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THE REGULATION OF PARATHYROID HORMONE-RELATED PROTEIN (PTHRP) GENE EXPRESSION IN HYPERCALCEMIA OF MALIGNANCY

by

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ABSTRACT

The studies included in this thesis were aimed at identifying the mechanisms that lead to aberrant expression of the PTHRP gene in cancer.

We have used the hepatocyte growth factor receptor oncogene, Tpr-Met, as a model and examined the effect of this oncogene on PTHRP expression. When transfected into Fisher rat 3T3 (Fr3T3) fibroblasts, Tpr-Met increased the transcription of PTHRP mRNA and secretion of the protein. To identify the signaling pathways involved we analyzed a mutant of Tpr-Met, Tyr489, that was impaired in activating a number of downstream effectors, including phosphatidylinositol-3 kinase, Grb2 and Shc. The ability of Tpr-Met/Tyr^{489} mutant to induce PTHRP expression was significantly reduced. Furthermore, inhibiting Ras using lovastatin, in wild-type Tpr-Met transfected cells, completely suppressed PTHRP levels, suggesting that the mechanism was Ras-dependent.

We next directly investigated the effect of Ras on PTHRP expression *in vitro*, and on hypercalcemia of malignancy *in vivo*. When transfected into cells the activated mutant of Ras (RasV12) potently increased PTHRP mRNA and protein levels. When RasV12 expressing cells were subcutaneously injected into BALB/c/*nu/nu* mice, the tumors developed rapidly, and signs of hypercalcemia were detected within 2 weeks. Inhibiting Ras using a specific inhibitor, B-1086, completely blocked expression of PTHRP, *in vitro*, and suppressed the signs of hypercalcemia *in vivo*. These results show that inhibiting Ras was sufficient to block tumor expression of PTHRP and development of hypercalcemia.

Using rat Leydig tumor H-500 cells, we next investigated effector pathways downstream of Ras that mediate serum stimulated PTHRP expression. PTHRP mRNA was decreased by a dominant negative mutant of Raf (Raf C4B) and by a MEK inhibitor (PD 098059), implicating the involvement of Ras-Raf-MEK pathway in the serum response. In addition, stimulation with UV light or expression of an activated form of Rac (Rac V12) was sufficient to increase PTHRP mRNA. Furthermore, a dominant negative mutant of Rac (Rac N17) also blocked serum induced expression of mRNA. This suggests that the stress-activated pathways may provide alternative mechanisms that can regulate the PTHRP gene. These pathways also appear to be important in the serum

induced response. Collectively, the results from these studies contribute to our limited knowledge of the mechanisms governing PTHRP expression in cancer. The findings also provide novel targets to explore for improved therapy of hypercalcemia.

RÉSUMÉ

Les travaux présentés dans cette thèse ont pour but d'identifier les méchanismes impliqués dans la dérégulation de l'expression du gène du PTHRP dans les cancers.

Nous avons utilisé comme modèle l'oncogène Trp-Met, récepteur du facteur de croissance hépatocytaire, et examiné ses effets sur le gène du PTHRP. Apres transfection dans les fibroblastes FR3T3, Trp-Met stimule la transcription de l'ARN messager codant pour le PTHRP et le sécrétion de la protéine. Pour identifier les voies de transduction impliquées, nous avons utilisé le mutant Trp-Met/Tyr^{489} incapable d'activer de nombreuses cibles comme PI 3-kinase, Grb2 ou encore Shc. De même, ce mutant présente une capacité réduite pour activer l'expression du gène du PTHRP. De plus, l'inhibition de Ras par la lovastatin dans les cellules surexprimant le récepteur Trp-Met sauvage conduit à la disparition complète du PTHRP, ce qui suggère un mécanisme dépendent de Ras.

Nous avons ensuite étudié directement les effets de Ras sur l'expression du PTHRP *in vitro*, et de la malignité sur l'hypercalcémie *in vivo*. Nous avons transfecté dans des cellules un mutant actif de Ras (RasV12) qui stimule très fortement l'expression de l'ARM messager codant pour le PTHRP ainsi que l'expression de la protéine correspondante. Quand ces cellules sont injectées de manière sous-cutanée à des souris BALB/c/nu/nu, des tumeurs se forment rapidement, et des signes d'hypercalcémie sont détectés en deux semaines. L'inhibition de RasV12 par l'inhibiteur spécifique B-1086, abolit l'expression du PTHRP *in vitro* et supprime les signes d'hypercalcémie *in vivo*. Ces résultats démontrent que l'inhibition de Ras est suffisante pour bloquer l'expression tumorale du PTHRP et le développement de l'hypercalcémie.

Nous avons aussi étudié dans les cellules tumorales H-500, les voies de transduction en aval de Ras qui médient la stimulation de l'expression du PTHRP par le serum. L'expression de l'ARN messager codant pour le PTHRP est inhibée par un mutant dominant négatif de Raf et par l'inhibiteur spécifique de MEK (PD098059), ce qui implique la voie Ras-Raf-MEK dans la réponse au serum. De plus, un traitement par les rayons UV ou la surexpression d'une forme constitutivement activée de Rac (RacV12) induisent l'expression de l'ARM messager codant pour le PTHRP. De même, un mutant dominant négatif de Rac (RacN17) inhibe l'expression de l'ARN du PTHRP. Ces

informations suggèrent que la voie de transduction du stress pourrait aussi être impliquée dans la régulation de l'expression du PTHRP.

Finalement, les résultats apportés par ces études contribuent à l'extension de notre connaissance des mécanismes régulant l'expression du PTHRP dans les cancers, ainsi qu'ils définissent de nouvelles cibles à atteindre pour améliorer les traitements de l'hypercalcémie dans les cas de tumeurs malignes.

FORWARD

The following excerpt is taken from the Guidelines Concerning Thesis Preparation, Faculty of Graduate Studies and Research, McGill University, and applies to this thesis. Candidates have the option of including, as part of the thesis, the text of paper(s) submitted or to be submitted for publication, or the clearly duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

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In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make it perfectly clear the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author or any component of such a thesis serve as an examiner for that thesis.

PUBLICATIONS

The work described in Chapters 2-4 of this thesis has been published in the following journals:

Chapter 2: Aklilu F., Park M., Goltzman D. and Rabbani SA. (1996) Am J Physiol 271:E277-83.

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Chapter 4: Aklilu F., Goltzman D. and Rabbani SA. J Biol Chem, submitted, 1998.

The work presented in Chapters 2-4 is my own. Dr. Morag Park kindly provided cell lines and cDNA constructs, and together with Dr. D. Goltzman and Dr. S.A. Rabbani provided input in designing the experiment and preparation of the manuscripts.

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ABBREVIATIONS

cAMP	cyclic adenosine 3,5-monophosphate
CREB	cAMP response element
C-terminal	carboxyl-terminal
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FGF	fibroblast growth factor
FPTase	farnesyl protein transferase
GM-CSF	granulocyte macrophage colony stimulating factor
G-protein	guanine nucleotide-binding protein
HGF	hepatocyte growth factor
HHM	humoral hypercalcemia of malignancy
HM	hypercalcemia of malignancy
HTLV-1	human T-cell lymphotrophic virus-1
IGF-I	insulin-like growth factor I
IP3	phophoinositol 1,4,5-trisphosphate
JE	monocyte chemoattractant protein-1
JNK	c-Jun N-terminal kinase
JNKK	c-Jun N-terminal kinase kinase
LOH	local osteolytic hypercalcemia

MBP	myelin basic protein
MEK	MAP kinase/ERK-activating kinase
MEKK	MEK kinase
MAP kinase	mitogen activated protein kinase
N-terminal	amino-terminal
OAF	osteoclast stimulating factor
PDGF	platelet derived growth factor
PI 3-kinase	phosphoinosite 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PTH	parathyroid hormone
PTHRP	parathyroid hormone-related protein
RTK	receptor tyrosine kinase
RSK	ribosomal S6 kinase
SAPK	serum activated protein kinase
SH	src homology
SCID	severe combined immunodeficiency
SRE	serum response element
SRF	serum response factor
TCF	ternary complex factor
TGF	transforming growth factor
TNF	tumor necrosis factor

TPA	phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate

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CHAPTER 1

INTRODUCTION

1.1 PARATHYROID HORMONE-RELATED PROTEIN AND HYPERCALCEMIA OF MALIGNANCY

Cancer is a disease of uncontrolled cell growth that produces symptoms through three mechanisms: by forming bulk mass at the primary tumor site, by invasion and metastasis to distant deposits and through obstruction of physiological functions. Additional symptoms can also occur as an indirect effect of the tumor or its metastases. These secondary features of cancer are collectively known as *paraneoplastic syndromes* of malignancy (Odell and Wolfen 1978). Paraneoplastic syndromes are caused by tumor-secreted proteins or by factors produced indirectly by normal cells in response to the tumor. The best-characterized paraneoplastic syndromes result from ectopic secretion by the tumor of polypeptide hormones. Among the metabolic complications caused by these tumor-secreted hormones, the most common is hypercalcemia of malignancy (HM) (Halloran and Nissenson 1992). A factor that closely resembles parathyroid hormone and occupies the same receptor, was discovered to be responsible for this secondary disease. In the last decade, the factor, termed parathyroid hormone-related protein (PTHRP), has become established as the major pathogenic agent in HM.

1.1.1 Discovery of PTHRP

A German group began the search for PTHRP in the early 1920s, reporting for the first time that cancer could be associated with elevated levels of serum calcium and low phosphorus concentrations (Zondek et al. 1923). Since bone is the most common organ to be affected by metastatic cancer, it was initially assumed that this syndrome arose

exclusively from cancer cells metastasizing to the skeleton. However, a later documentation of hypercalcemia in the absence of either osseous metastasis or parathyroid dysfunction led to the conception of a theory for ectopic tumor production of a calcium metabolizing agent (Gutman et al. 1936; Albright 1941).

The theory arose partly from a discrepancy between the expected symptoms that could arise from bone metastasis alone and clinical observations. The principal contradictions included an expected hyperphosphatemia in direct contrast to the observed occurrence of hypophosphatemia (Albright 1941). In addition, many of the characteristics of this syndrome resembled primary hyperparathyroidism (HPT) associated with parathyroid adenomas which is caused by excess production of parathyroid hormone (PTH). In HPT, PTH stimulates bone resorption and renal phosphate excretion, suggesting that the pathogenic factor in HM, if not PTH itself, was a PTH-like protein. These early studies laid the foundations for the current knowledge of the pathophysiology of this disease.

Landmark studies during the 1960s and 70s led to additional breakthroughs, defining the clinical and biochemical attributes of this disorder, as well as recognizing apparent differences between the clinical symptoms of HM and HPT. Among the distinguishing features, HM was associated with higher blood calcium concentrations and mild hypokalemic alkalosis, in direct contrast to mild metabolic acidosis that occurred in HPT (Lafferty 1966). In addition, circulating levels of 1,25 dihydroxyvitamin D3 were often low in HM and elevated in patients with HPT (Lafferty 1966; Stewart et al. 1980). Moreover, using sensitive radioimmuno-assay techniques, Powell et al. (1973)

established the absence of PTH in the plasma of HM patients. Furthermore, the PTH gene was not expressed in tumors associated with HM (Simpson et al. 1983). From such findings and from additional discoveries in parallel studies, it became clear that the agent in HM, although strikingly similar, was not PTH itself.

The biochemical properties of the putative factor were defined in the early 1980s using adenylyl cyclase bioassays that had been developed to detect PTH-like activity in renal and osteoblastic cells (Stewart et al. 1982). Subsequently, using these bioactivity assays together with high performance liquid chromatography (HPLC), a major bioactive form that was larger and more heterogeneous than PTH was isolated (Nissenson et al. 1981; Rodan et al. 1983). Other studies from the same period identified bioactive forms extracted from tumor tissues that were able to reproduce the physiological actions of PTH and restore plasma calcium and phosphorus levels in thyroparathyroidectomized rats (Rabbani et al. 1988). These findings eventually led to the cloning of a gene for a parathyroid hormone-related protein (PTHRP) (Burtis et al. 1987; Moseley et al. 1987; Strewler et al. 1987).

1.1.2 Pathophysiology of Hypercalcemia of Malignancy

HM occurs more frequently in clinically advanced malignancy and seldom in the early stages of cancer. The onset is acute and elevation of serum calcium is often profound and in a range greater than 12 mg/dL (normal range 8.8-10.2 mg/dL) (Mundy and Martin 1982). The symptoms associated with HM can be very drastic and include nausea, severe anorexia, extreme fluid loss leading to serious dehydration, constipation,

and kidney malfunction resulting from excess calcium load (Mundy and Martin 1982; Mundy and Guise 1997). Adverse effects on the central nervous system such as confusion, drowsiness and coma, and bone fractures as a consequence of systemic and massive destruction of bone have also been reported (Mundy and Martin 1982).

1.1.3 Pathogenesis of Hypercalcemia of Malignancy

It is well-established that two forms of HM exist (Stewart et al. 1980; Burtis et al. 1991). The first involves the ectopic production of PTHRP by tumors and its systemic distribution to target organs leading to diffuse osteoclastic bone resorption and retention of calcium by the kidneys. The second mechanism, which is associated with cancers such as breast and multiple myeloma that metastasize to bone, involves local osteolytic lysis mediated through factors directly secreted by the tumors and/or their surrounding cells. The former humoral mechanism has emerged as the major form of HM and is responsible for greater than 70% of HM (Stewart et al. 1980; Burtis et al. 1991).

a) Humoral Hypercalcemia of Malignancy

Humoral HM (HHM) is the direct consequence of PTHRP overproduced by tumors. This phenomenon typically occurs in patients with solid tumors and in the absence of extensive bone metastasis. There is a tendency for HHM to be associated with squamous, renal or genitourinary tumors (Yoshida et al. 1994; MacKenzie et al. 1994; Rudduck et al. 1993; Iguchi et al. 1993; Gotoh et al. 1993; Fujino et al. 1992; Richardson and Johnson 1992; Mitlakal et al. 1991). Virtually all cases of

hypercalcemia associated with the HTLV 1-associated adult T-cell leukemia/lymphoma also result in HHM caused by the overproduction of PTHRP (Watanabe et al. 1990).

First described by Stewart et al. (1980), HHM is characterized by increased circulating levels of PTHRP, elevated levels of serum calcium, increased nephrogenous cAMP excretion rate, and depressed levels of serum phosphorus, PTH levels, and 1,25-dihydroxyvitamin D3. The biochemical abnormalities in HHM are similar to primary hyperparathyroidism including increased nephrogenous cAMP and elevated serum calcium (Stewart et al. 1980). However, HHM differs from primary hyperparathyroidism in having normal or low levels of circulating PTH and 1,25 dihydroxyvitamin D3 and a relatively high fractional calcium excretion (Stewart et al. 1980). Quantitative bone histomorphometric studies have also disclosed increased osteoclast number and activity but a contrasting decrease in osteoblastic activity in HHM (Stewart et al. 1982). This phenomenon referred to as an *uncoupling* of osteoclast and osteoblast activity differs strikingly from the *coupled* events seen in primary hyperparathyroidism. The suppressed plasma PTH and 1,25 dihydroxyvitamin D3 levels in HHM result in reduced gastric calcium absorption and greater clearance through the kidneys (Stewart et al. 1980). The combined effect of these makes the degree of hypercalcemia in HHM as compared to primary hyperparathyroidism, more severe and associated with profound calcium loss.

b) Local Osteolytic Lesions

About 20-30% of cases of HM are caused by local osteoclastic bone resorption promoted by agents secreted in cancer cells embedded in bone tissue, or directly produced by the surrounding host cells (Stewart et al. 1980; Burtis et al. 1991; Budayr et al. 1989; Eilon and Mundy 1978; Mundy et al. 1974). Local osteolytic HM (LOH) is characterized by extensive bone metastasis and occurs typically in patients with myeloma, lymphoma or breast cancer (Coleman and Rubens 1987; Eilon and Mundy 1978; Mundy et al. 1974). Although associated with increased levels of serum calcium, serum phosphorous and renal phosphate clearance levels in LOH were normal (Stewart et al. 1980). The elevated levels of serum calcium inhibit the release of PTH and create a uniquely LOH phenomenon of high calcium level associated with reduced levels of nephrogenous cAMP excretion and circulating amounts of 1,25 dihydroxyvitamin D_3 (Stewart et al. 1980). In this event, calcium clearance in the kidneys is high due to the large calcium load, and in the absence of 1,25 dihydroxyvitamin D_3, intestinal calcium absorption decreases, and results in hypercalcemia with a high degree of calcium loss.

The major pathogenic mechanism in LOH involves stimulation of osteoclast activity by locally acting factors. A soluble factor, termed *osteoclast activating factor* (OAF), present in conditioned media from cultured myeloma cells, was shown to stimulate bone resorption in bone sections *in vitro* (Mundy et al. 1974). Candidate OAFs include lymphokines such as interleukin 1α, interleukin 1β, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β, which are all capable of stimulating osteoclastic activity *in vitro* and of causing hypercalcemia when infused into mice *in vivo*

(Dewhirst et al. 1985; Kawano et al. 1989; Yamamoto et al. 1989; Garrett et al. 1987; Mundy 1989). Initially, LOH was thought to result exclusively from the direct action of metastases on bone. Recent evidence suggests, however, the important role played by humoral factors such as PTHRP released by tumors that are commonly associated with LOH. For example, diverse hematological malignancies including multiple myeloma were reported to be associated with elevated circulating levels of PTHRP (Burtis et al. 1990; Henderson et al. 1990; Ikeda et al. 1994; Kremer et al. 1995). Elevated levels of plasma PTHRP was also observed in approximately 35% of hypercalcemic patients with breast cancer (Isales et al. 1987). The substantial overlap that exists in the pathogenic mechanisms of HM indicates that definition of cancers as either HHM or LOH can only serve to describe overall tendencies in a diverse continuum.

1.1.4 Animal Models of Hypercalcemia of Malignancy

The use of animal models has been a powerful tool in defining the pathophysiology of HM. A well-characterized animal model of HM is the rat H-500 Leydig tumor cell model. Animals bearing H-500 tumors were shown to rapidly develop the biochemical abnormalities associated with HM, and eventually die of complications arising from the disease (Sica et al. 1983). Early studies using this model demonstrated the major role of PTHRP in HM. Infusing tumor-bearing animals with PTHRP neutralizing antibodies markedly reversed the biochemical signs of HM and delayed the onset of hypercalcemia (Sato et al. 1993; Henderson et al. 1990). This animal model was also used to evaluate the *in vivo* anti-hypercalcemic effects of a vitamin D analogue,

EB1089 (Haq et al. 1993), and has contributed to the progress of this drug which is presently being tested in Phase I clinical trials (Gulliford et al. 1998). The rat Walker carcinoma 256 model is another animal model of hypercalcemia, associated with elevated plasma PTHRP and serum calcium as well as increased osteolytic activity and renal calcium reabsorption (Sharla et al. 1991; de Miguel and Esbrit 1993). Investigators have also made use of immunocompromised *nude* mice to study human tumor xenografts (Ikeda et al. 1988; Strewler et al. 1986). After transplantation with the tumor xenografts, the mice were reported to develop signs of HM, which could be delayed by passive immunization with neutralizing antibodies directed against PTHRP (Kukreja et al. 1988). In addition to these solid tumor associated models, a model of multiple myeloma has recently been developed in SCID mice (Alsina et al. 1995). This model was used to study the pathophysiology of severe osteolytic bone destruction associated with this disease. The use of animal models has contributed immensely to our understanding of HM and will continue to do so in the future. These models provide us with the opportunity to test and develop new therapeutic agents designed to treat hypercalcemia.

1.2 ORGANIZATION OF PTHRP GENE

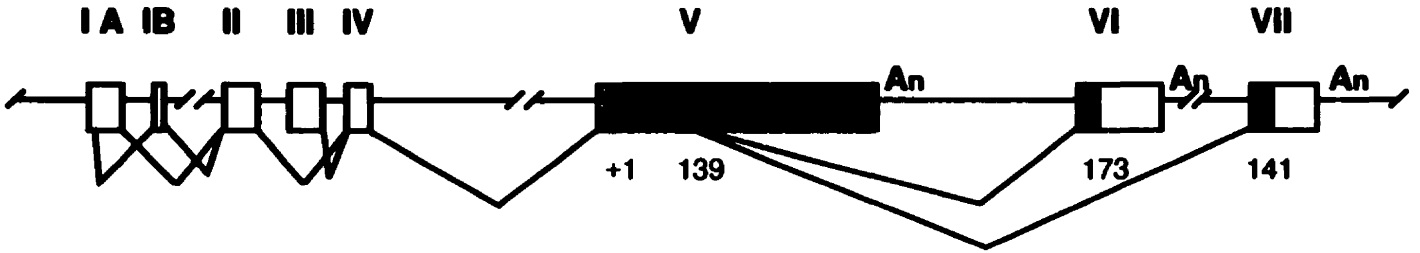
PTH and PTHRP genes are thought to have arisen from a common ancestral gene through an ancient chromosome duplication event (Halloran and Nissenson 1992; Broadus and Stewart 1994). The PTHRP gene is indeed very similar in organization and nucleotide sequence with the PTH gene (Strewler and Nissenson 1993). The two genes are also located within a cluster of related genes on homologous arms of chromosomes 12 and 11, respectively (Suva et al. 1989; Mangin et al. 1988). The human PTHRP gene with its three promoters, nine exons and complex pattern of alternative exon splicing, has, however, evolved to be far more complex than the PTH gene (Fig 1.1a).

