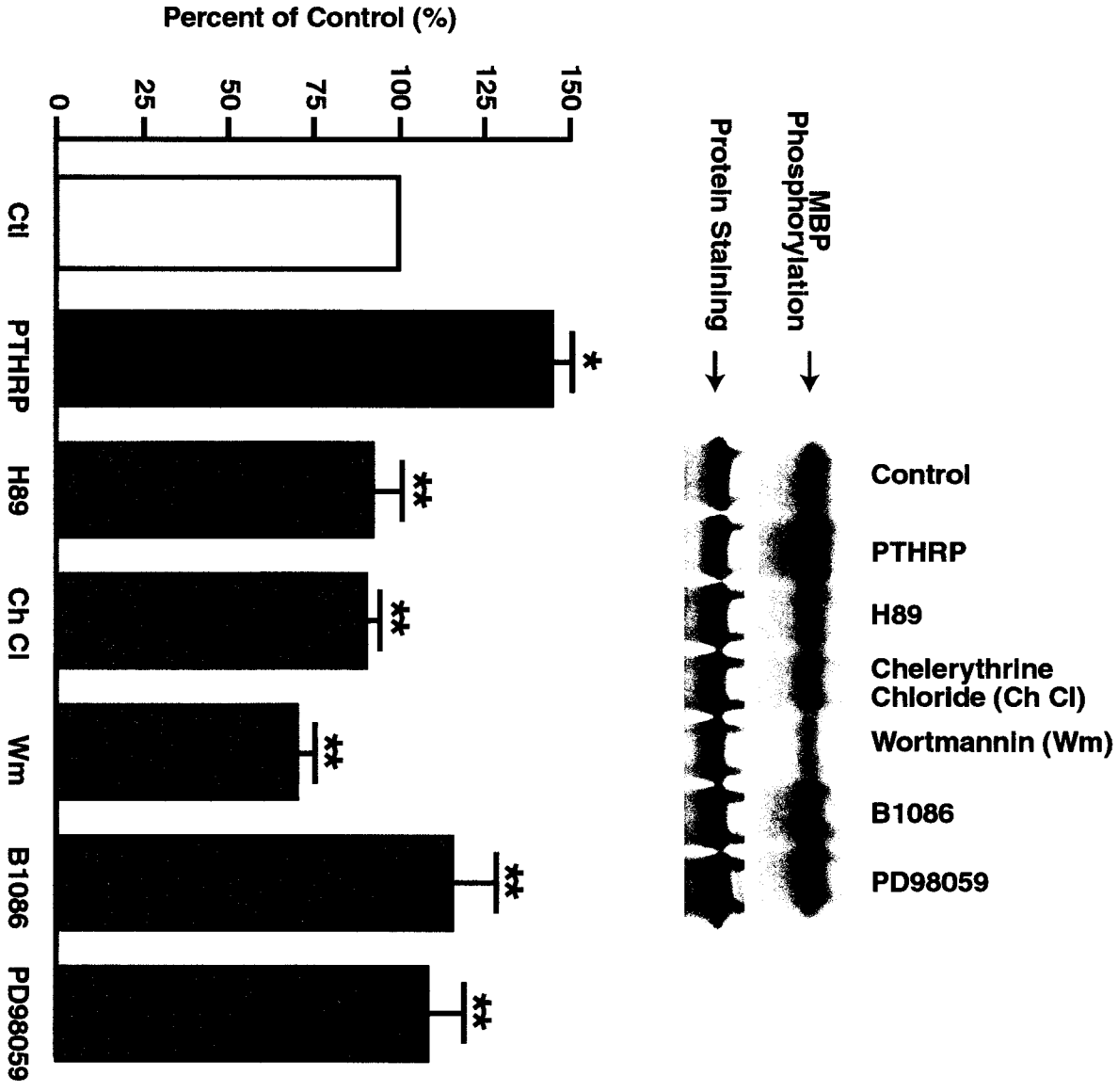


Figure 12: Effect of inhibitors of signal transduction on MAPK Activity in MG-63 cells