1.2.1 The structure of the PTHRP gene

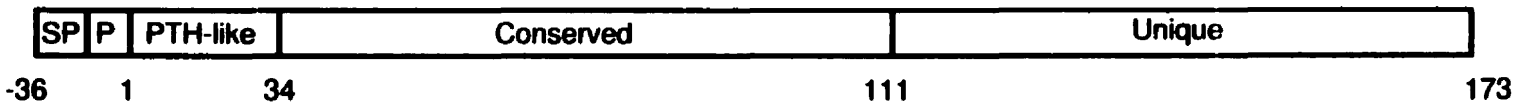
The human PTHRP gene is a complex transcriptional unit spanning approximately 15 kb (Yasuda et al. 1989a; Suva et al. 1989; Mangin et al. 1988). The first four exons of the gene are non-coding, and depending on which of the three promoters is used, the exons are differentially transcribed giving rise to variable 5' untranslated regions of the messenger RNA (mRNA). Exons V and VI contain most of the coding region of the gene. A transcribed consensus sequence between exons VI and VII later serves as a termination site during translation of the 139- amino acid isoform of the final protein product. While the 139 amino acid species ends here, exons VI and VII encode for the 173- and 141- isoforms. In addition, all mRNAs transcribed share a common AUUUA repeat a motif in their 3' untranslated regions. This AU-rich element is

Figure 1.1: Structure of human PTHRP gene; functional domains of the protein and post-translational processing.

A. The Structure of human PTHRP gene



B. Functional Domains of PTHRP



C. Post-translational processing of PTHRP

a well-established motif that confers rapid turnover in many genes (Ross 1995), and is presumed to impart the same to the PTHRP mRNAs.

1.2.2 PTHRP Promoter regions

The human PTHRP gene belongs to a relatively small number of genes that use both TATA and GC-rich promoter elements. Promoters P1 and P3, which lie upstream of exons IV and I respectively, contain classical TATA boxes (Yasuda et al. 1989; Suva et al. 1989; Mangin et al. 1990a; Campos et al. 1992). On the other hand promoter P2 that lacks a TATA box, is located in a GC-rich region enriched in Sp1 binding sites (Vasavada et al. 1993). The two TATA promoters P1 and P3 direct the majority of the expression of the PTHRP gene in human tissues (Campos et al. 1992). However, there are instances such as in human amniotic tissue and HTLV-1 transformed cells where the GC-rich P2 is the predominant promoter responsible for PTHRP gene expression (Dittmer et al. 1994; Brandt et al. 1992). Interestingly, a number of factors that regulate PTHRP gene expression affect the use of its promoters. Dexamethasone decreased transcription driven by all three promoters equally (Glatz et al. 1994). In contrast, EGF and TGFβ promoted transcriptional activity from the P2 and P3 promoters preferentially (Heath et al. 1995). Whether this preferential use of promoters by the different regulators of PTHRP extends to encompass a cell-type specificity, however, has yet to be determined.

1.2.3 Interspecies comparison of the different PTHRP genes

The PTHRP gene has been isolated from a number of species other than human including mouse, rat and chicken (Yasuda et al. 1989; Thiede et al. 1990; Karaplis et al. 1990; Mangin et al. 1990b; Schermer et al. 1991). The non-human genes are comparatively simpler in their organization than their human counterpart. The mouse and rat genes contain only a single promoter that is homologous with the downstream P3 promoter of the human gene (Karaplis et al. 1990; Mangin et al. 1990a). In addition, during mRNA transcription, there is only one 3' splicing pathway, leading to an mRNA form encoding a 141-amino acid mature protein (Karaplis et al. 1990; Mangin et al. 1990a). The chicken PTHRP gene, like the mouse and rat genes, also uses a single promoter that is equivalent to P3 of the human gene (Schermer et al. 1991). However, in contrast to the rat and mouse genes, it can encode two isoforms of PTHRP protein of 139- and 141-amino acids. A comparison of the different PTHRP genes reveals a striking homology especially in the coding exons (Goltzman et al. 1991). The conservation of the PTHRP sequence implies strong evolutionary selective pressures to maintain the function of the gene and supports the notion that PTHRP has an important physiologic role.

1.3 REGULATION OF PTHRP

A number of potential mechanisms have been invoked in an attempt to explain overexpression of PTHRP in malignant tissue including change in methylation status of the gene during neoplastic transformation and disruption of signal transduction pathways involved in regulation of the gene (Holt et al. 1993; Chapter 3). In general, paraneoplastic syndromes arise as a consequence of abnormally increased gene expression (Hocking et al. 1983; Ellis et al. 1987; Beutler et al. 1985; Schuler et al. 1988; Murphy et al. 1990; Texier et al.1991) which can occur through a diverse set of mechanisms. Increased stabilization of normally labile mRNA was reported to lead to overexpression of fibroblast growth factor (FGF) in astrocytoma (Schuler et al. 1988) and overexpresson of granulocyte-macrophage colony stimulating factor (GM-CSF) in lymphoid tumors (Murphy et al. 1990). A change in methylation status leading to a shift in promoter use was documented to result in overexpression of adrenocorticotropin (ACTH) by several human lung tumors (Texier et al.1991). In addition, deregulation of *trans*- activating factors in the promoter regions of GM-CSF and α-fetoprotein was shown to lead to the constitutive expression of these genes in human carcinomas (Nishizawa et al. 1990; Nakabayashi et al. 1991). A number of different mechanisms may likewise govern PTHRP overexpression in malignancy.

In addition to its expression in malignancy, PTHRP is expressed in a broad range of normal fetal and adult tissues including uterus, brain, placenta, lactating breast, pancreatic islets, kidney, bone, chondrocytes, urinary bladder and vascular smooth muscle (Daifotis et al. 1992; Kremer et al. 1991; Thiede and Rodin 1988; Yamamoto et

al. 1992; Mok et al. 1989a). Indeed, many tissues that normally express PTHRP account for the origin of most if not all hypercalcemic tumors. It is clear that understanding the regulation of PTHRP gene in these tissues will further our knowledge of why this gene becomes aberrantly expressed in cancer. As noted previously, the PTHRP gene is extraordinarily complex and studies on gene regulation are beginning to uncover a wide variety of physiological and pharmacological controls. The PTHRP gene is induced by a number of growth factors and cytokines and it is thought to belong to a family of genes including nuclear proto-oncogenes, cytokines, and growth factors that are collectively known as *immediate early* genes. This and other aspects of PTHRP gene regulation are described below.

1.3.1 Characteristics of PTHRP gene Regulation

The PTHRP gene shares many features in common with immediate early genes, and a number of investigators have reasoned that it belongs to this family of genes (Holt et al. 1994; MacIsaac et al. 1991; Allinson and Drucker 1992; Streuker and Drucker 1991; Thiede et al. 1991). Immediate early genes include transcription factors such as c-myc and c-fos, and cytokines such as macrophage colony stimulating factor and monocyte chemoattractant protein-1 (JE). The hallmark features of this family of genes are low basal expression and rapid and transient induction of gene expression by growth factors and cytokines which occurs independently of new protein synthesis (Heschman 1991). In addition, induction of expression is transient and the mRNAs transcribed from these genes are short-lived. These features are also present in the PTHRP gene. Growth

factors and cytokines induce a rapid and transient PTHRP gene expression (Kremer et al. 1991; Allinson and Drucker 1992; Liu et al. 1992; Sebag et al. 1992; Werkmeister et al. 1993, Ikeda et al. 1993). PTHRP is also known to have a transcript with a short half-life of 90-120 minutes (Ikeda et al. 1990, Allinson and Drucker 1992, Kiriyama et al. 1993). There is substantial evidence to suggest that an AUUUA motif located in the 3' untranslated region of mRNAs from immediate early genes confers instability (Shaw and Kamen 1986). Studies that experimentally deleted the AUUUA sequences of c-fos and c-myc mRNAs have found that subsequent to deletion of this sequence mRNA half-life was markedly prolonged (Jones and Cole 1987; Wilson and Treisman 1988). Likewise, PTHRP mRNAs contain multiple copies of this instability motif that are encoded by the exons which specify the 3' untranslated region (Ikeda et al. 1990; Allinson and Drucker 1992; Kiriyama et al. 1993).

Moreover, protein synthesis inhibitors are known to induce the transcription of many immediate early genes (Greenberg et al. 1986; Beutler et al. 1988; Wilson and Treisman 1988). This phenomenon can result either from increased gene transcription or increased mRNA stability, or both. Correspondingly, the protein synthesis inhibitor cycloheximide has been shown to induce PTHRP mRNA expression through both transcriptional and post-transcriptional mechanisms (Ikeda et al. 1990; Allinson and Drucker 1992, Kiriyama et al. 1993).

A short 7 base pair sequence TTTTGTA found in the 3' untranslated sequences was noted in as many as 25 immediate early genes including c-myc and JE and is also present in the PTHRP gene (Freter et al. 1992; Gillespie and Martin 1994). This

element was shown to be essential for the induction of JE by platelet derived growth factor (PDGF), serum, interleukin and double stranded RNA (Freter et al. 1992). Although its definitive function is not certain, it does not function in polyadenylation, splicing or destabilization of the transcript, but as suggested by the authors may be involved in trafficking of mRNA for protein synthesis (Freter et al. 1992).

Hence, there is substantial evidence implicating PTHRP as an immediate early gene. This pattern of expression that is characteristic of genes encoding transcription factors, growth factors and cytokines suggests that PTHRP may play an important role in cell proliferation and differentiation.

1.3.2 Regulation by Serum and Growth Factors

Serum, epidermal growth factor (EGF), Interleukin-2 (IL-2), transforming growth factor (TGF) β, insulin and insulin like growth factor type I (IGF-I) have all been shown to stimulate PTHRP gene expression in a variety of cell types including keratinocytes (Kremer et al. 1991; Allinson and Drucker 1992; Werkmeister et al. 1993), human osteosarcoma cell line (Rodan et al. 1989), human T-cell leukemia cells(Ikeda et al. 1993), human mammary epithelial cells (Sebag et al. 1994; Merryman et al. 1994), rat Leydig tumor cells (Liu et al. 1993), rat aortic smooth muscle cells (Hongo et al. 1991) and rat osteosarcoma cells (Falzon 1996). Serum, EGF, IGF-I and IL-2 induce a common pattern of PTHRP gene expression, a rapid and transient induction with a peak between 30 minutes to a few hours (Kremer et al. 1991; Allinson and Drucker 1992, Rodan et al. 1989; Liu et al. 1993; Sebag et al. 1992; Ikeda et al. 1993). PTHRP

expression in response to these factors appears to have both a transcriptional and post-transcriptional component. Many genes that are regulated by serum and growth factors contain serum response elements (SREs) in their 5' flanking sequences and these sequences are essential for gene induction in response to growth factors (Cochran 1993). Although putative SREs can be identified within the PTHRP promoter sequence by promoter sequence analysis the physical existence of these elements has yet to be confirmed.

In contrast to the above rapid and transient induction, the onset of TGFβ induced PTHRP gene expression is slow, peaks between 12 to 24 hours and persists for up to 72 hours (Zakalik et al. 1992; Casey et al. 1992). In spite of the kinetic differences, TGFβ also appears to act through both transcriptional as well as post-transcriptional mechanisms. The fact that TGFβ is one of the most commonly expressed growth factors in cancer suggests that regulation of PTHRP gene in this manner may be an important aspect in the pathogenesis of HHM.

1.3.3 Negative Regulation by 1,25-dihydroxyvitamin D3 and Glucocorticoids

Glucocorticoids and 1,25-dihydroxyvitamin D3 (vitamin D3) were found to produce a sustained inhibition of basal PTHRP gene transcription in various cell lines and tissues including human keratinocytes (Kremer et al. 1991) rat islet cells (Streuker and Drucker 1991) rat parathyroid cells (Ikeda et al. 1989) and in the rat uterus (Paspalaris et al. 1995). Both agents have been shown to casue a dose-dependent inhibition of basal or

serum induced PTHRP gene expression (Abe et al. 1998; Sebag et al. 1992; Liu et al. 1992). Glucocorticoids, but not vitamin D3, were shown to block estrogen stimulated expression of PTHRP in the uterus of ovariectomized rats (Paspalaris et al. 1995). These steroids are thought to exert their effects on gene expression through nuclear receptors that directly interact with elements located in the 5'-flanking region of the PTHRP gene (Kremer et al. 1991; Demay et al. 1992; Falzon 1996). Subsequent attempts to identify specific elements within the PTHRP promoter required for glucocorticoid down-regulation, however, were not successful (Glatz et al. 1994). Nevertheless, it has been shown that glucocorticoids downregulate PTHRP gene transcription from all three promoters (Glatz et al. 1994).

The ability of vitamin D3 to inhibit PTHRP expression has provoked interest for its use to target PTHRP overexpression in malignancy. However, vitamin D3 is itself a calcium mobilizing agent, and increases serum calcium by stimulating bone resorption, increasing intestinal calcium absorption and promoting renal tubular reabsorption (Horlick et al. 1980). Consequently, a rational-based approach to drug design was utilized to develop analogues of vitamin D3 that lacked calcemic bioactivity, while still maintaining the capacity to inhibit PTHRP expression. These analogs were reported to be potent inhibitors of PTHRP expression but had 10-fold less calcemic activity when administered to hypercalcemic animals bearing keratinocyte (Yu et al. 1995) and Leydig cell tumors (Haq et al. 1993). These findings hold therapeutic promise and are currently in Phase I clinical trials (Gulliford et al. 1998).

1.3.4 Regulation in Normal Physiology

The lactating mammary gland is an organ in which PTHRP expression is particularly high. Lactation, which is initiated during the last stage of pregnancy, can be divided into four phases: *milk synthesis* and *lactogenesis*, which involves the synthesis of milk; *galactopoeisis*, which involves maintenance of established lactation; and *milk ejection* involving the release of milk (Courie et al. 1980). The first three of these phases are controlled by prolactin, and milk ejection is mediated by oxytocin (Courie et al. 1980). In the rat, suckling induces a dramatic rise in prolactin synthesis which coincides with a rapid and transient increase in PTHRP expression in the early phase and more sustained increases in later phases of lactation (Courie et al. 1980; Thiede and Rodin 1988; Thiede 1992). Consequently, prolactin may be responsible for inducing production of PTHRP. However, prolactin treatment of non-pregnant rats could only reproduce the early transient phase of PTHRP and not the prolonged expression noted in later phases (Thiede 1989) suggesting that additional factors may interplay to produce sustained PTHRP expression seen in the latter stages of the physiological response.

Uterine occupancy is another physiological stimulus of PTHRP. Elevated levels of PTHRP persist throughout pregnancy and a large peak in both mRNA and protein levels is observed in the myometrium 48 hours before parturition (Thiede et al. 1990; Ikeda et al. 1990). This sudden increase in PTHRP production was reproduced by balloon-induced stretch of the uterus (Daifotis et al. 1992) implying that distension caused by the presence of the fetus may be sufficient to induce PTHRP expression. In addition to stretch, estrogens may regulate PTHRP expression in the myometrium

immediately prior to parturition. Thiede et al. (1991) showed that17 β-estradiol was able to induce a rapid and transient 6- to 8- fold increase in PTHRP mRNA in the uterus of ovariectomized rats. PTHRP expression is also subject to regulation by estrogens in other tissues, including the pituitary and the hypothalamus (Holt et al. 1994; Grasser et al. 1992).

Mechanical stretch has also been shown to induce PTHRP in the urinary bladder (Yamamoto et al. 1992). Distension of the bladder by the accumulation of urine led to dramatic increases in PTHRP mRNA which increased with time and was localized to the distended regions (Yamamoto et al. 1992). Since PTHRP is a potent smooth muscle relaxant (Martin et al. 1997) its induction by mechanical stretch may be linked to this function.

1.3.5 Regulation of PTHRP in Malignancy

a) Regulation by oncogenes

Understanding the molecular and cellular basis for PTHRP overexpression in malignancy is important in determining future avenues for therapy. To define the mechanisms involved in the deregulation of PTHRP production, a number of studies have examined regulation of PTHRP expression by oncogenes. Oncogenes and tumor suppressor genes play a fundamental role in the multi-step process of carcinogenesis and tumor progression. Oncogenes such as Ha- Ras, v-src, v-myc and p53 have been shown to co-operatively transform normal cells into tumor cells (Weinberg 1985; Land et al. 1983; Parada et al. 1984). The process of carcinogenesis can be recreated, *in vitro*, by

artificially transfecting these genes into normal cells. Li and Drucker (1994a) were the first to demonstrate that cellular transformation by oncogenes was correlated with increased PTHRP gene expression. They showed that transfection of oncogenes Ha-Ras and v-src into fibroblasts led to marked increases in levels of PTHRP mRNA (Li and Drucker 1994a). In a later study, Motokura and colleagues also noted that PTHRP expression was induced after co-transfection of oncogenic Ras and p53 genes into normal rat embryo fibroblasts (Motokura et al. 1995). In addition, when cells were only partially transformed by either Ras or p53 gene, only a modest increase in PTHRP secretion was observed (Motokura et al. 1995), suggesting that expression of this gene is closely linked to malignant transformation. Foley et al. (1996) similarly reported that a p53 oncogene increased the level of PTHRP expression in squamous carcinoma cells. Further, a comparison of PTHRP production with the either the presence or absence of a functioning p53 gene in a series of squamous carcinoma cell lines revealed a negative correlation between p53 function and PTHRP mRNA expression (Foley et al. 1996).

The majority of all viral HTLV 1-associated adult T-cell leukemia patients present with HHM (Prager et al. 1994). T-cells, which do not normally produce PTHRP, can be induced to do so by infection with HTLV 1 (Watanabe et al. 1990), indicating that viral infection leads to PTHRP overexpression. A number of investigators have documented that the viral Tax protein can act as a trans-activating factor to stimulate the expression of numerous cellular genes (Green et al. 1989, Green et al. 1991, Joshi and Dave 1992; Alexander et al. 1991). Tax protein has also been shown to stimulate the expression of the PTHRP gene (Dittmer et al. 1993, Ejima et al. 1993). The Tax

protein, which does not directly interact with DNA, is thought to lead to transcriptional activation of the PTHRP gene indirectly by promoting the activity of cellular transcription factors Ets-1 and AP-1 (Dittmar et al. 1993; Ejima et al. 1993). By facilitating the activity of transcription factors the viral transcription protein may lead to overactivity of the PTHRP gene and thus be responsible for the frequent incidence of HHM with adult T-cell leukemia.

These studies stress the important role that oncogenes and cellular transformation play in the overexpression of PTHRP in malignancy. Understanding these mechanisms is pertinent to providing potential targets for treatment of HHM. Investigating the molecular processes that induce PTHRP expression and exploring these as potential therapeutic targets thus became the primary focus of my Ph.D. study.

b) Regulation by methylation

DNA methylation has also been explored as a mechanism that could lead to overproduction of PTHRP in malignancy. Holt and colleagues have correlated the ability to secrete PTHRP with the methylation status of CpG islands within the PTHRP promoter in a series of renal and squamous carcinoma cells (Holt et al. 1993). Demethylation of this region by 5-azacytidine treatment led to increased gene transcription in the cells where expression was previously undetectable. Hypomethylation alone, however, does not always signify a higher level of PTHRP production. In a lung squamous cell carcinoma where the PTHRP gene is substantially methylated, expression of this gene is high. In contrast, expression in a normal lung cell

line where the gene is relatively unmethylated, gene expression is comparatively low (Ganderton et al. 1995; Ganderton and Briggs 1997). Although methylation of DNA can act as a switch to determine whether a gene becomes expressed or not, the process of gene regulation by this mechanism is likely to be more complex than the degree of methylation alone.

1.4 BIOSYNTHESIS AND PROCESSING OF PTHRP

The structure of the PTHRP gene predicts the existence of three initially translated protein products- PTHRP (1-139), PTHRP (1-141) and PTHRP (1-173), which are identical in the first 139 amino acids and differ only in their extended C-termini (Mangin et al. 1988; Yasuda et al. 1989; Broadus and Stewart 1994). It is predicted that these protein products undergo extensive post-translational processing (Skatch and Lingappa 1993; Fricker 1991) adding another layer of complexity to the already complex process of PTHRP gene expression. As in a host of other endocrine or neuroendocrine peptides including proopiomelanocortin (POMC), somatostatin (SRIF), atrial natriuretic peptide (ANP) and cholecystokinin (CCK) (Skatch and Lingappa 1993; Fricker 1991), it is likely that PTHRP may serve as a prohormone from which a family of secreted forms are derived. After translation, like many secreted proteins PTHRP enters the classical secretory pathway (Deftos et al. 1993; Plawner et al. 1995). This begins with signal peptide mediated entrance into the endoplasmic reticulum, a pro-peptide directed traverse through the Golgi stacks and the *trans*-Golgi network, and entry into the transport vesicles of the constitutive secretory pathway, or to the secretory granules where it is subject to regulated secretion (Deftos et al. 1993; Plawner et al. 1995).

1.4.1 Post-translational processing of PTHRP

Although pulse-chase studies have not confirmed endoproteolytic post-translational processing sites in PTHRP, the existence of a signal peptide and propeptide

have been inferred from the cDNA and from analogy with other secreted proteins (Yasuda et al. 1989b; Mangin et al. 1988; Broadus and Stewart 1994). As shown in Fig. 1.1b, the first 36 amino acids of the three PTHRP sequences are predicted to serve as the prepro-sequence. The N-terminal located signal peptide has a hydrophobic core of 10-15 amino acids flanked by charged amino acids. This hydrophobic region is important in permitting attachment to the signal recognition particle, docking and transport of the nascent peptide from the cytoplasmic compartment, where translation takes place, into the cisternae of the rough endoplasmic reticulum (Skatch and Lingappa 1993). As predicted by analogy with other secreted proteins, signal peptidase is thought to cleave the initial PTHRP chain at amino acid Gly^{-13} as it enters the cisternae of the rough endoplasmic reticulum. Cleavage of the signal peptide releases the pro-sequence starting at Arg^{-12}, which is thought to guide the mature protein through the Golgi apparatus to the secretory pathway, and to facilitate its proper folding (Skatch and Lingappa 1993). Pro-sequence cleavage most likely occurs in the Golgi apparatus, and probably involves cleavage at a target sequence Arg^{-4}-Leu^{-3}-Lys^{-2}-Arg^{-1}, which has been shown to be substrate for the furin prohormone convertase (Liu et al. 1995). The resulting mature protein, as predicted from the conserved pro-sequence cleavage site, as well as purified from different tissues by three individual investigators, begins with Ala^{1} (Moseley et al. 1987; Strewler et al. 1987; Stewart et al. 1987). In addition, this region is also homologous with PTH, and functional studies have shown that the first two amino acids Ala^{1} and Val^{2} of PTHRP are critical for its bioactivity (Rabbani et al. 1990).

1.4.2 Secreted forms of PTHRP

Mature PTHRP itself, is a prohormone capable of generating multiple mature daughter peptides (Fig 1.1c). The initial translated product contains several mono- and multi-basic sequences that can serve as cleavage sites for prohormone convertases (Liu et al. 1995; Broadus and Stewart 1994). Processing at these basic sites, which include Arg^{37} and Arg^{106}, is thought to generate at least three peptides each with its own distinct physiological function.

a) The N- terminal peptide.

The existence of an N-terminal PTHRP (1-36) secretory form that results from cleavage at the monobasic residue Arg^{37}, was first demonstrated by Soifer et al. (1992). The purification of a mid-region peptide, beginning at Arg^{38}, by affinity chromatography using anti-PTHRP (37-74) affinity columns, demonstrated that Arg^{37} is a monobasic processing site that can yield PTHRP (1-36). In addition, using monoclonal antibodies directed against the N-terminal synthetic PTHRP (1-34), intermediate and full length fragments, which co-migrated with molecular forms PTHRP (1-86) and PTHRP (1-141) were isolated by high performance liquid chromatography (Rabbani et al. 1992). These N-terminal fragments contain sequences that are structurally homologous to bioactive PTH (1-34) and important in the binding of PTHRP to the PTH/PTHRP receptor (Rabbani et al. 1990).

b) Mid-region peptide.

In addition to the studies of Soifer et al. (1992) described above, the existence of a mid-region peptide beginning at Ala^{38} was further defined in a later study by Wu et al. (1996), who purified the mid-region and by mass spectroscopic analysis and tryptic digestion, identified the fragments PTHRP (38-94), (38-95) and (38-101). Interestingly, the carboxy-terminal ends of these fragments correspond to a basic region between amino acids 80-106, which may serve as sites for endoproteolysis (Wu et al. 1996). Furthermore, the region 38-111 is a highly conserved domain among the many species for which the sequence is known (Halloran and Nissenson 1990; Broadus and Stewart 1994), and will likely have important physiological functions.

c) C-terminal peptide.

As described in the preceding section, a substrate site for prohormone convertases between regions 80-106 may exist, suggesting that a peptide beginning at position 107 is likely to exist. The region 107-111 is evolutionarily conserved suggesting that a peptide containing this region may be physiologically important. Confirming endoproteolytic processing at this site, PTHRP (109-138) fragments have been detected in the circulation of patients with chronic renal failure and HHM (Orloff et al. 1993; Burtis et al. 1990). Further substantiating the relevance of this finding, Fenton et al. (1991) have shown that a synthetic penta-peptide of the region 107-111 was able to inhibit bone resorption, and that N-terminal extension of this peptide reduced its bioactivity. The action of the C-terminal fragment on bone was shown to antagonize the effects of the N-terminal

fragment (Fenton et al. 1991), presenting the intriguing possibility that post-translational processing can produce contrasting effects on the biological activity of a protein.

1.4.3 Glycosylation of PTHRP

PTHRP has been shown to undergo glycosylation in epidermal keratinocyte cells, suggesting that it may exist as a glycoprotein (Wu et al. 1991). Conditioned media from the cells under protease-protection conditions was shown to contain a heavily glycosylated PTHRP form with a molecular weight of ~18 kDa. The glycoprotein nature of this secreted form was confirmed by treating the purified peptide with trifluoromethanesulfonic acid (TFMS), an O- and N-deglycosylating agent, which yielded a deglycosylated core protein with a molecular weight of ~10 kDa (Wu et al. 1991). Since it lacks a consensus sequence for N-glycosylation, PTHRP is thought to be O-glycosylated. O-linked glycosylation occurs in the Golgi apparatus and might play a role in the intracellular sorting and trafficking of proteins (Skatch and Lingappa 1993; Fricker 1991).

1.4.4 Secretion and Degradation of PTHRP

a) PTHRP Secretory Pathways

PTHRP is unusual in that it appears to use both regulatory and constitutive secretory pathways. Early studies on the biosynthesis of PTHRP reported a steady-state release of the protein with very little intracellular storage of newly synthesized forms (Rabbani et al. 1993). This pattern was suggestive of a constitutive mode of secretion

and was in keeping with the production of PTHRP by cell types such as vascular smooth muscle cells, hepatocytes, osteoblasts, keratinocytes, chondrocytes and renal tubular cells (Kremer et al. 1991 ; Rodan et al. 1989) that do not have a system of secretory granules necessary for regulated secretion. However, PTHRP is also produced by endocrine and neuroendocrine cells that use the regulated secretory pathway including pancreatic islet cells, parathyroid cells, adrenal medullar cells, pituitary cells and central nervous system neurons (Care et al. 1990; Drucker et al. 1989; Moseley et al. 1991; Asa et al. 1990; Pang et al. 1988). Furthermore, Brandt et al. (1991) have shown that phorbol ester and ionomycin treatment of carcinoid cell lines led to the secretion of PTHRP into conditioned medium within 10 minutes. This suggests that PTHRP can be stored in intracellular compartments and released upon stimulation of cells. More recently, Plawner et al. (1995) have demonstrated that PTHRP can be secreted via the regulated pathway in the neuroendocrine RIN cells and constitutively by non-neuroendocrine squamous carcinoma and fibroblast cells. A similar secretory "promiscuity" was reported by Yang et al. (1994), who compared secretion between RIN cells and chinese hamster ovary (CHO) cells. The basis for this difference in secretory pathway targeting is thought to lie in the proPTHRP sequence that targets the protein to the regulated pathway in cell lines that possess this mechanism. Secretion by the constitutive pathway in non-neuroendocrine cells, on the other hand, is thought to occur by default. This cell specific mechanism of secretion, which is extremely rare, seems appropriate for a multifunctional and widely expressed protein as PTHRP.

b) Protein Degradation

The highly basic region between amino acids 87 and 106, which serves as a prohormone convertase site for the carboxy-terminal peptide PTHRP (107-139), may also serve to target PTHRP through an intracellular degradation pathway. It has been shown that transfection of cDNA encoding truncated forms of this protein that lack the C-terminus into cells, results in secretion of greater quantity of protein, as much as 60-fold greater than after transfection with cDNA encoding the full length 1-173 protein (Ditmer et al. 1996). These findings suggest that the basic sequences within the carboxy-terminal region may function to direct the protein to a degradation pathway. PTHRP degradation is proposed to occur through the ubiquitin proteosome degradation system. In a cell-free translation system, ubiquitin was shown to bind and degrade prepro-PTHRP in an ATP dependent and proteosome inhibitor sensitive manner (Meerovitch et al. 1997). In transfected cells, degradation of PTHRP was shown to be proteosome-dependent. Proteosomal degradation of PTHRP is thought to involve binding to a chaperone protein, BiP, which facilitates the reverse transport of PTHRP out of the lumen of the endoplasmic reticulum into the cytosol where it accesses the proteosome (Meerovitch et al. 1998). So far, the ubiquitin-dependent mechanism is the only candidate pathway that has been proposed for degradation of PTHRP.

1.4.5 Intracellular targeting

In addition to being secreted through the classical secretory pathways and interacting with cell surface receptors in a paracrine/autocrine fashion, recent studies

have suggested that PTHRP may be able to enter the nucleus directly after translation (Henderson et al. 1994 ; Massfelder et al. 1997). The bi-partite clusters of basic residues 88-91 and 102-106, which, as described above, also serve as putative sites for cleavage by prohormone convertases, are homologous with nuclear targeting sequences found in viral and eukaryotic transcription factors, such as c-Jun and c-Fos, and in growth factors such as members of the fibroblast growth factor (FGF) family (Kiefer and Dickson 1993; Baldin et al. 1990). The paradoxical effects of PTHRP on cell growth, between exogenously added PTHRP (1-34) and stable transfected cDNA, observed in vascular smooth muscle cells, provided further evidence for the functional nuclear targeting of this protein (Pirola et al. 1993; Massfelder et al. 1996). PTHRP has also been identified in the nucleus by immunocytochemistry (Henderson et al. 1994; Massfelder et al. 1997), and nuclear localization was found to be dramatically increased in mitotic cells (Massfelder et al. 1997). Furthermore, deletion of the putative nuclear targeting mutibasic motifs decreased PTHRP staining in the nucleus, and deletion of the signal peptide, which directs the protein to the secretory pathway, but leaving the multibasic sequences intact increased the staining of PTHRP in the nucleus (Henderson et al. 1994). In their study, Massfelder and colleagues also demonstrated that removal of the nuclear targeting sequences reduced the biological effect of PTHRP gene transfection on the vascular smooth muscle cells. The question remains as to how a protein bearing a signal peptide can avoid the endoplasmic reticulum and enter the cytoplasm. However, bFGF and FGF-3, which also have signal peptide sequences, have been reported to escape the endoplasmic reticulum and localize to the nucleus (Kiefer et al. 1993; Baldin

et al. 1990). Studies on processing of PTHRP through proteosome-mediated degradation pathway have suggested that association of PTHRP with a chaperone protein BiP may facilitate its entrance into the cytosol (Meerovitch et al. 1998). An analogous mechanism may be in place to target PTHRP to the nucleus. An alternative mechanism observed with FGF-3 involves a switch from the classic AUG translation initiation site to an upstream CUG codon, resulting in exclusion of the signal peptide (Kiefer and Dickson 1993). An alternative translation initiation site (CUG) exists in exon V of the PTHRP gene, which if used, would allow the signal peptide to be bypassed (Wyslomerski and Stewart 1998). Alternatively, PTHRP protein may be secreted through the secretory pathway, and upon binding to the cell surface receptor be directed to the cytoplasm through receptor-mediated endocytosis and released into the cytoplasm. Intracellular targeting through this mechanism has been reported for other molecules including angiogenin, and acidic and basic FGF (Moroianu et al. 1994; Kiefer and Dickson 1995; Baldin et al. 1990).

Collectively, these findings describe the biochemical properties of a very unique protein. Either the regulated or the constitutive secretory pathway is used depending upon the cell in which it is expressed can secrete PTHRP. The multibasic clusters within PTHRP appear to act as putative sites for enzyme cleavage as well as to control its rate of intracellular degradation. Finally, more C-terminally located multibasic sequences seem to direct the protein into the cytosol and then to the nucleus where it can act through an intracrine mechanism. This phenomenon is highly unusual and has not yet been described for any other protein.

1.5 RECEPTORS FOR PARATHYROID HORMONE-RELATED PROTEIN

Implicit in the preceding sections is the concept that PTHRP is a widely expressed protein with multiple mature secretory forms, each with its own intrinsic bioactivity, specific receptor and signal transduction pathway. A receptor that binds the PTH-like N-terminal peptide has indeed been cloned and putative receptors for the mid- and carboxy-terminal PTHRP secretory forms have been characterized.

1.5.1 Classical PTH/PTHRP receptor

The best-documented effects of PTHRP involve the binding of the N-terminal peptide with the classical PTH/PTHRP receptor. This receptor is thought to mediate the pathogenic effects of PTHRP in HHM. The PTH/PTHRP receptor, which binds both PTH and PTHRP with equal affinity, was originally cloned from the classical PTH targets of opossum kidney, rat bone, and human bone and kidney cells (Jüppner et al. 1991; Abou-Samra et al. 1992; Schipani et al. 1993). Although the cDNA has not been isolated from these tissues, a PTH/PTHRP receptor has also been characterized in non-classical target cells including dermal fibroblasts, embryonic carcinoma cells, vascular smooth muscle cells and human keratinocytes (Pun et al. 1988; Chan et al. 1990; Nickols et al. 1990; Henderson et al. 1991). The receptors in these target tissues are most likely intended for autocrine/paracrine action by PTHRP.

The cDNAs of the PTH/PTHRP receptor isolated from the different mammalian species encode homologous proteins that range between 585-593 amino acids in length. The receptor contains an extracellular amino-terminus, seven transmembrane-spanning

helices and an intracellular carboxy-terminal tail (Jüppner et al. 1991; Abou-Samra et al. 1992; Schipani et al. 1993). Although its overall structure is similar to that of other of G-protein coupled receptors, the PTH/PTHRP receptor has limited sequence homology and a unique gene organization, and belongs to a new family of membrane proteins including calcitonin and secretin receptors, whose functions in mammals have only recently been defined (Jüppner 1995). When expressed in COS cells, the PTH/PTHRP receptor was reported to associate with two second messenger systems, adenylyl cyclase and PLC-β, leading to increases in intracellular cAMP, inositol trisphosphate and free calcium (Abou-Samra et al. 1992). Interaction of the PTH/PTHRP receptor with G-proteins Gs, Gq/11 and to a lesser extent Gi has recently been confirmed by agonist stimulated GTPγ-azidoanilide incorporation in human embryonic kidney cells (Schwindinger et al. 1998). The importance of the C-terminus for interactions with G-proteins was demonstrated by the failure of a C-terminally truncated receptor (515 amino acids) to stimulate an intracellular calcium increase in COS cells (Abou-Samra et al. 1992). Mutation studies revealed additional sites required for effector activation including a requirement for Lys^{319} in the second intracellular loop that is critical for activating phospholipase C, and a region between amino acids 377-384 in the third intracellular loop required for activating both adenylyl cyclase and phospholipase C.

The ligand binding domains of the PTH/PTHRP receptor have been mapped by a series of studies that have used various approaches. Mutation as well as photoaffinity cross-linking studies have identified two regions, one in the extracellular N-terminal domain and another in the third extracellular loop that are essential for ligand binding

(Lee et al. 1994; Mannstadt et al. 1998; Zhou et al. 1997). Jüppner et al. (1994), using receptor chimeras between the rat and human receptors, which exhibit differences in binding affinities for PTH (7-34), found that the N-terminal domain confers most of the higher binding affinity for the ligand. Studies by Fukayama et al. have shown that antibodies directed against the N-terminus of the receptor interfere with ligand binding (Fukayama et al. 1998), confirming the importance of this region in receptor-ligand interactions.

Early studies on the structure-function of the ligand have shown that deletion of the two N-terminal amino acids of PTH or PTHRP results in dramatic loss of adenylyl cyclase-stimulating ability (Rabbani et al. 1988), without adversely affecting activation of phospholipase C and phosphoinositide synthesis (Cosman et al. 1989). More recently, the C-terminal truncated PTH (1-31) has been shown to activate adenylyl cyclase, but not phospholipase C (Takasu and Bringhurst 1998).

In addition to second messenger systems involving adenylyl cyclase and phospholipase C, the interaction of G-protein coupled receptors with mitogen activated protein (MAP) kinases has recently become recognized. The activated PTH/PTHRP receptor has been shown to interact with the MAP kinase pathway; on the one hand to antagonize growth factor mediated MAP kinase activation in a cAMP/PKA-dependent manner in osteosarcoma cells (Verheijen and Defize 1995), and also to lead to activation of MAP kinases when expressed in chinese hamster ovary and prietal yolk sac carcinoma cells (Verheijen and Defize 1997). Activation of MAP kinases has similarly been reported for many G-protein coupled receptors including angiotensin II, somatostatin,

endothelin-1, thromboxane A2, prostaglandin H2, interleukin-8 and LHRH (Sadoshima et al. 1995; Sakanaka et al. 1994; Wang et al. 1992, 1993; Cazaubon et al. 1993; Koide et al. 1992; Morinelli et al. 1994; Watanabe et al. 1995; Knall et al. 1996; Sim et al. 1995). It is possible that activation of MAP kinases by the PTH/PTHRP receptor may mediate its action on such cellular processes as cell growth and differentiation.

1.5.2 Evidence for a Novel N-terminal Receptor

In addition to the classical PTH/PTHRP receptor, a number of findings suggest the existence of other unique N-terminal PTHRP binding receptors. Receptors which are like the PTH/PTHRP receptor by coupling to protein kinase C but differ by not activating adenylyl cylcase have been characterized in pancreatic islet cells, lymphocytes, and mouse epidermal keratinocytes (Gaich et al. 1993; McCauley et al. 1992; Whitfield et al. 1992). The likelihood of another receptor is also suggested by activation of only adenylyl cyclase by PTHRP (1-34) in smooth muscle cells (Orloff et al. 1994). Hence, additional N-terminal PTH/PTHRP binding receptors may exist in these cell types which not only produce and but also respond to PTHRP.

1.5.3 Evidence for Receptors that interact with Mid- and C-terminal Regions

PTHRP undergoes extensive post-translational processing that yields multiple secretory forms. Given that the mid- and C-terminal regions are highly conserved and

share no homology with other known proteins, it is possible that each of these products has its own unique receptor.

a) Mid-region receptor

The intracellular signal transduction pathways of a putative receptor that binds mid-region PTHRP peptides have been characterized (Orloff et al. 1994, Wu et al. 1996). Orloff et al. reported that the mid-region PTHRP (67-86) could mobilize cytosolic calcium and stimulate the formation of inositol trisphosphate in squamous carcinoma cells (Orloff et al. 1994). The cytosolic calcium response was also shown to be additive with PTHRP (1-34), and was not inhibited by the N-terminal receptor antagonist PTHRP (7-34), suggesting that this response may not be mediated by the classical PTH/PTHRP receptor. Expression studies in Xenopus oocytes have shown that mRNA prepared from squamous carcinoma cells leads to the translation of a receptor protein that could mediate an intracellular calcium response to PTHRP (67-86) (Orloff et al. 1996). Similarly, Wu et al. have reported that a mid-region PTHRP (38-94) increases cytosolic calcium levels in pancreatic islet β cells, human carcinoma cells and vascular smooth muscle cells (Wu et al. 1996). Unlike the classical PTH/PTHRP receptor. the cytosolic calcium responses of this putative mid-region receptor are not accompanied by adenylyl cyclase activity or increases in cAMP.

The physiological role of mid-region PTHRP in maintaining maternal-fetal calcium gradient has been known for some time (Care et al. 1990). Collectively, these

findings strongly suggest the existence of a unique mid-region peptide binding receptor that mediates these effects.

b) C-terminal receptor

A putative receptor for the carboxy-terminal form PTHRP (107-111) has been inferred from the inhibitory effect of this region on basal as well as PTHRP (1-34) stimulated osteoclast activity (Fenton et al. 1991). This action of PTHRP was localized to the region 107-111 termed *osteostatin*. This C-terminal region has also been shown to increase intracellular calcium in osteoclasts, and to stimulate membrane-associated protein kinase C activity in lymphocytes and osteosarcoma cells (Fenton et al. 1991 ; Gagnon et al. 1993 ; Whitfield et al. 1994). Peptides that encompassed this region, PTHRP (1-141) and (1-108) were also shown to be more effective in stimulating prostaglandin E2 synthesis in SaOS2 cells, in primary bone cell cultures and cultured fetal rat long bones (Mitnick et al. 1992). Like the mid-region receptor, a C-terminal recognizing receptor with these characteristics has yet to be identified.

1.6 ACTIONS OF PARATHYROID HORMONE-RELATED PROTEIN

The extraordinarily wide tissue distribution of PTHRP, the complex structure of the gene, the high degree of evolutionary conservation, and the unusual abundance of post-translational processing sites all attest to the fundamental role of PTHRP in biology. PTHRP gene ablation in mice that was found to be lethal (Karaplis et al. 1994), further substantiating this claim. In addition to the actions of PTHRP in HHM, multiple physiological roles for PTHRP have been elucidated and many more have yet to be identified. PTHRP has been shown to stimulate transepithelial calcium transport in a wide variety of tissues including renal tubules, mammary gland, placenta, and shell gland (Rizzoli et al. 1989; Rakopoulos et al. 1992; Kovacs et al. 1996; Thiede et al. 1991). PTHRP is also a potent smooth muscle relaxant. This effect has been shown in smooth muscle of the uterus (Shew et al. 1991; Barri et al. 1992), urinary bladder (Yamamoto et al. 1992), arteries (Pirola et al. 1994), stomach and small intestines (Mok et al. 1989a, 1989b). PTHRP may also regulate cellular proliferation, differentiation and apoptosis in a various tissues (Kaiser et al. 1992, 1994; Liu et al. 1995; Pirola et al. 1993; Amling et al. 1997). Furthermore, the abundant expression of PTHRP in fetal tissue, notably in the parathyroids, dermis and epidermis, implies that this protein may have a critical function as a developmental factor.

1.6.1 Actions of PTHRP on Bone and Kidney

It has long been established that PTHRP causes HHM through the direct consequence of its action on bone and in kidney. In HHM, PTHRP increases bone

resorption and renal calcium retention by interacting with the classical PTH/PTHRP receptor. PTHRP has been shown to act on osteoblast-like osteosarcoma cells to increase expression of osteocalcin, cytokines and neutral proteases (Kano et al. 1992). In addition, production of collagen, alkaline phosphatase and osteopontin were shown to be suppressed by PTHRP (Kano et al. 1992). PTHRP has also been shown to inhibit collagen synthesis in avian epiphyseal cartilage cells (Pines et al. 1990). These effects of PTHRP may be mediated through its activation of protein kinase C and inositol trisphosphate second messenger systems that were observed in UMR-106 and ROS 17/2.8 osteoblast-like osteosarcoma cells (Babich et al. 1990; Cosman et al. 1989). PTHRP also interacts with its receptors in bone to promote the proliferation and differentiation of osteoclast precursors resulting in increased osteoclastic bone resorption (Weir et al. 1993). Since osteoclasts do not possess the PTH/PTHRP receptor, their activation by PTHRP is thought to occur indirectly through stimulation of osteoblastic cells to produce osteoclast-activating cytokines (Weir et al. 1993).

In addition to its effect on bone, the action of PTHRP in the kidney is an essential component of HHM. PTHRP was shown to impair sodium-phosphate cotransport, to increase nephrogenous cAMP excretion and reduce the fractional excretion of calcium in renal cortical membranes isolated from rats bearing hypercalcemic Leydig H-500 tumors (Sartori et al. 1988; Sica et al. 1984; Nagasaki et al. 1989). PTHRP has also been shown to stimulate adenylyl cyclase activity and cAMP production in rat and human renal membranes (Orloff et al. 1991). Subsequent release of cAMP into the nephritic lumen is thought to account for the increased nephrogenous cAMP excretion in response

to PTHRP. Intravenous administration of PTHRP (1-34) has been shown to lead to elevated serum vitamin D3 (Fraher et al. 1992), suggesting that PTHRP may also regulate renal 1α-hydroxylase enzyme activity. In contrast to infusion of the N-terminal peptide, elevated plasma PTHRP in HHM is associated with suppressed synthesis of vitamin D3 (Stewart et al. 1980). It is possible that non N-terminal regions of PTHRP may have an inhibitory effect on renal 1α-hydroxylase enzyme.