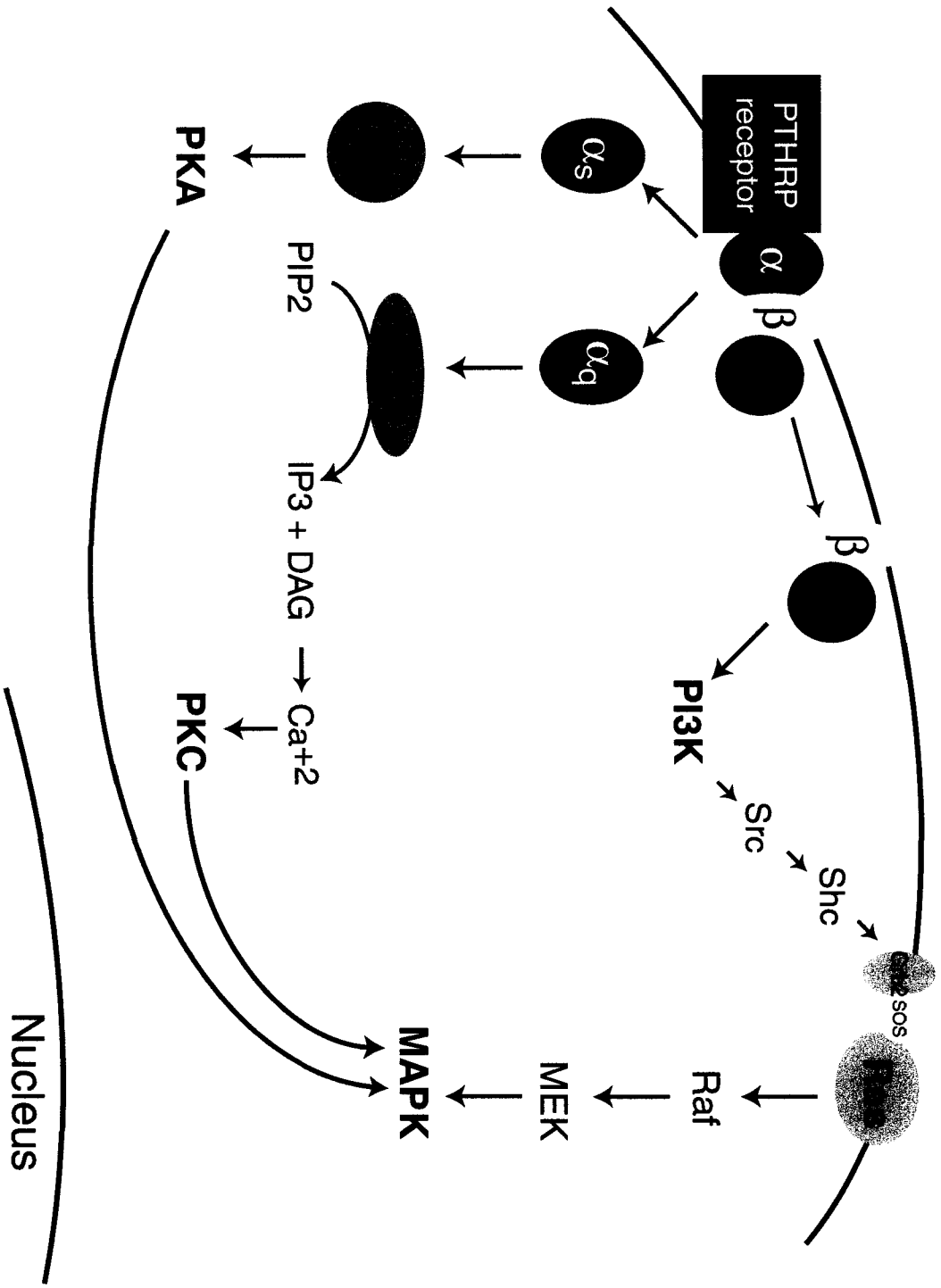


Figure 13: A schematic diagram of PTHRP receptor signaling involved in regulating differentiation in MG-63 osteoblastic cells