Renal actions of PTHRP are thought to account for the initial rise in serum calcium in the early stages of HHM (Ralston et al. 1989). In the advanced stages of HHM, the dual action of PTHRP on bone and kidney are combined to produce the more severe hypercalcemia (Ralston et al. 1989).

1.6.2 PTHRP and placental calcium transport

There is substantial evidence supporting a role for PTHRP in regulating placental calcium transport. A gradient of serum calcium exists between the circulation of the fetus where serum calcium is maintain at a higher level, and the mother (Williams et al. 1991). The gradient is sustained by active transport of calcium across the placenta (Williams et al. 1991). It has been known for sometime that PTH is not the primary calcitropic hormone in the fetus, since immunoreactive levels of this hormone are low, while at the same time, PTH-like bioactivity is high. PTHRP produced by the fetal parathyroid glands, has since been identified as the active component that maintains the calcium gradient (Rodda et al. 1988; MacIsaac et al. 1991). In addition to the fetal parathyroids, the placenta is another source of PTHRP. PTHRP produced by the

placenta, is thought to facilitate calcium transport in the early stages of gestation when the parathyroids are not fully functional (Care et al. 1990). There is convincing evidence that the region of PTHRP involved in calcium transport is a mid-region between amino acids 35-85. In parathyroidectomized fetal sheep that are hypocalcemic and lack the fetal-maternal calcium gradient, infusion with a fragment that encompasses the mid-region of PTHRP, PTHRP (1-86), (1-108) or (1-141), but neither PTH nor N-terminal PTHRP (1-34), was able to restore the placental gradient and normo-calcemia (Care et al. 1990). Additional evidence for the role of the mid-region of PTHRP in calcium transport comes from knockout studies of either PTHRP or PTH/PTHRP receptor genes (Kovacs et al. 1996). Lack of a functional PTHRP gene was shown to abolish the fetal-maternal calcium gradient and to slow transplacental transport of calcium. Infusion of PTHRP peptides (1-86) or (67-86), but not (1-34), was able to increase calcium transport in these mice (Kovacs et al. 1996). In mice that lacked the classical PTH/PTHRP receptor gene, Kovacs and colleagues found that placental calcium transport was even more efficient than in the wild type littermates. This indicates that placental calcium transport occurs independently of the PTH/PTHRP receptor, and further implicates the mid-region peptide as the candidate factor.

High levels of PTHRP that are present in the mammary glands during pregnancy and lactation and are also found in milk, imply that this protein may be involved in the transport of calcium into milk (Rakopoulos et al. 1992; Grone et al. 1994; Budayr et al. 1989; Khosla et al. 1990; Law et al. 1991). Clinical studies have shown that lactation is associated with maternal bone loss and renal calcium retention (Kalkwarf and Specker

1995). The level of plasma PTHRP was also high in the circulation of lactating women, reaching levels of up to 10-fold greater than measured in HHM (Grill et al. 1992; Bucht et al. 1995). It is likely that PTHRP acts as an endocrine factor to mobilize maternal bone and promote the retention calcium in the kidneys, for milk production. The induction of PTHRP production elicited by lactation, is thought to occur through stimulation of prolactin synthesis. A correlation has been documented between the circulating levels of prolactin and PTHRP (Sowers et al. 1996), and high levels of circulating PTHRP together with significant bone loss were also reported in non-lactating subjects with hyperprolactinemia (Stiegler et al. 1995).

In addition to its function in mammalian systems, PTHRP is highly expressed in the shell gland of chicken (Theide et al. 1991) and may be involved in the transfer of calcium from the circulation of the hen, across the oviduct to the developing eggshell (Theide et al. 1991).

These studies strongly suggest a role for PTHRP in calcium transport across membranes. The cellular and molecular mechanisms involved in this process have yet to be determined.

1.6.3 PTHRP and Smooth Muscle Relaxation

Mok et al. were the first to show that PTHRP acts as a potent smooth muscle relaxant on gastric and duodenal smooth muscle (Mok et al. 1989a, Mok et al. 1989b). Expression of PTHRP in a variety of smooth muscle beds and the role this protein in relieving muscle contraction has since become established.

a) Vasculature

The action of PTHRP on vascular tissue has fast become an active area of investigation. PTHRP is expressed in arterial smooth muscles in response to mechanical distension of the arterial wall as a result of increased arterial pressure or in response to vasoconstrictors such as angiotensin II (Pirola et al. 1994; Pirola et al. 1993; Winquist et al. 1987; Massfelder et al. 1996). PTHRP is a potent vasodilator in a number of arterial beds including renal, coronary, pulmonary and mesenteric vessels (Massfelder et al. 1996; Mok et al. 1989b; Winquist et al. 1987). This suggests that PTHRP produced by vascular smooth muscle cells and by adjacent endothelial cells, may act locally to counteract vasoconstrictive effects and to regulate regional blood flow.

b) Uterus

PTHRP may also act to induce uterine smooth muscle relaxation during gestation. In the rat uterus, PTHRP expression can be detected as early as day 6 of gestation and increases rapidly during the last stages of pregnancy when fetal growth is rapid (Thiede et al. 1990). This effect has been reproduced by artificially induced stretch, and has also been reported using uterine smooth muscle segments subjected to mechanical stretch in a culture setting (Daifotis et al. 1992). The N-terminal peptide PTHRP (1-34) but not a mid-region peptide was able to induce smooth muscle relaxation in the uterus (Barri et al. 1992) suggesting that this action PTHRP is mediated by the PTH-like domain and possible through the PTH/PTHRP receptor.

c) Oviduct, Bladder and Stomach

Additional targets for PTHRP induced muscle relaxation include the oviduct, bladder and stomach. PTHRP has been shown to relax chicken oviduct muscle (Thiede et al. 1991) and may modulate egg transit through the oviduct. It is intriguing that PTHRP may have a dual function in the oviduct, first to modulate egg laying and also to facilitate calcium transport during calcification of the eggshell as described in section 1.6.2.

In the urinary bladder, where PTHRP production directly correlates with bladder volume, infusion with the N-terminal peptide was found to induce relaxation of bladder muscle strips (Yamamoto et al. 1992). This effect has also been observed in excised gastric strips that have been pre-contracted with acetylcholine or cholecystokinin (Botella et al. 1994). Collectively, these observations implicate a physiological role for PTHRP in regulating of smooth muscle tone and in accommodating mechanical distension.

1.6.4 Role of PTHRP in Skeletal Development

The most convincing evidence for the role of PTHRP in skeletal development comes from knockout studies. Mice homozygous for PTHRP gene disruption exhibit gross abnormalities of endochondral bone formation and disruption of skeletal growth (Amizuka et al. 1994; Karaplis et al. 1994). Accelerated chondrocyte differentiation and early ossification resulting in phenotypic short limbs are among the disturbances in growth and differentiation of chondrocytes observed in the PTHRP knockout mice

(Amizuka et al. 1994). In the same vein, targeted overexpression of PTHRP gene in chondrocytes using the mouse type II collagen promoter, inhibited chondrocyte differentiation, delayed the onset of osteogenesis and the mice were born with a cartilaginous endochondral skeleton (Weir et al. 1996).

A recent study has found that Indian Hedgehog, a protein that is expressed during bone formation and acts to repress terminal differentiation during cartilage development and chondrocyte differentiation, requires PTHRP and the PTH/PTHRP receptor for its action. The evidence for this comes from knockout studies of either PTHRP or PTH/PTHRP receptor genes (Lanske et al. 1996; Vortkamp et al. 1996). Ablation of either PTHRP or PTH/PTHRP receptor genes was shown to confer resistance to Indian Hedgehog and this protein was unable to delay the expression of differentiation markers in *in vitro* bone explants from the deficient mice. These findings indicate that PTHRP and its receptor mediate the effects of Indian Hedgehog, and provide additional proof that PTHRP is critical in bone development.

The role of PTHRP in bone growth and differentiation is also implicit in the clinical observation of short limbed dwarfism in patients with Jansen's metaphyseal chondrodysplasia. This disease has been linked to a mutation within the PTH/PTHRP receptor gene, which results in its constitutive activation (Schipani et al. 1995).

1.6.5 A role in cell growth and differentiation

There is increasing evidence that PTHRP can modulate proliferation and differentiation in a variety of cell types. An interesting correlation exists in the action of PTHRP in promoting cell growth and its role in inducing differentiation. Generally, PTHRP appears to elicit differentiation in non-malignant cells and cell proliferation in malignant cells.

PTHRP was found to induce cell proliferation and to suppress the expression of keratinocyte differentiation markers in a non-malignant human keratinocyte cell line by using antisense RNA technology (Kaiser et al. 1992, 1994). Furthermore, differentiation was induced by PTHRP (1-34) and suppressed by treatment with a PTH/PTHRP receptor antagonist in cells of epidermal lineage (Horlick et al. 1994).

The role of PTHRP in cellular differentiation and morphogenesis is also supported by transgenic studies that examined the effect of targeted overexpression of this gene in the developing epidermis and mammary gland (Wysolmerski et al. 1994; Wysolmerski et al. 1995). Overexpression of PTHRP in basal keratinocytes resulted in arrest of ventral hair follicle development, reduced density of dorsal hair follicles, hyperkeratosis and pigmentation (Wysolmerski et al. 1994). In the mammary gland, overexpression of PTHRP has been documented to prevent ductular proliferation and elongation. This manifests *in vivo* as impairment of side-branching morphogenesis during sexual maturation, and absence of terminal ductule formation during early pregnancy (Wysolmerski et al. 1995). These results suggest that PTHRP may determine morphology and development in these organs.

PTHRP appears to inhibit vascular smooth muscle cell proliferation. PTHRP has been shown to be induced in response to vascular injury (Massfelder et al. 1996; Ozeki et al. 1996). Using immunohistochemical and *in situ* hybridization techniques, Ozeki et al. (1996) have shown that PTHRP expression increased gradually between days 2 to 14 after vascular injury in the rat carotid artery. However, PTHRP has a paradoxical effect in this tissue. Exogenously added PTHRP (1-34) was shown to inhibit the proliferation of cultured primary rat aortic cells (Pirola et al. 1993). In contrast, introduction of the cDNA by stable transfection and endogenous expression of PTHRP was shown to stimulate growth (Massfelder et al. 1996). Nevertheless, these findings may be less of a contradiction and more the outcome of the differential processing and targeting of the endogenously synthesized PTHRP.

The growth promoting effects of PTHRP are often observed in malignant systems. Burton et al. (1990) have shown that inhibiting the actions of PTHRP with receptor antagonists decreased the growth of a human renal carcinoma cells. A study by our laboratory has similarly showed that blocking PTHRP synthesis by expressing antisense orientation RNA in a rat Leydig tumor cell, H-500 resulted in a significant reduction in cell proliferation *in vitro*, and decreased the growth of transplanted tumors *in vivo* (Rabbani et al. 1995). Expression of PTHRP has frequently been shown to accelerate with tumor progression in human carcinomas (Hidaka et al. 1998; Alipov et al. 1997; Nagataki et al. 1995) presenting the possibility that PTHRP may be involved in promoting tumor progression. PTH/PTHRP receptor was found to be co-expressed with

PTHRP in the vast majority of breast and colon carcinomas (Iezzoni et al. 1998) making an autocrine tumor promoting activity possible.

1.6.6 PTHRP and apoptosis

PTHRP has also been implicated as an anti-apoptotic agent. The ultimate fate of hypertrophied chondrocytes is to undergo apoptosis prior to calcification of the cartilaginous matrix. This process was disrupted in transgenic mice with targeted overexpression of PTHRP (Weir et al. 1996). It has been suggested that PTHRP prevents apoptotic death by inducing the expression of Bcl-2 gene, an anti-apoptotic gene, in the differentiating chondrocyte (Amling et al. 1997). Targeted overexpression of PTHRP has indeed been shown to cause the retention of a large proportion of hypertrophic chondrocytes in the developing bones in the transgenic mice (Weir et al. 1996; Amling et al. 1997).

These findings make a convincing argument that PTHRP plays multiple physiological roles. It suffices to say that PTHRP is critical for the growth and development of a healthy organism.

1.7 GROWTH FACTOR SIGNAL TRANDUCTION AND CANCER

Growth factors, cytokines and hormones are players in a highly intricate and coordinated system of cellular control. They are critical mediators of cellular processes including growth, differentiation, migration, apoptosis and transformation, and biological processes including embryogenesis, immune response and wound healing (Schlessinger and Ullrich 1992; Aaronson 1992). The largest group of polypeptides is the family of growth factors including platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin, insulin-like growth factor-1 (IGF-1) and TGFβ. Genetic aberrations affecting growth factor signaling pathways have been linked to a variety of chronic and developmental diseases including a key role in carcinogenesis. Many proteins involved in growth factor signaling were initially identified as oncogenes or tumor suppressor genes in cancer. In this section, I will describe the different components of growth factor signal transduction as they are known today. I will begin with the transmembrane receptor tyrosine kinases (RTKs) that initiate the growth factor signals, the Ras guanine nucleotide (GTP) binding protein where multiple signals that originate at the cell surface converge, and finally the downstream targets of Ras, the mitogen activated protein (MAP) kinase pathways, Rho family of proteins and PI 3-kinase which mediate the growth factor-induced biological response.

1.7.1 Receptor Tyrosine kinases

Growth factors bind and activate a unique set of transmembrane receptors distinguishable by their intrinsic tyrosine kinase activity. These RTKs comprise the largest family of receptors and are divided into more than twelve categories based on their sequence similarity (Ullrich and Schlessinger 1990). The family of RTKs include receptors for PDGF, EGF, FGF, insulin, hepatocyte growth factor (HGF), neurotrophins such as nerve growth factor (NGF) and orphan receptors with unidentified ligands (Aaronson 1992). Many RTKs were initially identified as viral or cellular oncogenes, and still others were cloned by virtue of their sequence or structural homology to other receptors (Sherr et al. 1985; Dickson et al. 1989; Taira et al. 1987; Shibuya et al. 1990; Kraus et al. 1989; Ullrich et al. 1985). All members of this receptor family share a similar molecular structure consisting of an extracellular ligand-binding domain, a hydrophobic transmembrane domain, and an intracellular domain made up of a highly conserved enzymatic tyrosine kinase domain and a target binding C-terminal region (Ullrich and Schlessinger 1990).

a) Receptor tyrosine kinase activation

Growth factor binding to the extracellular domain of RTKs induces receptor aggregation, a molecular interaction that leads to the activation of the receptor tyrosine kinase enzyme (Ullrich and Schlessinger 1990). Although receptor mediated dimerization appears to be a general property of all growth factor receptors, the different receptors undergo dimerization by a variety of mechanisms. Certain growth factors, such

as PDGF, are dimeric molecules, and binding of PDGF to its receptor is thought to bridge two receptors together to form a dimer (Hart et al. 1988). A different mechanism is involved in the activation of the insulin/IGF-1 receptor which already exists as a dimer in its rest state (Ullrich and Schlessinger 1990). Binding of insulin or IGF-1 to this receptor is thought to induce allosteric changes within the already dimerized form In the case of the EGF receptor, binding of a single EGF molecule to one receptor may induce conformational changes that favors the formation of dimers with another ligand occupied receptor (Greenfield et al. 1989; Lax et al. 1991; Weber et al. 1984). The role of receptor aggregation in mediating activation has been demonstrated for EGF (Kashlas et al. 1991), PDGF (Ueno et al. 1991) and FGF receptors (Amaya et al. 1991), by the ability of a kinase defective mutant receptors to act as a dominant negative when co-expressed with the wild-type receptor. This is thought to occur throught the formation of inactive heterodimers.

In addition to activating the receptor, dimerization is critical for intermolecular phosphorylation of tyrosine residues in the C-terminal tail of the receptor (Honeggar et al. 1990). The function of the C-terminal domain is to present substrates for phosphorylation by the adjacent kinase. Phosphorylation of the receptor in this region triggers the recruitment of cytoplasmic target proteins containing Src homology 2 (SH2) domains to specific phosphotyrosine residues (Koch et al. 1991; Heldin 1991; Margolis 1992). These form a complex on the inner surface of the plasma membrane. C-terminal domain mediated translocation of target proteins to the plasma membrane may also act to

bring them closer to molecules involved in transmitting the subsequent signaling steps (Aaronson 1992).

b) Receptor tyrosine kinase substrate interaction

Target proteins that directly associate with the active receptor are activated by phosphorylation. These target proteins include phospholipase Cγ (PLCγ) (Meisenhelder et al. 1989), phophatidylinositol-3-OH kinase (PI 3-kinase) (Kaplan et al. 1987), Ras GTP activating protein (GAP) (Molloy et al. 1989), Src and Src-like kinases (Ralston and Bishop 1985), Shc and Grb2 adaptor proteins (Rozakis-Adcock et al. 1992).

SH2 domains, which represent a conserved region of about 100 amino acids, are hallmarks of tyrosine kinase signaling. Located on the target proteins, they mediate the interaction with the activated receptor (Koch et al. 1991; Heldin 1991; Margolis 1992). In addition to SH2 domains, receptor substrates often contain another conserved motif termed SH3, presumed to act as a membrane or cytoskeletal anchor (Koch et al. 1991). SH2 containing proteins belong to two main classes (Schlessinger and Ullrich 1992). Type I proteins, such as PLCγ and Src, contain SH2 and SH3 domains and have intrinsic enzymatic activity. Upon tyrosine phosphorylation, type I proteins exert their enzymatic activities and transmit the signal to downstream messenger proteins. Type II SH2 containing proteins include Shc and Grb2 and are comprised of only SH2 and SH3 sequences and do not possess kinase enzymatic activity. Type II proteins function as adapters to bring two messenger proteins together or to bind and modulate the activity of other enzymes.

c) Signal specificity of receptor tyrosine kinases

Various aspects of RTK function may contribute to the specificity of the signal generated by a particular receptor. The target binding motifs on RTKs are conserved and selective for specific substrates (Waksman et al. 1993). For instance, the binding motif YMXM is a conserved high-affinity binding site for PI 3-kinase, whereas YVNI motif preferentially binds Grb2 (Seedorf 1995). The presence of different binding motifs may thus be crucial in impart signaling specificity to the different RTKs.

Indeed under physiological conditions, there are major differences in function between RTKs. The insulin receptor controls metabolic processes such as glucose and lipid metabolism in muscle, fat and liver (White and Kahn 1993), whereas NGF is essential for development and survival of sympathetic nerves and a certain population of nerves in the central nervous system (Chao 1992). PDGF and EGF on the other hand, seem primarily to mediate mitogenic responses in variety of different cell types (Heldin and Westermark 1990; Smits et al. 1991; Chao 1992). Despite the different functions displayed by these receptors in their native tissues, when expressed in NIH 3T3 fibroblast cells, they all activate mitogenic signaling pathways (Chao 1992). Tissue distribution of growth factors and their receptors and the cellular context in which they are expressed may thus also contribute to the specificity of signaling and the resulting biological response.

1.7.2 Ras family of proteins

Ras proteins represent a major point of convergence of multiple signal transduction pathways including growth factor receptors and G-protein coupled receptors (Schlessinger 1993). They serve to link transmembrane events to such intracellular messenger proteins as MAP kinases. Since their discovery in human cancers in the 1980s, Ras family of proteins have been intensively studied and are now known to play a critical role in cancer as well as in normal biology (Der et al. 1982; Santos et al. 1982; Parada et al. 1982). As a result of these studies, we now know that Ras proteins control an extraordinarily diverse set of cellular processes in biology, including regulating many aspects of growth and differentiation, cytoskeletal organization and vesicle trafficking (Bourne et al. 1987; Kosravi-Far and Der 1994).

a) Biochemistry of Ras

Ras proteins belong to a superfamily of small molecular weight guanine nucleotide binding proteins with intrinsic GTPase activity (Gilman 1984; Temeles et al. 1985; Gibbs et al. 1984; McGrath et al. 1984; Sweet et al. 1984). Members of this superfamily are tightly regulated binary switches that are turned on by GTP binding, and turned off by intrinsic GTPase mediated hydrolysis of GTP to GDP. The GTPase activity of these proteins is subject to regulation by guanine nucleotide releasing factors (GNRFs) such as son of sevenless (SOS), guanine nucleotide exchange factor (GRF) and Ral-GDS that catalyze formation of the active GTP-bound form of Ras (Maekawa et al. 1993; Feig 1994; Bollag and McCormick 1991). Inactivation of Ras is mediated by factors that

stimulate the intrinsic GTPase activity of the protein and the conversion of the GTP-bound active form to the GDP-bound inactive form. In mammalian systems these GTPase activating proteins (GAPs) include rasGAP and neurofibromin (NF1) GAP (Boguski and McCormick 1993).

Mammalian Ras proteins are encoded by three highly homologous genes, H-*Ras*, N-*Ras* and K-*Ras*, and two pseudogenes H-*Ras*-2 and K-*Ras*-1 that all encode a 21 kDa protein product (DeFeo et al. 1981; Ellis et al. 1981; Parada et al. 1982; Santos et al. 1982; Der et al. 1982). For signaling purposes, there are no known differences in the function of these proteins. However, differences in their transforming potential has been disclosed and appears to be cell type dependent (Maher et al. 1995). Ras proteins are expressed in virtually all cells, although to a certain extent, cell type preferences exist between the different isoforms (Barbacid 1987). Also, there are cell cycle associated differences in the expression of these genes, such that the highest level of Ras is expressed in proliferating cells (Denhardt et al. 1994).

Ras proteins are relatively simple in structure. They consist of a conserved catalytic domain, a GTP binding site and two 'switch' regions close to the γ-phosphate group of the bound GTP (Barbacid 1987). Upon GTP binding, these switch motifs undergo conformational change. The GTP binding region is close to the N-terminus where the Ras effector binding site is also located. Substrates of Ras such as rasGAP, Raf-1, and PI 3-kinase have been shown to directly interact with this region in a GTP-dependent manner (Duchesne et al. 1993; van Aelst et al. 1993; Rodriguez-Viciana et al. 1994).

Ras genes can become persistently activated by single point mutations in their coding sequences (Tabin et al. 1982; Reddy et al. 1982; Taparowski et al. 1982). These include codons12,13, 59, 61, located in the GTP-phosphate binding region of Ras (Tabin et al. 1982; Reddy et al. 1982; Taparowski et al. 1982; Bos et al. 1987; Tsuchida et al. 1982; Yuasa et al. 1983). *In vitro* mutagenesis studies have shown that mutations in codons 63, 116-119 and 146 in the GTP-base binding region can also confer insensitivity to GTPase stimulation by rasGAP (Fasano et al. 1984; Walter et al. 1986; Sigal et al. 1986). Consequently, these mutant Ras proteins are locked in the active GTP-bound state and are constitutively activated.

b) Membrane attachment of Ras

Ras is a membrane anchored protein, and its localization to the inner surface of the plasma membrane is critical for its function (Willingham et al. 1980; Willumsen et al. 1984; Fujiyama et al. 1986). Attachment to the membrane requires a post-translational modification and the acylation of Cys^{186} located in a C-terminal CAAX motif (a cysteine followed by two aliphatic amino acids) (Sefton et al. 1982; Chen et al. 1985; Buss et al. 1986; Fujiyama et al. 1986). In the case of Ras proteins, these modifications involve the addition a farnesyl group, and for other members of the superfamily such as Rap and Rho proteins a geranylgeranyl group (Lowy and Willumsen 1993). Subsequent to the addition of these polyisoprenyl moieties, the three carboxy-terminal amino acids -AAX are removed and post-translational processing is completed by carboxylmethylation of the cysteine (Takai et al. 1992). The resulting hydrophobic

tail of processed Ras is then inserted into the plasma membrane and anchors the protein in the proximity of its regulators and effectors (Takai et al. 1992).

c) Activation of Ras by Receptor Tyrosine Kinases

The best characterized signaling event downstream of RTKs involves the activation of Ras (Leevers and Marshall 1992; Ruderman 1993). The activated receptor creates a phosphotyrosyl binding site for SH2 containing proteins such as the adaptor proteins Shc and Grb2 (Ullrich and Schlessinger 1990; Schlessinger and Ullrich 1992). Upon association with the activated receptor, Shc becomes phosphorylated creating a recognition site for the SH2 domain of Grb2. Grb2 can either bind the phosphorylated SH2 binding site on Shc or associate directly with the activated receptor. Nevertheless Grb2, which is stably associated with the Ras guanine nucleotide exchange factor SOS, translocates to the plasma membrane to where Ras is anchored. SOS stimulates a GDP to GTP exchange and leads to the activation of Ras. Activated Ras then relays the signal into the cytoplasm.

d) Role of Ras in cancer

Ras genes are the most frequently identified oncogenes in human cancer (Bos 1989; Lemoine 1990; Rodenhuis 1992; Hunter 1997). The frequency of Ras mutations varies considerably between tumor types. Ras mutations occur in greater than 90% of pancreatic tumors (Almoguera et al. 1988), 40-50% of colorectal carcinomas (Bos et al. 1987), 30% of lung adenocarcinomas (Rodenhuis and Slebos 1990) and about 25% of

acute myeloid leukemias (Ahuja et al. 1990). In contrast, only 5% of breast and renal carcinomas have been associated with mutations in this gene (Dawkins et al. 1993; Rochlitz et al. 1992). The role of Ras in cancer, however, reaches far beyond the occurrence of activating mutations. Since Ras proteins a point of convergence for multiple intracellular signals, deregulation of multiple second messenger proteins can also lead to Ras activation.

The important role of Ras in cancer has spurred many drug companies to search for specific inhibitors of this protein. Since membrane attachment of Ras is essential for its function, this step has been the target of attempts to interfere with Ras function. Indeed, many successful inhibitors of Ras have thus been developed.

1.7.3 Ras signal transduction pathway

Ras proteins are well known for their regulatory role in oncogenic, mitogenic and developmental signaling. The downstream signal transduction cascades initiated by Ras that mediate the actions of Ras are beginning to be understood. GTP-bound form of Ras has been shown to directly bind and/or activate a number of effector proteins. These include Raf-1 (van Aelst et al. 1993), the catalytic subunits of PI 3-kinase (Rodrigues-Viciana et al. 1994), PKCζ (Diaz-Meco et al. 1994), Ral GDS (Urano et al. 1996), rasGAP (Adari et al. 1988) and MEKK1 (Russell et al. 1995). A number of the downstream targets of Ras including MAP kinases, Rho GTPases, and PI 3-kinase, have been shown to be required for Ras-dependent cellular transformation. In the following section, these three targets of Ras are described.

a) MAP kinases

The MAP kinase family of proteins are comprised of dual serine/threonine and tyrosine phosphorylated signaling enzymes that link cellular signals to changes in gene expression and protein synthesis (Fig. 1.2) (Lewis et al. 1998; Blenis 1993). Members of this serine/threonine family of kinases include i) extracellular signal-regulated kinases (ERKs) 1 and 2 (Boulton et al. 1991; Boulton and Cobb 1991), ii) NH_2-terminal Jun kinase/stress-activated protein kinases (JNKs) α, β, δ (Derijard et al. 1994a; Kyriakis et al. 1994) and iii) p38 MAPKs α, β, δ, γ (Rouse et al. 1994; Han et al. 1994). Activation of the ERK group of MAP kinases by Ras has been shown to require the direct interaction of Ras with Raf proteins (van Aelst et al. 1993; Vojtek et al. 1993; Warne et al. 1993; Zhang et al. 1993). Relatively less understood is the mechanism whereby Ras can activate JNK and p38 kinases. Several lines of evidence suggest that GTPases of the Rho family may be downstream targets of Ras that mediate the activation of JNK and p38 pathways (Qui et al. 1995; Khosravi-Far et al. 1995; Prendergast et al. 1995; Coso et al. 1995; Minden et al. 1995).

i) ERK pathway

The ERK pathway may participate in the regulation of a variety of cellular processes. Activation of this pathway was shown to be necessary for Ras-induced transformation (Troppmair et al. 1993; Kosravi-Far et al. 1996). Constitutive activation of ERKs was sufficient to induce cells to acquire a transformed phenotype and become tumorigenic (Mansour et al. 1994). GTP-bound Ras initiates activation of the ERK

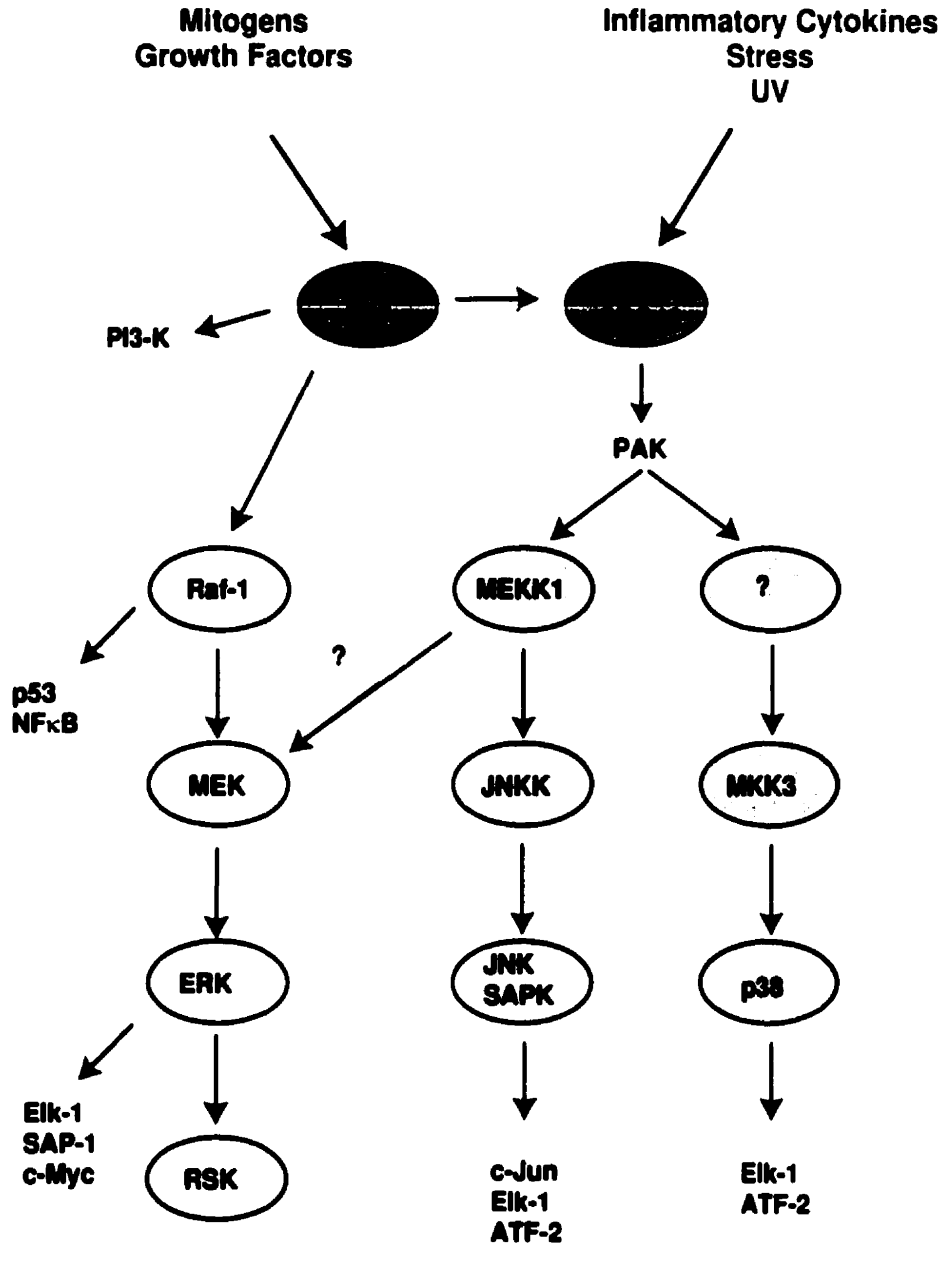


Figure 1.2: Multiple Mitogen Activated Protein (MAP) Kinase Pathways.

pathway by direct interaction with the serine/threonine kinase, Raf-1 (van Aelst et al. 1993; Vojtek et al. 1993; Warne et al. 1993; Zhang et al. 1993). Association of Raf-1 with Ras promotes membrane translocation of the otherwise cytoplasmic Raf-1, where subsequent events lead to activation of its kinase function. This process is complex and incompletely understood. Once activated, Raf-1 stimulates the downstream dual-specificity kinase. MAP kinase kinases (MEK)1 and 2, which leads to threonine/tyrosine phosphorylation and activation of ERKs 1 and 2 (Minden et al. 1994). Following their activation, ERKs translocate to the nucleus and regulate the activity of key enzymes and transcription factors which in turn regulate the expression of target genes (Marais et al. 1993). ERK substrates include nuclear transcription factors Elk-1, c-Myc, a serum response factor accessory protein (SAP)-1 and the cAMP response element binding protein (CREB) (Marais et al. 1993; Hipskind et al. 1994). Elk-1 and SAP-1 are thought to form a complex with the serum response factor (SRF) on the serum response element (SRE), to regulate the expression of immediate early genes such c-*fos* (Marais et al. 1993). ERKs can also activate other kinases, termed MAP kinase activated protein (MAPKAP) kinases, such as the p90-ribosomal S6 kinase (RSK) serine/threonine kinase (Sturgill et al. 1988). RSK may phosphorylate several nuclear and cytosolic proteins such as c-Fos, CREB and ribosomal protein S6, to regulate gene expression and protein synthesis (Ginty et al. 1994; Blenis 1993).

ii) JNK pathways

Whereas the ERK pathway is classically associated with growth stimulatory responses, the JNK pathway is strongly activated by various stresses and cytokines that result in apoptosis (Xia et al. 1995; Verheij et al. 1996; Chen et al. 1996; Yang et al. 1997). JNK proteins are activated in response to inflammatory cytokines, such as lipopolysaccharides (LPS), proinflammatory cytokines interleukin-1(IL-1) or tumor necrosis factor (TNF) α, ultra violet (UV) radiation, heat shock or osmotic stress and protein synthesis inhibitors such as cycloheximide and anisomycin (Derijard et al. 1994a; Kyriakis et al. 1994; Han et al. 1994; Raingeaud et al. 1995; Chen et al. 1996; Newton et al. 1997). Oncogenic Ras can also augment JNK activity (Denhardt et al. 1994a). Ras induced activation of JNK has been shown to require two members of the Rho family of GTPases, Cdc42 and Rac (Coso et al. 1995; Minden et al. 1995).

A sequential cascade of MEKK1 and SEK1 activates JNK (Yan et al . 1994; Minden et al. 1994; Lin et al. 1995). $P21^{ras}$-activated kinases (PAKs), which are stimulated by Rac and CDC42, have been implicated to link the Rho proteins to the MEKK1-SEK1-JNK cascade (Bagrodia et al. 1995; Zhang et al. 1995; Frost et al. 1996). Other Rho proteins such as Rho A have been shown to activate JNK independently of PAK, suggesting that activation of PAK may not be the only mechanism of activating JNK (Teramoto et al. 1996).

JNK may also have functions beyond responding to cellular damage and apoptosis. Indeed, there is a considerable body of evidence supporting the role of JNK in cell growth and oncogenic transformation. JNK has been shown to be necessary for cell

growth (Bost et al. 1997), and for transformation induced by Met, Bcr-Abl, and Ras oncogenes (Rodrigues et al. 1997; Raitano et al. 1995; Clark et al. 1997)

Once activated, JNK protein kinases translocate to the nucleus where they phosphorylate the transcription factor domains of various nuclear factors including activating transcription factor (ATF)-α and -2 , Elk-1, SAP-1, c-Jun and JunD nuclear transcription factors (Derijard et al. 1994a; Derijard et al. 1994b; Coso et al. 1995; Gupta et al. 1995). Phosphorylation of these nuclear factors by JNK is thought to increase their transcriptional activity and mediate expression of JNK regulated genes.

iii) P38 MAP kinase

Another stress-activated pathway that is regulated downstream of Ras, is the p38 MAP kinase pathway. P38 is a MAP kinase with a unique Thr-Gly-Tyr motif that is Thr/Tyr phosphorylated by the MKK3 dual specificity kinase (Rouse et al. 1994). Like JNKs, p38 kinases are activated in response to osmotic shock, UV light, and inflammatory cytokines (Raingeaud et al. 1995). Like JNK, p38 is activated by Rac1, CDC42 and PAK proteins (Bagrodia et al. 1995; Zhang et al. 1995). Although tremendous analogy exists between JNK and p38 pathways, several key differences imply that these two pathways may be regulated independently. Unlike JNK, p38 is not significantly activated by Ras (Raingeaud et al. 1996). Furthermore, MEKK3 and MEKK6 preferentially phosphorylated p38 kinase in response to osmotic stress whereas JNK is preferentially activated by MEKK4 (Yan et al. 1994; Raingeaud et al. 1995). A recently identified MEK, Tpl-2, which has been shown to activate ERK and JNK through

MEK1 and SEK1, failed to activate p38 kinase (Salmeron et al. 1996). In the same vein, p97-germinal center kinase (GCK) which leads to MEKK4 and JNK activation, does not activate p38 MAP kinase (Pombo et al. 1995). Finally, although p38 kinases can activate both ATF-2 and Elk-1, they do not phosphorylate c-Jun or JunD (Raingaud et al. 1995).

b) Cross-talk between MAP kinase pathways

The delineation of these linear MAP kinase signaling pathways linking cell surface receptors to the nucleus is a remarkable achievement in cellular signal transduction. The identification of these pathways is however only the first step in understanding the complexity of serine/threonine kinase signaling. The linear MAP kinase pathways represent a minor component of a more complex circuitry, with the frequent occurrence of cross-talk between members of distinct pathways. For example, in addition to regulation by activated growth factor tyrosine kinase receptors, MAP kinase activity can be modulated by receptor coupled to heterotrimeric G-proteins. In G-protein signaling, the Gα subunit generated cyclic adenosine 3'- 5'-monophosphate (cAMP) and protein kinase C pathways have been shown to modulate the activity of Raf-1 kinase independently of Ras (Stevenson et al. 1993; Kolch et al. 1993). Cross-talk between the mitogen activated ERK and stress activated JNK pathways has been documented. MEKK1, which classically activates JNK, was shown to activate the MEK-ERK pathway. Although this was observed with a high concentration of MEKK1 in an *in vitro* kinase assay, the existence of this interaction *in vivo* has not been excluded (Xu et

al. 1995). Further upstream, Rac1 and Cdc42 have been shown to cooperate with Raf-1 to sequentially activate MEK and ERK (Lewis et al. 1997). In the opposite direction, constitutively active MEK1 has been shown to stimulate JNK activity in U937 human leukemic cells (Franklin and Kraft 1995). Adding to the complexity of serine/threonine pathways, new Raf-1 substrates have been identified including transcription factors NFkB and p53, which may provide an alternative mechanism linking Raf-1 to the nucleus to directly influence the expression of genes (Li and Sedivy 1993; Jamal and Ziff 1994).

c) Inhibitors of MAP kinase pathways

Two kinds of inhibitors have been used in studies involving MAP kinase. The first are inactive mutants that function as dominant negatives. These have been developed for MEKKs 1- 4 and 6, ERKs, JNK and p38 MAP kinase (Alawi et al. 1993; Frost et al. 1994; Raingeaud et al. 1996). The second group of MAP kinase inhibitors are cell permeable chemical compounds. For example, PD098059 is a selective inhibitor of MEK1 and 2, and SB203580 and SB202190 are anti-inflammatory drugs, which were found to inhibit p38 activation (Lee et al. 1994; Bayaert et al. 1996). Given the importance of MAP kinase pathways in developmental, mitogenic and oncogenic signaling, the development of new ways to inhibit these pathways will aid signal transduction research and may provide clinical strategies for treatment of diverse diseases including cancer.

d) Rho family of proteins and the cytoskeleton

Rho family of small GTPases are a recent addition to the downstream effectors of Ras. The Rho family of proteins, which are a major branch of the Ras superfamily, share approximately 30% amino acid homology with Ras, and 50% within its own family (Khosravi-Far and Der 1994; Khosravi-Far et al. 1998). This group of small GTPases are implicated in inflammation response, apoptosis and the organization of actin cytoskeleton (Hall 1992). Mammalian Rho proteins represent more than 11 members, including RhoA, RhoB, RhoC, RhoD, RhoE, RhoG, Rac1, Rac2, CDC42, TC10 and TTF (Kosravi-Far et al. 1998). These members of the Rho family can each generate unique changes in cytoskeletal elements. For instance, activation of CDC42 promotes the formation of shaft-like filopodia and microspikes (Kosma et al. 1995), activation of Rac induces membrane ruffling and formation of curtain-like lamellipodia and activated Rho promotes stress fibers and focal adhesions (Gauthier-Rouviere et al. 1998). Since transformed cells are associated with a disarrayed actin cytoskeleton and are, as a result, capable of anchorage-independent growth (Khosravi-Far 1998; Hunter 1997), the importance of these proteins that control cytoskeletal arrangement in cell transformation and the invasive phenotype has become recognized.

The interaction between Ras and Rho proteins has come to be referred to as the 'GTPase cascade' since it involves a sequential activation of more than one member of these GTPases (Khosravi et al. 1998). Although a direct interaction between Ras and Rho proteins has not been shown, Ras induced changes in actin cytoskeletal organization that is necessary for transformation, was shown to require the activity of Rac1 and

sequentially, those of other Rho GTPases (Ridley and Hall 1992, Ridley et al. 1992). In addition, co-expression of dominant negative mutants of the following Rho proteins: Rac1, RhoA, RhoB, or CDC42 with activated Ras resulted in a suppression of Ras-induced transformation (Qui et al. 1995; Khosravi-Far et al. 1995; Prendergast et al. 1995). Activation of Rac1, RhoA, RhoB, and CDC42 have also been shown to be sufficient for transformation in Rat1 and NIH3T3 fibroblasts (Qiu et al. 1995; Khosravi-Far et al. 1995; Prendergast et al. 1995).

Rho GTPases have been shown to activate JNK and p38 MAP kinase pathways (Coso et al. 1995; Minden et al. 1995). Their function in Ras-induced cytoskeletal changes, however, is thought to occur independently of these pathways (Khosravi-Far et al. 1998). It appears that Ras activated multiple Rho proteins, acting through a mechanism independent of JNK or p38, are responsible for Ras-induced changes in cellular morphology.

e) PI 3-kinase

Phophoinositide-3-OH kinase (PI 3-kinase), a lipid kinase, is another candidate to mediate the effects of Ras on actin cytoskeleton. PI 3-kinase is activated by many growth factor receptors and is thought to exert its cellular effects through the elevation of phosphoinositol 3,4,5-trisphosphate levels in the cell (Carter and Downes 1992). Structurally, PI 3-kinase consists of two related subunits: a p85 subunit, which regulates the enzymatic activity of the kinase, and a catalytic p110 subunit required for kinase function. Growth factor receptors, by interacting with the p85 subunit, are thought to

allosterically increase the activity of the p110 subunit (Carter and Downes 1992). Interaction of PI 3-kinase with growth factor receptors leads to translocation to the plasma membrane where its lipid substrates are located. It has recently been suggested that growth factor receptors may only modestly stimulate PI 3-kinase activity and that interaction of the p110 subunit with Ras is required for optimal activation (Rodrigues-Viciana et al. 1994). In support of this, Rodrigues-Viciana et al. (1994) have shown the direct high-affinity interaction of Ras with p110 subunit of PI 3-kinase. They also showed that the interaction was GTP-dependent and was attenuated by an effector domain mutation of Ras. This was the initial evidence suggesting that PI 3-kinase may be a downstream target of Ras. It has since been substantiated by additional findings. For instance, Gold et al. demonstrated that expression of dominant negative Ras could block growth factor and cytokine stimulated phosphorylation of inositides by PI 3-kinase (Gold et al. 1994). Hence, it appears that PI 3-kinase is a downstream target of Ras.