DISCUSSION

Previous studies in our laboratory and others have demonstrated the capacity of PTH and PTHRP to affect the differentiation of a number of cell types, including osteoblasts. The current evidence indicates that PTHRP can act as an autocrine or paracrine growth and/or differentiation factor in a number of tissues (125, 126, 127). The existence of both PTHRP and the PTH/PTHRP receptor in bone, and the ability of a variety of bone-derived cells to produce PTHRP both in culture and in vivo, strongly suggest a local role in bone. Evidence for such a role was also provided by the effects of targeted disruption of the PTHRP gene in mice (46). Thus, mice heterozygous for the null mutation displayed haploinsufficiency and decreased bone volume as the mice aged. Such observations, confirmed by targeted overexpression of the same gene (122), suggest that PTHRP may modulate the maturation and differentiation of osteoblasts.

Treatment of MG-63 cells with 100 nM PTHRP (1-34) was seen to induce increased expression of the osteoblastic differentiation markers type I collagen and alkaline phosphatase. Although production of type I collagen is not exclusive to the differentiating osteoblast, but is also produced by fibroblastic cells, type I collagen is considered a useful osteoblast differentiation marker when expressed in an established sequence with other bone markers such as alkaline phosphatase (5). Both catabolic and anabolic effects of PTHRP and PTH may be observed in bone via the PTH/PTHRP receptor (14). Ishizuya et al (39) have shown that these discrepancies appear to be a function of exposure time, such that intermittent exposure of cells to PTH (1-34) of approximately 6 hours will cause osteoblast differentiation. Treatment of MG-63

osteoblastic cells with PTHRP (1-34) were consistent with these observations, in that levels of type I collagen mRNA were seen to rise at approximately 6 hours, peak at 8 hours, and begin to decline with extended exposure time. These results would suggest that there may exist an inhibitory effect of prolonged exposure of osteoblasts to PTHRP. Subsequent experiments were therefore performed with 8 hour incubation periods in keeping with these observations. The ability of the MG-63 cells to withstand prolonged exposure to each inhibitor was first verified by trypan blue dye exclusion viability experiments, as well as close observation for any adverse changes in cell morphology. It seems possible that receptor desensitization due to internalization did occur during this time interval but this was not examined, and signaling events leading to eventual differentiation would to appear to have adequately been initiated during the incubation times that were employed.

It is known that PTH/PTHRP receptor activation can lead to activation of multiple G proteins, namely Gαq and Gαs, with subsequent activation of Phospholipase C (PLC) and adenylate cyclase (76), respectively. Stimulation of Phospholipase C (PLC) will result in subsequent production of 1, 4, 5 -trisphosphate and diacylglycerol (1, 3, 124), leading to mobilization of calcium and PKC respectively. While there are varying reports regarding the involvement of PLC in osteoblastic differentiation, we have seen that inhibition of PKC, by treatment with chelerythrine chloride can block induction of type I collagen and alkaline phosphatase. Stimulation of the PTH/PTHRP receptor also results in activation of adenylate cyclase followed by a rapid increase in intracellular cAMP, and subsequent activation of PKA. We show that the inhibition of adenylate

cyclase by treatment with H-89, results in an abrogation of the PTHRP (1-34) stimulated increase in type I collagen mRNA expression and also interrupts the production of the later marker of MG-63 cell differentiation, alkaline phosphatase.

While activation of PKA and PKC is known to occur in minutes, the ability of these short term signals to influence such late developing cellular phenomenon as differentiation is well documented in the literature. Previous studies by Tsai et al (118) show that 100 nM PTHRP induces cAMP in UMR106 osteosarcoma cells in minutes, with subsequent occurrence of differentiation at later time points. In several studies investigating the effects of PTH on osteoblast activity, the PKC and PKA signaling pathways appear to be simultaneously activated, and seem to co-operate in the anabolic effects of the shared receptor in bone cells. Cross-talk between these two signal transduction systems may therefore occur in osteoblasts following PTH stimulation. Further studies to elucidate this possibility were also undertaken by combined inhibitor treatment of MG-63 cells, but these show additive and not synergistic effects (data not shown).