PI 3-kinase has been shown to be required for growth factor induced changes in actin cytoskeleton similar to those mediated by the GTPases Rac and Rho. Membrane ruffling induced by PDGF (Wennstrom et al. 1994), insulin and IGF-1 (Kotani et al. 1994) and oncogenic Ras (Rodrigues-Viciana et al. 1994) were all sensitive to inhibition by the PI 3-kinase inhibitor wortmannin and blocked by overexpression of a dominant negative mutant of p85 subunit. Furthermore, expression of an activated PI 3-kinase was shown to induce actin reorganization analogous to Rac-mediated lamellipodia and focal complexes, and Rho-mediated stress fibers and focal adhesions (Reif et al. 1996). It is

thus likely that PI 3-kinase exerts its cytoskeletal effects though stimulation of Rac and Rho mediated pathways.

The several lines of evidence I have outlined suggest the involvement of PI 3-kinase in mediating Ras-induced changes in cell morphology. PI 3-kinase is also required for other cellular events including insulin stimulated glucose transport (Yamamoto-Honda et al. 1994), NADP-oxidase function in neutrophils (Downard 1997), and for DNA synthesis stimulated by a number of growth factors (Roche et al. 1994). The extent to which these functions contribute to Ras-induced cellular effects has yet to be determined.

1.8 Objective of Thesis

Understanding the mechanisms which determine the inappropriate expression of the PTHRP gene in malignancy is important for providing the tools with which to approach therapy of HM. Growth factors and oncogenes, which are key players in malignant transformation, have been shown to activate PTHRP gene expression and may lead to its inappropriate expression of in malignancy. The objective of this thesis is to confirm previous findings, that components of the growth factor signaling pathway are involved in the regulation of PTHRP gene expression and to further extend their findings by identifying critical cross-roads in growth factor signal transduction required for the regulation of this gene. Inhibition of important regulatory pathways is an approach that can be used to interfere with the production of PTHRP in malignancy and provides a novel tool for the treatment of HM.

In chapters 2, 3 and 4 of the thesis are presented three studies that investigate the regulation of PTHRP gene expression. The objectives of these three studies are as follows:

1) To investigate the role of a growth factor receptor derived oncogene in the regulation of PTHRP production and to explore intracellular signaling pathways that may be involved.

2) To directly examine and establish the role of Ras in the production of PTHRP *in vitro* and on the development of hypercalcemia *in vivo*, and to explore the use of a potent and specific Ras farnesylation inhibitor as a potential therapeutic agent.

3) To examine the pathways that mediate serum activation of PTHRP gene expression, to further confirm the role of Ras in this process and explore the requirement of downstream MAP kinase signaling pathways.

CHAPTER 2

INCREASED PARATHYROID HORMONE-RELATED PEPTIDE (PTHRP) PRODUCTION BY A TYROSINE KINASE ONCOGENE, TPR-MET: ROLE OF THE RAS SIGNALING PATHWAY

2.1 PREFACE

PTHRP is produced in most tissues and is overproduced by certain types of tumors. Understanding the events that lead to the overexpression of this gene is of great clinical significance. This study was aimed at elucidating the molecular mechanisms that govern PTHRP overexpression in cancer. To approach this question, we chose as our model the hepatocyte growth factor (HGF) receptor derived oncogene, Tpr-Met, transfected into fibroblast cells. Tpr-Met was originally identified as a result of gene rearrangement of the HGF receptor, in a human osteosarcoma cell line treated *in vitro* with a chemical carcinogen (Cooper et al. 1984; Park et al. 1986). Tpr-Met provided an ideal oncogene model for study of PTHRP gene expression because its intracellular signaling mechanisms have been extensively delineated and Tpr-Met mutants impaired in specific signal transduction pathways were available for study. The fact that the hepatocyte growth factor receptor, Met, and PTHRP are involved in similar physiological processes including branching morphogenesis and bone remodelling was also taken into consideration. In this Chapter, the mechanism of PTHRP gene regulation by Tpr-Met was investigated. This study is presented herein in the form of a published paper (*Am J Physiol* 271:E277-83, 1996). Tpr-Met and mutant Tpr-Met/Tyr^{489} transfected fibroblasts were kindly provided by Dr. Morag Park, McGill University, Montreal, Canada.

2.2 ORIGINAL ARTICLE

2.2.1 Abstract

We have used the Tpr-Met oncogene as a model to examine signaling pathways of growth factors and tyrosine kinase oncogenes, which can increase parathyroid hormone-related peptide (PTHRP) production. PTHRP production, in Tpr-Met transfected cells, when assessed by Northern blot analysis and radioimmunoassay, was increased 4-8 fold. Treatment of these cells with the transcriptional inhibitor, actinomycin D, and nuclear run-off assays showed that the major cause of increased PTHRP mRNA was enhanced gene transcription. To analyze the intracellular signaling molecules involved in PTHRP production, stable cell lines expressing a Tyr^{489} Phe mutant of the Tpr-Met oncoprotein were examined. The mutant fails to activate phosphatidylinositol (PI)-3 kinase or associate with the Grb-2 adaptor protein and caused a significant reduction in PTHRP production. Treatment of wild-type Tpr-Met transfected cells with wortmannin, a PI 3-kinase inhibitor, had no effect on PTHRP production, however, treatment of these cells with lovastatin, an inhibitor of $p21^{ras}$ isoprenylation, significantly reduced PTHRP expression. These results show that PTHRP is a downsteam target of the Tpr-Met oncogene and indicate that the PTHRP stimulating activity is mediated via the Ras signaling pathways.

2.2.2 Introduction

Parathyroid hormone related peptide (PTHRP) is the major pathogenetic factor responsible for the syndrome of hypercalcemia of malignancy. In addition to its production by tumors associated with hypercalcemia such as squamous cell carcinomas of the lung, head and neck, and renal cell carcinomas, PTHRP has also been identified in fetal and adult non-neoplastic tissues (Moseley et al. 1987; Rabbani et al. 1986; Stewart et al. 1987). In these tissues PTHRP is believed to play an important role in various physiological functions including a role in modulating cellular growth and differentiation (Burton et al. 1990; Li and Drucker 1994a). PTHRP is the product of a complex gene which can result in the production of various isoforms of 139, 141 and 173 amino acids in length which arise due to alternate splicing in humans (Yasuda et al. 1989a). In contrast, only a single 141 amino acid isoform is present in rodents (Karaplis et al. 1990; Yasuda et al. 1989b). Within its amino terminal region, PTHRP has a high degree of sequence homology with parathyroid hormone (PTH), and as a result PTHRP and PTH act through a common PTH/PTHRP receptor and share similar biological effects (Jüppner et al. 1991; Rabbani et al. 1988). One frequent characteristic of a variety of human and rodent cell lines, is the capacity of growth factors and cytokines to regulate PTHRP production (Haq et al. 1993; Liu et al. 1993). In these cells, PTHRP gene expression is rapidly induced and the mRNA encoding PTHRP then rapidly disappears. This rapid turnover of the transcript is presumably due to the presence of multiple AUUUA motifs located in the 3′ untranslated region of PTHRP mRNA which are commonly found in mRNAs involved in cell proliferation and differentiation (Shaw and

Kamen 1986). These kinetics of PTHRP gene expression in response to growth factors is analogous to that of early response genes (Cochran 1993).

In order to investigate the molecular mechanisms of PTHRP induction by growth factors, we have examined the signal transduction pathways involved in its stimulation by an oncogenic derivative of the hepatocyte growth factor/scatter factor receptor (*met*), Tpr-Met. Tpr-Met was first isolated from human osteosarcoma cells treated with N-methyl-N'-nitro-N-nitroso-guanidine (Park et al. 1986) and is the product of a genomic rearrangement event which juxtaposes the tyrosine kinase domain of the *met* receptor downstream of sequences encoded by the translocator promoter region (*tpr*) gene. Physiologically, ligand activation of receptor tyrosine kinases promotes receptor dimerization and autophosphorylation on tyrosine residues. Autophosphorylation increases the catalytic activity of the receptor and provides docking sites for substrate proteins containing SH2 domains which transduce the signal to the interior of the cell (Park et al. 1986; Rodrigues and Park 1994). However, in the case of Tpr-Met, the ligand binding and transmembrane domains of *met* have been replaced with *tpr* sequences which contain leucine zipper motifs predicted to mediate protein dimerization (Rodrigues and Park 1994). *Tpr* mediated dimerization of Tpr-Met appears to be sufficient to maintain autophosphorylation and constitutive activation of the kinase.

In the present study we have used the Tpr-Met oncogene as a model to investigate (i) the capacity of a protein tyrosine kinase oncogene to induce PTHRP production and (ii) to examine intracellular signaling pathways which are important for increased PTHRP production. To examine the intracellular signaling molecules involved in

activating the PTHRP gene we have employed tyrosine to phenylalanine mutants of Tpr-Met that have been previously shown to be impaired in their ability to interact with the substrates Grb-2, phosphatidylinositol (PI)-3 kinase and an unknown protein of 110 kDa (Fixman et al. 1995). Furthermore, the effect of inhibiting PI 3-kinase, or blocking isoprenylation (farnesylation) of Ras on PTHRP production by wild-type Tpr-Met was determined.

2.2.3 Materials and Methods

Cell culture

Cell lines expressing the wild-type and mutant forms of Tpr-Met were generated by ecotrophic retroviral infection as previously described (Rodrigues and Park 1993). Transient transfection of the pLXSN retrovirus vector encoding the Tpr-Met gene and G418 resistance, and of the pSV-Y-E-MLV plasmid encoding retroviral packaging proteins into COS-1 cells was performed by the DEAE-dextran method. At 12 h after transfection, cells were treated with 100mM chloroquine for 3 h and incubated for a further 48 to 72 h before harvesting. Transfected COS-1 cells were lysed and the supernatant containing the packaging virus at a titre of 5×10^3 plaque forming units was used to infect Fischer rat (Fr) 3T3 cells. The mutant form Y489F was prepared by site directed mutagenesis of the Tpr-Met to change tyrosine 489 to phenylalanine (Fixman et al. 1995). Experimental cell lines were then selected for G418 resistance and foci formation.

For growth curves, Fr 3T3 cells were plated in 9.6 cm^2 petri dishes (Falcon) at seeding densities of 50,000 cells/plate. For 1 week, every 24 h, cells from replicate dishes which were cultured in the presence of 2% fetal bovine serum (FBS) were trypsinized, resuspended, and counted in a model Z Coulter counter (Coulter Electronics, Beds, United Kingdom). Medium was changed in all plates every 2 days.

PTHRP Radioimmunoassay (RIA)

Immunoreactive (i) PTHRP secreted into culture medium by control and exprimental cells was measured using an NH_2-terminal RIA (Henderson et al. 1989). Cells were grown to 80% confluence and incubated with medium containing 0.1% FBS for 48h. The conditioned medium was collected and frozen immediately and cells were trypsinized and counted to determine cell number using a Coulter counter. Immediately before the assay, aliquots of conditioned medium were dried in a SpeedVac (Savant Instruments Inc., Hicksville, NY) and then reconstituted with serum for quantitation in an RIA using ^{125}I-labelled [Tyr^0]PTHRP (1-34) and an antiserum with specificity for the NH_2-terminal fragment PTHRP(1-34). Synthetic rat PTHRP (1-34) was used as the assay standard. PTHRP levels were expressed as nanogram (ng) equivalents relative to PTHRP (1-34). All PTHRP levels were corrected for cell number.

Northern blot analysis

Total cellular RNA was isolated from the control and experimental cells by acid guanidium thiocyanate-phenol-choroform extraction as previously described (Torczynski

et al. 1983). Twenty μg of total cellular RNA was electrophoresed on a 1.1% agarose-formaldehyde gel, transfered to a nylon membrane (Nytran) by capillary blotting and then fixed by air drying and UV cross-linking for 10 min. The integrity of the RNA was assessed by ethidium bromide staining. Hybridization was carried out with ^{32}P-labeled PTHRP cDNA and with 18S RNA probe using α^{32}P dCTP and γ^{32}P ATP as previously described (Liu et al. 1993; Torczynski et al. 1983; Yasuda et al. 1989a). After a 24 h incubation at 42^{0}C, filters were washed twice under low stringency conditions (1xSSC and 1% SDS; at RT for 40 mins) and under high stringency conditions (0.1xSSC, 0.1% SDS; at 55^{0}C for 40 mins). Autoradiography of filters was carried at -70°C using XAR film (Eastman Kodak Co., Rochester, NY) with two intensying screens or by using phosphorimager screen (Eastman Kodak Co., Rochester, NY). The level of PTHRP expression was quantified by densitometric scanning using the Mac BAS V1.01 alias program.

Nuclear run-off assay of gene transcription

For nuclear run-off assays cells were harvested, after treatment with various agents, in cold PBS. These cells were then lysed in cold NP-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40) for 5 min on ice. Cell nuclei were collected by centrifugation at 4 C, and resuspended in storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol). The nuclei were either used immediately or frozen in liquid nitrogen for later use. Nuclear run-off assays were carried out by adding 100 μl nuclear suspension (2-4 x 10^7 nuclei) to

100 µl reaction buffer (50 MM Tris-HCl, pH 7.5, 0.3 M KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM each of ATP, CTP, and GTP) and 50-100 µCi [^{32}P]α-uridine trisphosphate, >600 Ci/mmol, (ICN, Costa Mesa, CA) for 60 min at room temperature. After the incubation, DNase I (150 U per reaction) and proteinase K (0.2 mg/ml) were added sequentially and incubated for 30 min at 37°C, respectively (Liu et al. 1993). Newly synthesized RNAs were isolated by spin column and ethanol precipitated, and pelleted by centrifugation. RNAs were hybridized with PTHRP and an 18S RNA probe and with pGEM vector DNA (Stratagene, La Jolla, CA) previously immobilized on Nytran membranes, using a slot blot manifold (Bio-Rad, Richmond, CA). These membranes were incubated in the hybridization solution (6 x SSC, pH 7.4, 50% formamide, 1% SDS, 0.1 mg/ml sonicated salmon sperm DNA) at 42°C for 48 h. After hybridization, membranes were washed in a final wash solution of 0.1 x SSC, 0.1% SDS at 42°C, and exposed to Kodak XAR film (Eastman Kodak Co, Rochester, NY) with intensifying screens. The intensity of each band was quantified by scanning the films by laser densitometry.

Statistical Analysis

Statistical analysis was done by one way analysis of variance or by Student's *t* test.

Figure 2.1. Comparison of growth curves of wild-type Fr 3T3 cells (●), Fr 3T3 cells infected with vector only (■), and Fr 3T3 cells infected with the experimental vector Tpr-Met (♦).

Cultures were seeded at a density of 5.0×10^4 cells per dish and incubated for the indicated periods of time in standard culture medium. Cells from triplicate dishes were trypsinized and counted as described in "Materials and Methods". Each point represents the mean ± SEM of four different experiments. Significant differences from wild-type Fr 3T3 cells and Fr 3T3 cells infected with the control vector are represented by asterisks * ($^*p<0.05$).

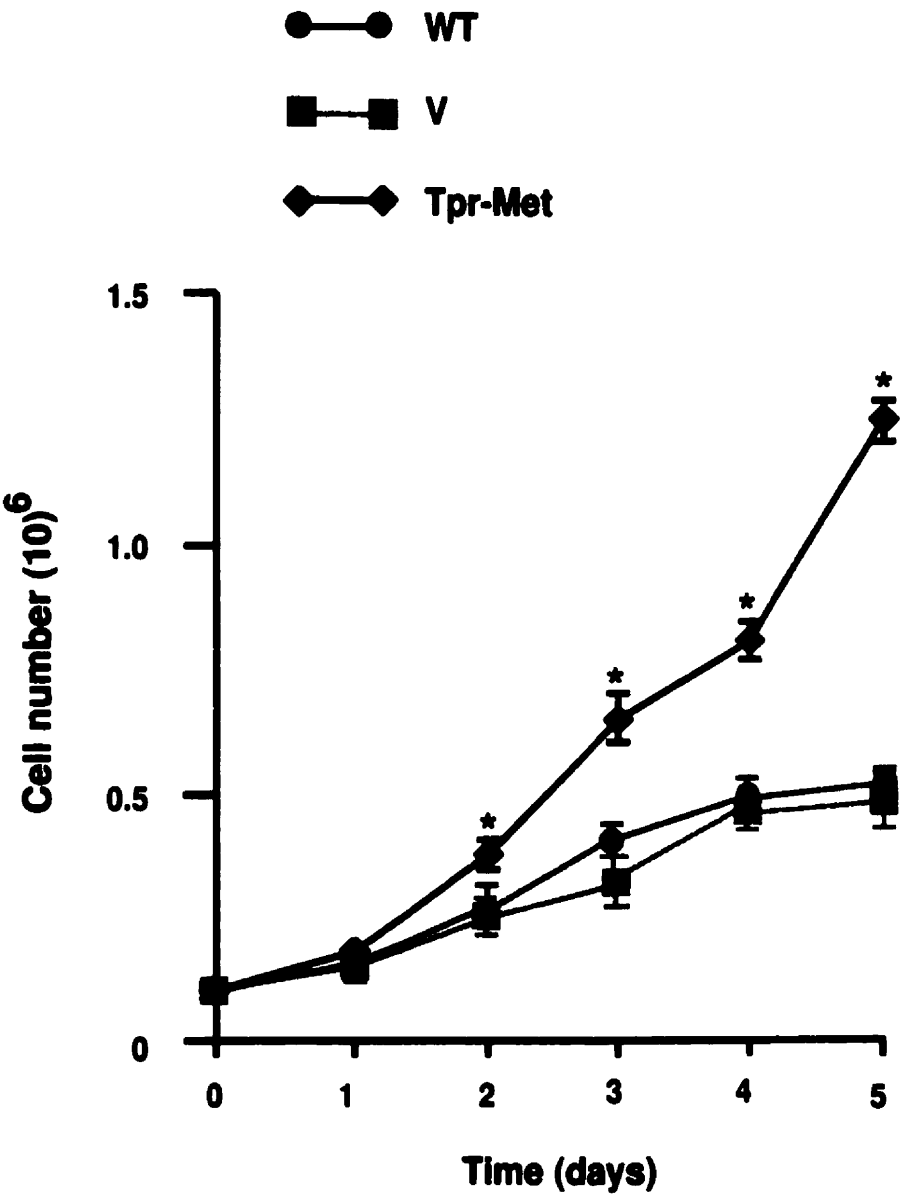

WT
V
Tpr-Met
1.5
1.0
0.5
0
Cell number (10)6
0
1
2
3
4
5
Time (days)
*
*
*
*

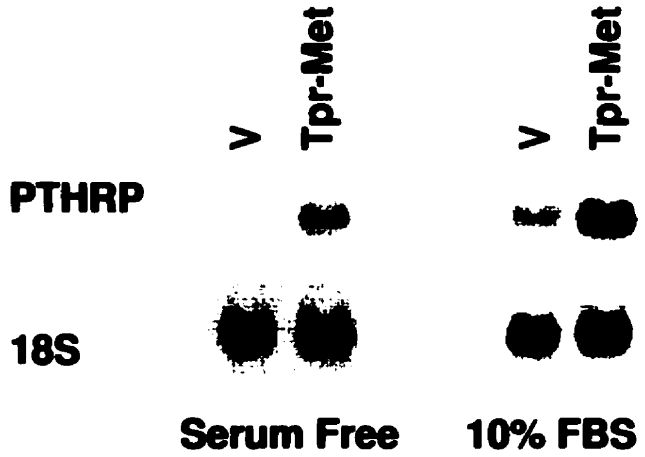
Figure 2.2. Effect of Tpr-Met expression on PTHRP production.

Panel A: Northern blot analysis of Fr 3T3 cells infected with vector alone (V) or with experimental vector (Tpr-Met). Cells were grown in the absence of serum (serum free) or in the presence of 10% fetal bovine serum (FBS). Twenty μg of total cellular RNA was extracted and electrophoresed on a 1.1% agarose formaldehyde gel. Filters were probed with a ^{32}P labelled PTHRP cDNA or with a ^{32}P labelled 18 S RNA probe as described in "Materials and Methods". All blots were quantified by densitometric scanning (lower panel)

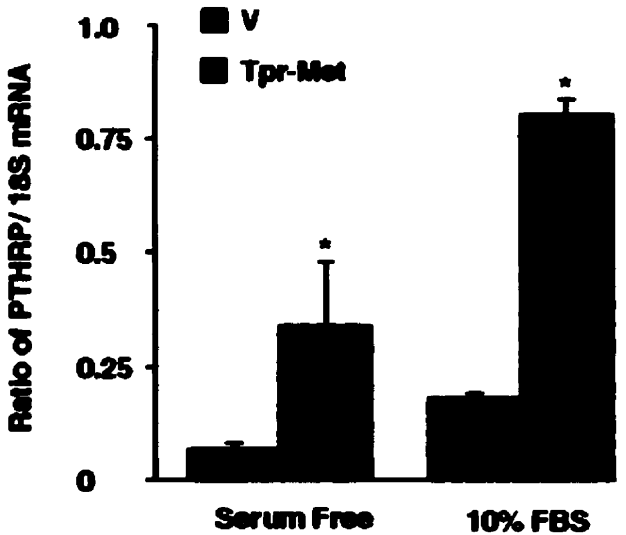
Panel B: Conditioned culture medium from Fr 3T3 cells infected with vector alone (V) or with experimental vector (Tpr-Met) was collected after 48 hrs and analyzed for PTHRP immunoreactivity as described in "Materials and Methods".

Significant differences from control cells are represented by asterisks * ($^{*}p<0.05$).

A.


V
Tpr-Met
V
Tpr-Met
PTHRP
18S
Serum Free
10% FBS

B.


V
Tpr-Met
Ratio of PTHRP/ 18S mRNA
1.0
0.75
0.5
0.25
0
*
*
Serum Free
10% FBS

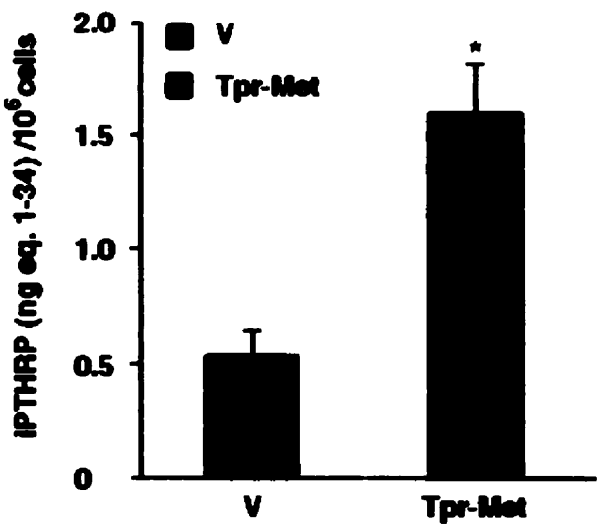
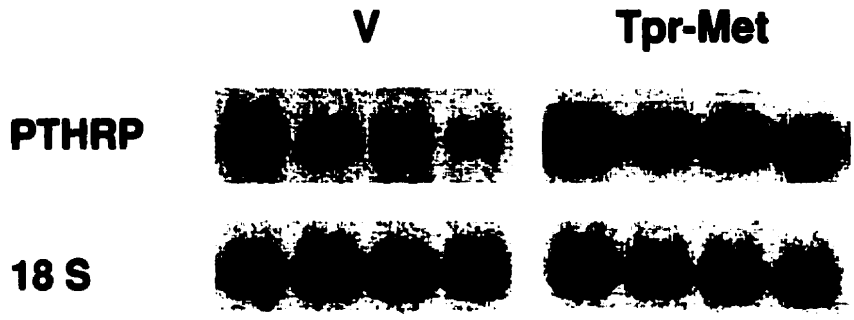

V
Tpr-Met
iPTHRP (ng eq. 1-34) /10^6 cells
2.0
1.5
1.0
0.5
0
*
V
Tpr-Met

Figure 2.3. Stability of PTHRP mRNA transcripts following incubation of cells with the transcriptional inhibitor actinomycin D.

Fr 3T3 cells infected with vector alone (V) or with the experimental vector (Tpr-Met) were grown to 70% confluence and incubated with 5 μg/ml of actinomycin D for the indicated time periods. 20 μg of total cellular RNA was extracted from different cells at the various times and analyzed by Northern blot analysis for PTHRP mRNA expression and for 18S mRNA as a control for RNA loading (upper panels). Ratio of PTHRP/18S mRNA was assessed by densitometric scanning and plotted as a function of time of exposure to actinomycin D to determine the half-life of PTHRP transcripts (lower panel).


V
Tpr-Met
PTHRP
18 S

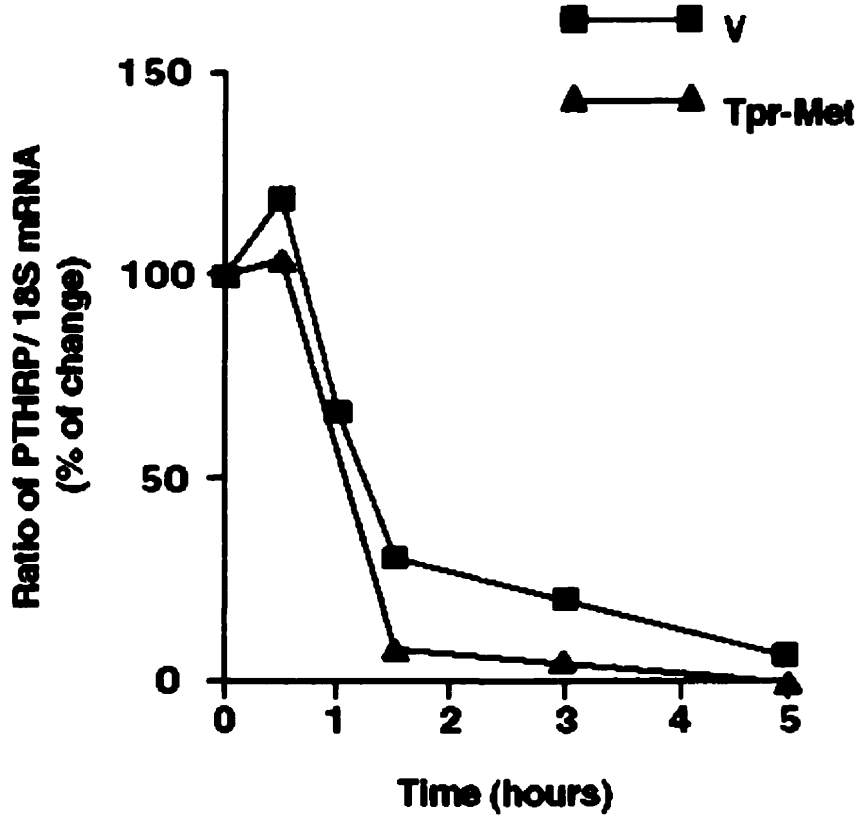
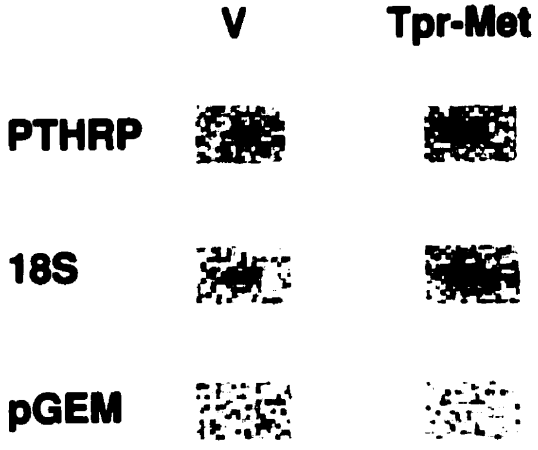

V
Tpr-Met
150
100
50
0
Ratio of PTHRP/ 18S mRNA
(% of change)
0
1
2
3
4
5
Time (hours)

Figure 2.4. Effect of Tpr-Met on PTHRP gene transcription.

Nuclear run-off assays were performed as described in "Materials and Methods". ^{32}P labelled run off transcripts were prepared from nuclei isolated from Fr 3T3 cells infected with vector alone (V) or with experimental vector (Tpr-Met). Probes used were PTHRP cDNA, pGEM and 18 S RNA (upper panels). All autoradiographs were scanned by laser densitometry and the % increase in transcription of PTHRP relative to 18S RNA was determined (lower panels).

Significant differences from control cells are represented by asterisks * ($^{*}p<0.05$).


V
Tpr-Met
PTHRP
18S
pGEM

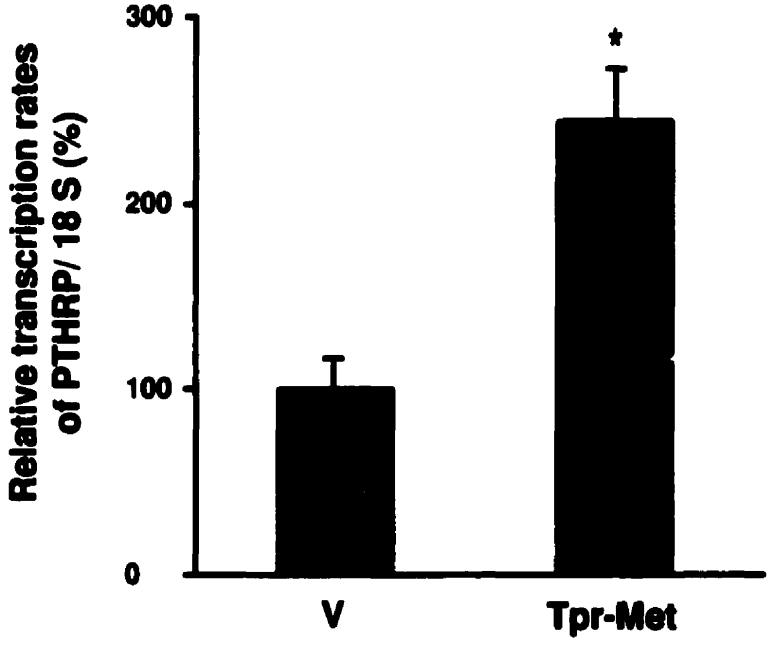

Relative transcription rates of PTHRP/ 18 S (%)
300
200
100
0
*
V
Tpr-Met

Figure 2.5. Effect of mutation of Tpr-Met at tyrosine 489 on PTHRP production.

Panel A : Northern blot analysis of 20 μg of total cellular RNA from Fr 3T3 cells infected with vector alone (V), from cells infected with vector containing wild-type Tpr-Met (Tpr-Met) and from cells infected with vector containing mutant Tpr-Met (Y489F). All blots were probed with an 18S cDNA as control for RNA loading. The ratio of PTHRP/18S mRNA was assessed by densitometric scanning.

Panel B : Conditioned culture medium from vector infected cells (V), cells infected with vector containing wild-type Tpr-Met and cells infected with vector containing the mutant (Y489F) Tpr -Met oncogene was collected and tested for PTHRP immunoreactivity as described in "Materials and Methods".

Significant differences from control Tpr-Met transfected cells are represented by asterisks (*) (*$p<0.05$).

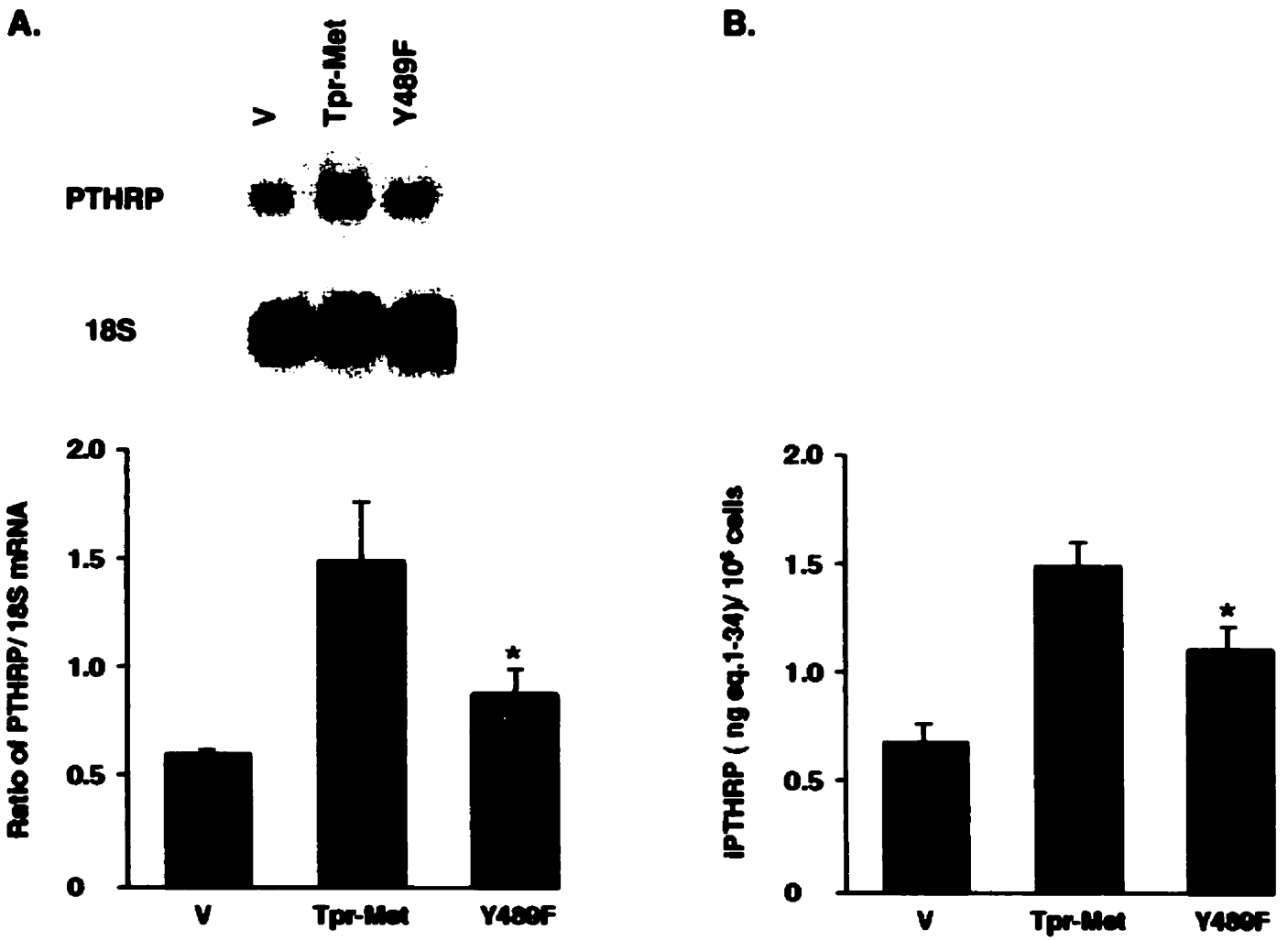

A.
V
Tpr-Met
Y489F
PTHRP
18S
Ratio of PTHRP/18S mRNA
2.0
1.5
1.0
0.5
0
V
Tpr-Met
Y489F
*
B.
IPTHRP (ng eq.1-34)/ 10⁶ cells
2.0
1.5
1.0
0.5
0
V
Tpr-Met
Y489F
*

Figure 2.6. Effect of a PI 3-kinase inhibitor on PTHRP gene expression.

Fr 3T3 cells expressing Tpr-Met were grown to 70% confluence. After 24 h of incubation in serum free medium, cells were treated with the PI 3-kinase inhibitor wortmannin (50-100 ng/ml) in Dulbecco's modified Eagle's medium (supplemented with 10% fetal calf serum) for 6 h. The medium was replaced with fresh medium containing wortmannin after 3 h. 20 μg of total cellular RNA was extracted and analyzed by Northern blot analysis to determine any change in PTHRP mRNA expression from control cells treated with vehicle alone (0). The ratio of PTHRP/18S was assessed by densitometric scanning.

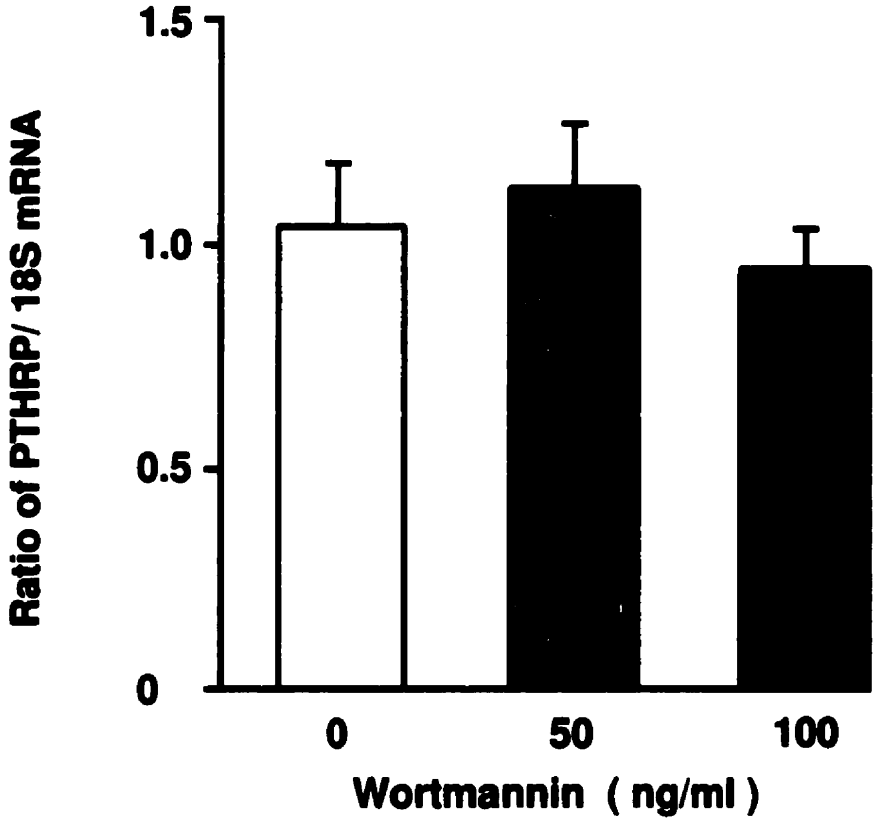
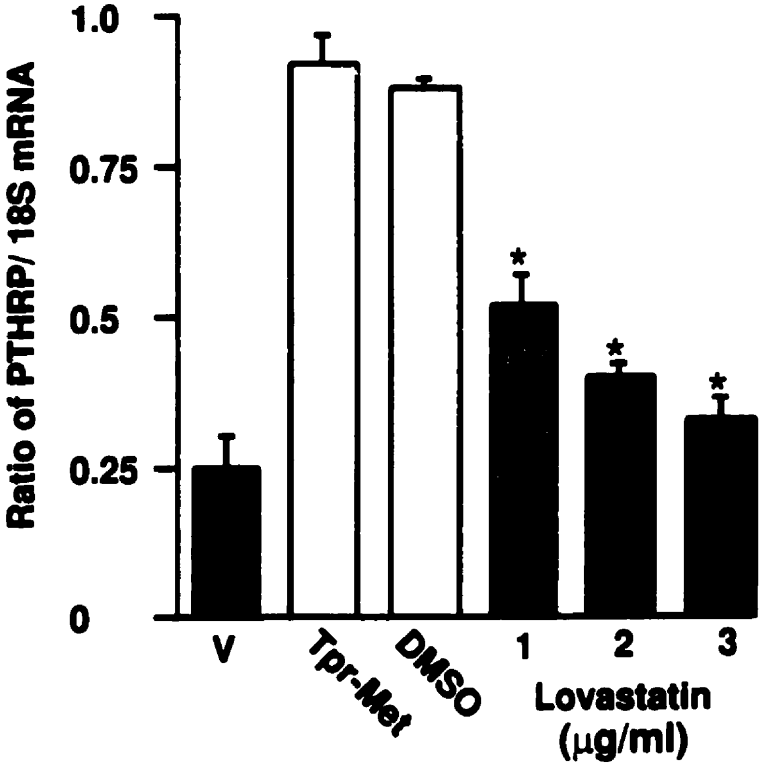

1.5
1.0
0.5
0
Ratio of PTHRP/ 18S mRNA
0
50
100
Wortmannin (ng/ml)

Figure 2.7. Effect of a Ras inhibitor, lovastatin, on the capacity of the Tpr-Met oncogene to induce PTHRP gene expression.

Fr 3T3 cells expressing Tpr-Met were grown to 70% confluence. After 24 h incubation in serum free conditioned medium, cells were treated for 24 h with an inhibitor of *Ras* farnesylation, lovastatin (1-3 μg/ml), in Dulbecco's modified Eagle's medium (supplemented with 10% FBS). DMSO (0.1%) was used as a vehicle for lovastatin and was included as a control. 20 μg of total cellular RNA was extracted and analyzed by Northern blot analysis to determine any change in PTHRP mRNA expression from control untreated cells or from Tpr-Met expressing cells treated with vehicle (DMSO) only. V refers to cells infected with vector alone. The ratio of PTHRP/18S mRNA was assessed by densitometeric scanning. Significant differences from control cells are represented by asterisks (*) (*$p<0.05$).


Ratio of PTHRP/ 18S mRNA
1.0
0.75
0.5
0.25
0
V
Tpr-Met
DMSO
1
2
3
Lovastatin
(μg/ml)
*
*
*

2.2.4 Results

Effect of Tpr-Met on cell growth

The effect on Fr 3T3 cell growth was examined *in vitro* following infection with the retroviral vector containing the Tpr-Met oncogene. Comparison of cell growth was made with uninfected wild-type 3T3 cells and with cells infected with vector alone. Infection of vector alone into Fr 3T3 cells did not produce any significant change in their growth as compared to wild-type cells. In contrast, cells infected with the experimental vector showed a marked increase in cell growth (Fig. 1). This increase in cell growth due to expression of Tpr-Met was evident after 2 days of culture and cells continued to show reduced cell doubling time over the course of experiment.

Effect of Tpr-Met expression on PTHRP production

To determine whether expression of Tpr-Met oncogene resulted in any change in PTHRP production, we examined the level of PTHRP mRNA in Fr 3T3 fibroblasts infected with experimental vector or with the vector alone. Both control and experimental cells were grown in the absence of serum or in the presence of 10% FBS and screened with PTHRP cDNA as a hybridization probe. Expression of Tpr-Met in Fr 3T3 cells caused a significant (4-fold) increase in PTHRP mRNA, both in the absence and in the presence of serum (Fig. 2A). Consequently the effect of Tpr-Met was independent of growth factors present in serum. This increase in PTHRP mRNA was accompanied by a

similar increase in PTHRP released into the cell conditioned culture medium as determined by an NH_2-terminal PTHRP RIA (Fig. 2B).

Analysis of the stability of PTHRP mRNA transcripts

A change in mRNA production can result from alteration either in its mRNA stability or in its rate of gene transcription. In order to explore the mechanism of the Tpr-Met associated increase in PTHRP mRNA in Fr 3T3 fibroblasts, we first examined the effect of Tpr- Met on the stability of PTHRP mRNA. Both control and experimental cells were treated with the transcriptional inhibitor, actinomycin D for 1-5 hours. Total cellular RNA was extracted and the effect of Tpr-Met transfection on mRNA stability determined. PTHRP mRNA transcripts displayed a relatively short half life (75-90 minutes) in Fr 3T3 fibroblasts with no significant change in stability following transfection with Tpr-Met oncogene (Fig. 3). These results demonstrate that induction of PTHRP production in these experimenal cells involved a mechanism other than an increase in mRNA stability.

Effect of Tpr-Met on PTHRP gene transcription

To explore other molecular mechanisms involved in Tpr-Met induced PTHRP mRNA expression, we have determined the rate of PTHRP gene transcription by nuclear run off assays. These were performed in cellular nuclei isolated from Fr 3T3 cells transfected with vector alone or with experimental plasmid. Nascent transcripts from control and experimental Fr 3T3 cell nuclei were isolated and labelled with ^{32}P followed

by hybridization with PTHRP cDNA. A significant increase in PTHRP gene transcription was seen in experimental cells transfected with Tpr-Met as compared to control cells transfected with vector alone (Fig. 4). These results demonstrate that enhanced PTHRP production in experimental Fr 3T3 cells is due to the ability of Tpr-Met to cause an increase in the rate of PTHRP gene transcription.