While it has been well established that the PTH/PTHRP receptor can signal through both $G\alpha s$ and $G\alpha q$ (1), the question of PTHRP signal transduction via $\beta\gamma$ subunit dependent pathways remains to be definitively answered. The ability of the transient transfection of a $G\alpha s$ mutant (97) which sequesters $\beta\gamma$ subunits to abrogate the effects of PTHRP treatment on osteoblastic differentiation markers may implicate $\beta\gamma$ signaling. However, because the dominant negative $G\alpha s$ (14) mutant utilized also inhibits signaling via the endogenous $G\alpha s$ subunit, the results obtained from our

experiment may have simply confirmed results obtained by inhibition of adenylate cyclase by H-89. Nevertheless, data obtained by chemical inhibition of PI3 kinase are consistent with a role for the βγ subunits in mediating some of the effects of PTHRP. Further studies in which βγ subunits are selectively inhibited will be required but such studies are currently technically challenging.

PI3 kinase is a heterodimeric cytosolic protein composed of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit (16). PI3 kinase is stimulated by both G-protein coupled receptors and receptor tyrosine kinases. Stephens et al (102) first discovered that PI3 kinase is specifically activated by purified βγ subunits. The ability of the chemical inhibitor of PI3 kinase, Wortmannin, to cause a significant reduction in the expression of both osteoblast differentiation markers in our studies, suggests that PI3 kinase is involved in PTHRP mediated MG-63 cell differentiation. Because PI3 kinase inhibition is seen to decrease βγ dependent MAPK activation, it is commonly presumed that βγ subunits activate PI3 kinase, and thus initiate a cascade of events that leads to the phosphorylation of the protein Shc. Whether Rac or other GTP-exchange proteins are involved in PI3 kinase-mediated pathways was not tested. Nevertheless, the interaction of βγ with SH2 containing proteins, can be mediated by PI3 kinase, such that Shc complexes to the SH2 containing adaptor protein Grb2. Grb2 can then stably associate with the Ras guanine nucleotide exchange factor, Sos. Ras is anchored to the plasma membrane, where Sos can stimulate the exchange of GDP for GTP, thereby leading to Ras activation. We have found that chemical inhibition of Ras by treatment with B1086, a farnesyl transferase

inhibitor, also diminished the effect of PTHRP (1-34) on induction of both type I collagen levels and alkaline phosphatase levels.

One of the best characterized downstream targets of Ras is the family of mitogen activated protein kinases (52). MAP kinases are a family of serine/threonine kinases involved in the transduction of cellular signals to the nucleus. These proteins regulate a vast range of cellular processes including proliferation, differentiation, transformation, inflammation, apoptosis, and cytoskeletal rearrangement. Previous data also support the pivotal role of MAPK in the induction of phenotypic indices of osteoblast differentiation (114, 128). Upstream of MAPK, the activation of Ras will lead to the sequential activation of Raf-1, a serine/threonine kinase, and MAPK kinase (MEK), whose substrates are the extracellular signal regulated kinases ERK1 and ERK2 of the MAPK family. It has also been shown that activation of MAPK can occur in a manner independent of Ras activation, presumably via direct activation of Raf or MEK by PKA (121) or PKC. Treatment of MG-63 cells with the MAPK inhibitor PD-98059 interrupted PTHRP stimulation of indices of osteoblastic differentiation, thereby suggesting that MAPK is involved in this process. The ability of PTHRP to induce MAPK activity in MG-63 osteoblastic cells complements the results of the studies on the effect of PD-98059 on PTHRP induction of markers of differentiation. Furthermore, the capacity of multiple inhibitors of upstream signaling to diminish MAPK activity assay seems to suggest that these multiple signaling pathways converge to some extent at MAPK. Whether other members of the MAPK

family, such as p38K or JNK, are also involved in PTHRP signaling remains to be determined.

It is probable then that PTH/PTHRP activation in response to PTHRP initiates parallel signaling via Gαs, leading to PKA activation, Gαq, leading to activation of PKC, and βγ, leading to activation of PI3K and Ras (Figure 9). Convergence of these signals may then occur via activation of MAPK. Further study is now required in order to extend these observations to other osteoblastic cell models and to determine if other kinases, such as the stress activated kinases, play a role in PTHRP-mediated osteoblastic differentiation. The elucidation of the signaling pathways involved in the effects of PTHRP on enhancing differentiation of osteoblastic cells clearly has important implications with regards to our understanding of the actions of this hormone in normal physiology and particularly with respect to its anabolic actions in bone.

CONCLUSION

The formation of bone begins with an intricate series of events that involves the tight control of proliferating mesenchymal cells and their commitment and differentiation to cells of the osteoblast lineage. These steps in commitment are followed by the maturation of the osteoblast and the subsequent formation and mineralization of the bone matrix. These events are under the control of both systemic hormones and cytokines generated in the bone microenvironment. While PTHRP was first discovered in association with hypercalcemia of malignancy, and the greatest efforts have been expended in the elucidation of its role in disease, much yet is left to be discovered with respect to its roles in normal physiology.

The roles of PTHRP in bone development have been explored at many different levels. The clear evidence of its unquestionable necessity in embryonic skeletal development was provided by the phenotype of the PTHRP knockout. The role of the classical receptor, the shared PTH/PTHRP receptor, in this function of PTHRP was in that the null mutant of the PTH/PTHRP receptor was almost phenotypically identical to the PTHRP null mutant animal. Such observations were confirmed by experiments in the targeted overexpression of the PTHRP gene, and globally suggested the involvement of PTHRP as a modulator of bone cell maturation and differentiation.

While this thesis has dealt with the role of PTHRP in the induction of osteoblast differentiation, the role of PTHRP in bone can in fact be viewed as both multi-faceted and seemingly contradictory. In essence this is due in great part to the intricacies of the control of osteoblastic differentiation. There exists a fine balance between the hormonal

influences in the bone microenvironment, the characteristics of bone as a heterogeneous population of cells, the constant remodelling of bone, and the effects of mechanical strain. While it is clearly stated in the preceding publication that the conclusions drawn with respect to the role of PTHRP in bone cells may well only be applied to the effects of the N-terminal portion of PTHRP on osteoblast-like osteosarcoma derived MG-63 cells one must note the necessity of such control over the multifarious nature of bone through artificial experimental conditions.

It is of particular importance to note that this "snapshot" of the role of PTHRP in bone serves as a model of PTHRP receptor activation. The classical involvement of both PLC and adenylate cyclase downstream of PTH/PTHRP receptor activation has been well documented in the literature. While the particular points of involvement and the balance between these two pathways appear to vary from study, this is but a reflection of the need for more elegant study models. What is as yet only beginning to be explored is the role of βγ in PTH/PTHRP receptor signaling. This thesis has explored the possibility of βγ involvement in bone cell differentiation, and suggests the involvement of PI3 kinase-mediated pathways, which initiate a cascade of signaling events to the ultimate phosphorylation of Shc, followed by Ras activation via the adaptor proteins Grb2-Sos. It is obvious that this may be the focus of future work in that the involvement of Rac or other GTP-exchange proteins involved in PI3 kinase mediated pathways was not further explored. Such a focus would clarify the role of βγ signaling downstream of the PTH/PTHRP receptor, particularly in light of the recent report that deletion or reduction of Src expression can also be seen to contribute to an increase in bone mass (72). The

targeted disruption of *c-src* gene had been demonstrated to cause osteopetrosis (100), and further work by Marzia et al (72) has demonstrated that underlying this pathological imbalance in bone formation versus bone breakdown lies an upregulation of various positive markers of osteoblast differentiation, including the PTHRP receptor. Given the evidence of MAPK activation in the induction of markers of bone cell differentiation, it is clear that there exists a point of convergence of multiple signaling pathways. Whether the major signaling cascade involves the βγ pathway and subsequent activation of the classical Ras-Raf-MAPK pathway is as yet unclear. There is obviously an intricate control of bone cell growth and differentiation, I that remains to be elucidated in order to reconcile these scenarios.

The intricacies of the networks of signal transduction in not only the developing bone, but in all developmental processes, particularly with respect to their tight control and regulation, is just now beginning to be unravelled. The elucidation of the normal control in growth and developmental processes will be the clues we need in order to harness, and perhaps some day control, such diseases of dysregulation in bone development as the chondrodysplasias, osteogenesis imperfecta, and even primary and metastatic bone tumors.

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