Effect of the Tpr Met mutant Y489F, on PTHRP production

Substitution in Tpr-Met of tyrosine 489 to phenylalanine (Y489F), has no significant effect on its tyrosine kinase activity (Fixman et al. 1995). However, the Y489F mutant is impaired in its ability to bind and activate PI-3, in its capacity to associate with Grb2 adaptor protein and in its ability to transform Fr 3T3 cells (Fixman et al. 1995). We therefore assessed this mutant in stimulating PTHRP production. The Y489F mutant displayed a significantly reduced capacity to induce PTHRP mRNA when compared to the wild-type Tpr-Met (Fig. 5A). Analysis of conditioned culture medium from control and experimental cells also revealed a significant decrease in the amount of PTHRP released into conditioned culture medium as determined by an NH_2-terminal RIA (Fig. 5B).

The effect of wortmannin, a PI 3-kinase inhibitor on PTHRP induction by Tpr-Met

To determine if PI 3-kinase was specifically involved in Tpr-Met oncogene induction of PTHRP, we assessed the capacity of wortmannin, an inhibitor of PI 3-kinase to alter PTHRP production in Tpr-Met infected Fr 3T3 cells. Incubation of cells with 50 ng/ml wortmannin for 30 minutes has previously been reported to be sufficient for

significant PI 3-kinase inhibition (Powis et al. 1994). However, in our study treatment of experimental Fr 3T3 cells with 50-100 ng/ml wortmannin for up to 6 hr failed to cause any significant decrease in PTHRP mRNA expression (Fig. 6). Furthermore, a concentration of wortmannin as high as 500 ng/ml for as long as 9 h was without effect on PTHRP mRNA (results not shown).

Effect of an inhibitor of isoprenylation on PTHRP production

To investigate the importance of the Ras signaling molecule in mediating induction of PTHRP by Tpr-Met, Fr 3T3 fibroblasts transfected with Tpr-Met were treated with either vehicle alone (0.1% DMSO) or with various concentrations (1-3 μg/ml) of lovastatin. Lovastatin has previously been shown to inhibit farnesylation of Ras, in the presence of 10% FBS for 24 h (Sinensky et al. 1990). In experimental Fr 3T3 cells, lovastatin inhibited the capacity of Tpr-Met to induce PTHRP mRNA expression in a dose dependent manner. Treatment of experimental cells with 3μg/ml of lovastatin reduced the level of PTHRP mRNA to close to that observed in control 3T3 cells infected with vector alone. At all concentrations of lovastatin used in this study there was no evidence of cellular toxicity and lovastatin had no effect on PTHRP gene expression in control vector-transfected cells (data not shown).

2.2.5 Discussion

Previous studies from our laboratory and from others have demonstrated the capacity of growth factors, particularly EGF and IGF to enhance the production of PTHRP in various cell lines (Liu et al. 1993; Sebag et al. 1994). The effect of these ligands via their receptor tyrosine kinases appears to occur at least in part through enhancement of gene transcription (Liu et al. 1993). Hepatocyte growth factor (HGF) also known as scatter factor (SF) is a multifunctional cytokine which exerts pleiotropic effects in a wide variety of normal and neoplastic cells. The receptor for HGF/SF has been identified as the product of the Met proto-oncogene (Bottaro et al. 1991), a receptor tyrosine kinase initially isolated as the cellular homologue of an activated oncogene, Tpr-Met (Park et al. 1994). We have employed the Tpr-Met oncogene to examine the mechanisms and signaling pathways involved in enhancing PTHRP production in a model cell line, Fr 3T3 fibroblasts. Constitutively active Tpr-Met substantially increased PTHRP mRNA expression as well as immunoreactive PTHRP secretion in this cell line. The mechanism of this effect appeared to be largely enhanced gene transcription rather than reduced mRNA degradation. In this respect the effect of Met appears similar to that of other protein tyrosine kinases although recent studies have also shown an additional influence of EGF on stabilizing PTHRP mRNA (Heath et al. 1995).

Previous studies from our laboratory demonstrated the relative loss of the capacity of exogenous growth factors to stimulate PTHRP gene expression as the cells become malignant (Henderson et al. 1991). The present study demonstrates that the

constitutively active growth factor receptor, Tpr-Met, which is oncogenic, can stimulate PTHRP production and thus provides a mechanistic explanation for the reduced requirement for a growth factor ligand as cells become transformed.

Mutation of a single tyrosine residue (Y489) in the COOH terminus of the Tpr-Met oncoprotein, to a conserved phenylalanine, does not affect endogenous kinase activity but severely impairs the capacity of Tpr-Met to transform Fr 3T3 cells (Fixman et al. 1995). Concomitantly, we have demonstrated a marked drop in the capacity of this mutant to increase PTHRP production. Activated protein tyrosine kinases such as Tpr-Met bind to proteins with Src homology 2 (SH2) domains via their phosphorylation sites (Park et al. 1986). While all SH2 domains appear to require a phosphotyrosine residue for binding, specific, high affinity binding is directed by the amino acid sequence motif surrounding the phosphotyrosine residue (Pawson 1994). Wild-type Tpr-Met can thus bind to a series of intracellular protein substrates and thereby engage several downstream signaling pathways. The Y489-mutation decreases the association between Tpr-Met and several proteins including PI3 kinase, the Grb2 adaptor protein and an unknown protein of 110kDa (Fixman et al. 1995). We therefore determined whether signaling pathways mediated by PI3 kinase or Grb2 might be involved in transducing the Tpr-Met induced increase in PTHRP.

PI3 kinase is a heterodimeric cytosolic protein consisting of a regulatory 85kDa subunit and a catalytic 110kDa subunit. The regulatory unit contains two SH_2 domains which can bind to specific activated tyrosine kinases such as Tpr-Met (Fixman et al.

1995). This association serves to increase the PI3 kinase catalytic activity toward the D-3 position of several phosphatidylinositols generating the signaling molecules phosphatidylinositol 3-phosphate, phosphatidylinositol 3, 4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate. Wortmannin, a fungal metabolite, is known to inhibit only the PI3 kinase at nanomolar concentrations, although at micromolar concentrations, it also inhibits protein kinases (Powis et al. 1994) and proteins of the nuclear lamina (Reusch et al. 1995). Nanomolar concentrations of wortmannin failed to cause any significant reduction in PTHRP mRNA suggesting that in the cell model used the PI3 kinase pathway fails to play a significant role in Tpr-Met-induced augmentation of PTHRP production.

Grb2, a 23kDa SH2-containing adaptor protein binds to phosphotyrosine motifs in specific activated protein tyrosine kinases such as Tpr-Met (Fixman et al. 1995) Grb2 links protein tyrosine kinases to cellular proliferation by mediating the activation of Ras. Grb2 appears to activate Ras by forming a stable complex through its Src homology 3 (SH3) domains, with a proline-rich region in a Ras-specific GDP/GTP exchange factor, mammalian Son of Sevenless (mSOS). Consequently Grb2, binding leads to increases in Ras GTP levels and to activation of the Ras signaling pathway. The reduced capacity of the Tpr-Met mutant Y489F to associate with Grb2 (Fixman et al. 1995) and also to stimulate PTHRP production is consistent with a role for Grb2 in the signaling pathway from Tpr-Met through ras to enhanced transcription of the PTHRP gene.

A critical requirement for Ras function is appropriate localization to the inner surface of the plasma membrane, and post-translation addition of a polyisoprenoid (farnesyl) to Ras is obligatory for membrane localization and bioactivity. Biosynthesis of the required polyisoprenoid intermediates involves the mevalonic pathway of cholesterol biosynthesis and an early and rate-limiting step in this pathway is catalyzed by 3-hydroxy-3 methylglutaryl coenzyme A (HMG-CoA) reductase. This enzyme is inhibited by lovastatin, which has been shown to block Ras isoprenylation in several cell types (including rat-1 fibroblasts which are similar to Fr3T3 fibroblasts), without interfering with other signal transduction processes (Fenton et al. 1992; Reusch et al. 1995). Although lovastatin can cause a marked inhibition of Ras activity, it may also however, modify processing of proteins of the nuclear lamina (Reusch et al. 1995). In the current study, lovastatin markedly reduced the capacity of Tpr-Met to increase PTHRP demonstrating a critical role for Ras in the signaling pathway leading from Tpr-Met to PTHRP production.

We previously demonstrated a marked increase in PTHRP production by a Ras-transformed malignant keratinocyte cell line, HPKIA-Ras, relative to the established parent cell line HPKIA (Henderson et al. 1989). NRK 49F fibroblastic cells and RCB 2.2 osteoblastic cells transfected with Ras oncogene also produce increased amounts of PTHRP (Li and Drucker 1994b) and these oncogene transformed cells show an increased rate of PTHRP gene transcription. These observations therefore support our current findings that the gene encoding PTHRP is a downstream target for Ras. Furthermore our

present studies indicate that Ras may play a central role in transducing the PTHRP-inducing signal from protein tyrosine kinases and suggest that Grb2 is involved in this pathway.

2.2.6 Acknowledgements

This work was supported by Medical Research Council of Canada Grants MT-10630, MT-12609, MT-11545 and MT-5775 and by a grant from the National Cancer Institute of Canada. M. Park is a scholar of the National Cancer Institute of Canada and S.A. Rabbani is a recipient of a scholarship from MRC and the Cancer Research Society of Canada.

CHAPTER 3

INDUCTION OF PARATHYROID HORMONE RELATED PEPTIDE BY THE RAS ONCOGENE: ROLE OF RAS FARNESYLATION INHIBITORS AS POTENTIAL THERAPEUTIC AGENTS FOR HYPERCALCEMIA OF MALIGNANCY

3.1 PREFACE

The work in Chapter 2 demonstrated that Tpr-Met induced PTHRP gene expression through a Ras-dependent mechanism. The study in this Chapter is aimed at directly investigating the the role of Ras in PTHRP gene regulation *in vitro* and in development of hypercalcemia *in vivo*, and tested the effect of inhibiting Ras with a specific peptidomimetic inhibitor of Ras, B-1086. Results from this study demonstrate the central role played by Ras in PTHRP gene regulation, and present Ras as a potential target in treatment of hypercalcemia of malignancy. The study is presented herein in the form of a published paper (*Cancer Res* 57(20):4517-22, 1997). The Ras inhibitor B-1086 was kindly provided by Dr. Michael Lewis, Eisai Research Institute, Andover, MA. I was responsible for all the experimental work presented in this chapter. I greatly appreciate the input of Drs. D. Goltzman and M. Park in the design of the experiments and preparation of the manuscript.

3.2 ORIGINAL ARTICLE

3.2.1 Abstract

Parathyroid hormone related peptide (PTHRP) is the major causal agent in the syndrome of humoral hypercalcemia of malignancy (HHM). Several studies have shown that PTHRP production is increased in response to growth factors and oncogenes, such as Tpr-Met, that are associated with the tyrosine kinase signalling pathway. Using site directed mutagenesis of Tpr-Met and chemical inhibitors of phosphotidylinositol (PI)-3 kinase and Ras isoprenylation, we previously demonstrated that induction of PTHRP is mediated via the Ras signalling pathway. In the present study we have directly investigated the role of the Ras oncogene in HHM. As a model system we used Fisher rat 3T3 fibroblasts stably transfected with a Ras oncogene (Ras-3T3). Ras transfection enhanced PTHRP production 5-10 fold in these cells and inoculation of this cell line into nude mice led to the development of hypercalcemia within two weeks. We used this system to evaluate the effect of a potent inhibitor of Ras processing, B-1086 on cell growth, PTHRP production, plasma calcium, and tumor growth. Treatment of Ras-3T3 cells *in vitro* with B-1086 at 0.1-10 μg/ml produced a significant reduction in PTHRP mRNA expression and PTHRP secretion, and a significant decrease in cell proliferation. Treatment *in vivo* of BALB/c/*nu/nu* mice bearing Ras-3T3 tumors, with B-1086, resulted in a significant inhibition in tumor growth. In addition, this treatment produced near normalization of serum Ca^{++}, a significant decrease in plasma PTHRP and a reduction in tumoral PTHRP mRNA levels. These results show that the Ras pathway is involved in

PTHRP production by tumors, identifies Ras as a potential target for treatment of HHM, and demonstrates Ras processing inhibitors as candidate therapeutic agents against this syndrome.

3.2.2 Introduction

Humoral hypercalcemia of malignancy (HHM) is a major complication in a significant portion of cancer patients and parathyroid hormone related peptide (PTHRP) has been identified as the major pathogenic factor for this disease (Moseley et al. 1987; Stewart et al. 1987; Strewler et al. 1987; Rabbani et al. 1986; Li and Drucker 1994a). PTHRP shares a high degree of homology in its NH_2-terminal region with parathyroid hormone (PTH) and because of this sequence homology, is able to mimic the actions of PTH by interacting with a common PTH/PTHRP receptor (Rabbani et al. 1988; Jüppner et al. 1991). In normal physiology, PTHRP is widely expressed, acts in an autocrine or paracrine manner, and has been shown to have a broad spectrum of functions especially related to modulating cell growth and differentiation (McAuley et al. 1990; Burton et al. 1990; Rabbani et al. 1995; Kremer et al. 1991). PTHRP appears to be a key regulator of skeletal development in the fetus but has also been implicated in transepithelial calcium transport in such sites as mammary epithelium, kidney and placenta (Karaplis et al. 1994; Thiede and Rodan 1988; Sebag et al. 1994; Soifer et al. 1993; Abbas et al. 1990). Furthermore, PTHRP has been shown to be a potent muscle relaxant acting on the smooth muscle of the uterus, urinary bladder and vascular wall (Mok et al. 1989b). In

HHM, PTHRP acts primarily as an endocrine factor (Phibrick et al. 1996). When large quantities of PTHRP are produced by cancer cells, PTHRP is secreted into the circulation, and acts at the level of bone to stimulate bone resorption and Ca^{++} release, and on the kidneys to increase Ca^{++} reabsorption (Guise et al. 1993). The outcome of these actions is the development of hypercalcemia, which may be fatal.

Increasing number of studies on the regulation of PTHRP gene expression are beginning to shed light on the molecular mechanisms responsible for PTHRP overproduction by cancer (Stewart et al. 1980). We have previously shown the critical role played by growth factors, acting on tyrosine kinase receptors, in stimulating PTHRP production (Aklilu et al: Chapter 2). We subsequently demonstrated the effect of the oncogenic derivative of the hepatocyte growth factor/scatter factor (HGF/SF) receptor, Tpr-Met, on increasing PTHRP production and demonstrated that the mechanism of this stimulation involves the intracellular Ras signalling pathway (Aklilu et al: Chapter 2). Previous studies by us and others, examining the relationship between tyrosine kinases and PTHRP, have indeed indicated that Ras is at the cross-roads through which the signal for PTHRP induction is propagated.

Ras genes play a fundamental role in basic cellular functions(Bos 1989). Ras proteins are GDP/GTP regulated switches that in their mature functional form are anchored to the cell membrane by an isoprenyl tail (Casey et al. 1989; Kato et al. 1992). In normal physiology, Ras functions in transducing developmental and proliferative signals from the cell surface to the nucleus. In addition to its role in growth and

differentiation, Ras is also important in controlling cytoskeletal protein expression and in regulating vesicle trafficking within the cell (Boguski and McCormick 1993). Perhaps owing to its central role within the cell, alteration of this gene is often associated with the initiating steps that lead towards carcinogenesis. Indeed, activating mutations in the Ras gene account for at least 10% of human neoplasia and play a significant role in progression of these malignancies (Barbacid 1987). In light of this, Ras is an ideal target for therapeutic intervention to combat both the establishment of the primary cancer and for the prevention of secondary syndromes (Kohl et al. 1995). One approach to target Ras has been the development of peptidomimetic inhibitors of Ras, which mimic the farnesylation site of the Ras protein, a CAAX consensus sequence at the carboxy-terminal, and act by competitively inhibiting Ras farnesylation (Nagasu et al. 1995; Gibbs et al. 1993). Farnesylation is essential for subsequent modifications of Ras, for membrane localization and ultimately for functioning of this oncogenic protein (Sepp-Lorenzino et al. 1995).

In the current study, using Fisher rat 3T3 fibroblasts transfected with the Ras oncogene, we have examined the role of Ras in PTHRP production and in the development of hypercalcemia *in vivo*. We have also tested the ability of a Ras farnesylation inhibitor B-1086, to alter the course of the tumor and its production of PTHRP, and assessed the effects on the hypercalcemic syndrome *in vivo*.

3.2.3 Materials and Methods

Ras farnesylation inhibitor B-1086

The Ras farnesyl transferase inhibitor, B-1086, was kindly provided by Dr. Michael Lewis of Eisai Research Institute, Andover, MA.

Animal Protocols.

Female BALB/c/*nu/nu* mice (Charles River Breeding Laboratories, Wilmington, MA), weighing 15-20 g (4-5 weeks of age) were injected on day 0 subcutaneously into the left and right flanks with Ras-3T3 cells (1×10^5) suspended in 0.1 ml sterile saline. Animals were housed individually and fed *ad libitum* on auotoclaved standard rodent chow (rat chow 5012, Ralston-Purina Canada, Inc., Lasalle, Quebec, Canada) containing 1% calcium and 0.74% phosphorus and sterilized tap water (Rabbani et al. 1993). On day 5, vehicle, 50 mg/kg B-1086, or 100 mg/kg B-1086 was administered daily intraperitoneally into anaesthetized animals. All animals were examined for the development of tumors daily for up to 15 days. On the day of sacrifice, animals were bled by intracardiac puncture and tumor volume of control and experimental animals was measured in two dimensions by callipers and the tumor volume calculated ($\{\text{width} + \text{length}/2\}^3$). On the day of sacrifice, animals were bled by intracardiac puncture and tumor tissue was extracted and immediately frozen in an ethanol bath for RNA analysis.

Cells and Cell growth

The Fisher rat (Fr) 3T3 fibroblasts expressing the Ras oncogene were generated by ecotropic retroviral infection as previously described (Chapter 2). Transient transfection into COS-1 cells of the pLXSN retroviral vector encoding the Ras gene and G418 resistance, and of the pSV-Y-E-MLV plasmid encoding retroviral packaging proteins was performed by the DEAE-dextran method. At 12 h after transfection, cells were treated with 100mM chloroquine for 3 h and incubated for a further 48 to 72 h before harvesting. Transfected COS-1 cells were lysed and the supernatant containing the packaging virus, at a titre of 5 x 10^3 plaque forming units, was used to infect Fr 3T3 cells. Experimental cell lines were then selected for G418 resistance and foci formation

For growth curves, Fr 3T3 cells were plated in medium containing 2% fetal bovine serum (FBS) in 9.6 cm^2 petri dishes (Falcon) at seeding densities of 5 x10^3 cells/plate. Cells from replicate dishes were trypsinized and counted, daily, in a model Z Coulter counter (Coulter Electronics, Beds, United Kingdom). Medium was changed in all plates every 24 hours.

Northern blot analysis

Total cellular RNA was isolated from the control and experimental cells, following treatment with vehicle alone or graded concentration of B-1086, by acid guanidium thiocyanate-phenol-chloroform extraction as previously described (Torczynski et al. 1983). Twenty μg of total cellular RNA was electrophoresed on a 1.1% agarose-

formaldehyde gel, transferred to a nylon membrane (Nytran) by capillary blotting and then fixed by a drying and UV cross-linking for 10 min. The integrity of the RNA was assessed by ethidium bromide staining. Hybridization was carried out with ^{32}P-labelled PTHRP cDNA and with an 18S RNA probe using a^{32}P dCTP as previously described (Torczynski et al. 1983; Thomas 1980). After a 24 h incubation at 42°C, filters were washed twice under low stringency conditions (1xSSC and 1% SDS; at RT for 40 mins) and under high stringency conditions (0.1xSSC, 0.1% SDS; at 55^{0}C for 40 mins). Autoradiography of filters was carried out at -70°C using XAR film (Eastman Kodak Co., Rochester, NY) with two intensifying screens or by using a phosphorimager screen (Eastman Kodak Co., Rochester, NY). The level of PTHRP expression was quantified by densitometric scanning using the Mac BAS V1.01 alias program.

Ras processing Assay.

Ras-3T3 cells were plated in medium containing 10% calf serum and 24h later, treated with 0, 10 or 50 μg/ml of B-1086 for 72h and harvested in lysis buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml PMSF, 30 μg/ml Aprotinin, 10 μg/ml sodium orthovanadate). The lysates (25 μg) were electrophoresed on a 15% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and immunoblotted with anti-ras antibody (Santa Cruz biotechnology). The signals were visualized using chemiluminescence (Amersham).

Other Analytical Methods.

For radioimmunoassay (RIA), conditioned medium (2 ml/well of a 6-well cluster plate) was removed from control and experimental 3T3 cells treated with vehicle alone or with graded doses of B-1086 for 24 hrs. Duplicate 200-500 µl aliquots of cell conditioned culture medium were evaporated to dryness in a Speedvac (Savant Instruments, Inc., Hicksville, NY) and stored at -20°C until assayed. Dried medium was reconstituted with 200 µl of outdated blood bank plasma and radioimmunoassayed as described previously using ^{125}I-labelled [Tyr] PTHRP (1-34) as a tracer and PTHRP (1-34) as a standard (Henderson et al. 1989). Results are expressed as nanogram equivalents of PTHRP(1-34)/10^6 cells.

Serum calcium levels were determined by atomic absorption spectrophotometry (model 703, Perkin-Elmer, Norwalk, CT) and plasma PTHRP was assayed using a commercial 2-site immunoradiometric assay IRMA (Nichols Institute Diagnostics, San Juan Capistrano, CA)

Statistical Analysis. Results are expressed as the mean ± S.E. of at least triplicate determinations and statistical comparisons are based on the Student's *t* test or analysis of variance. A probability value of <0.05 was considered to be significant.

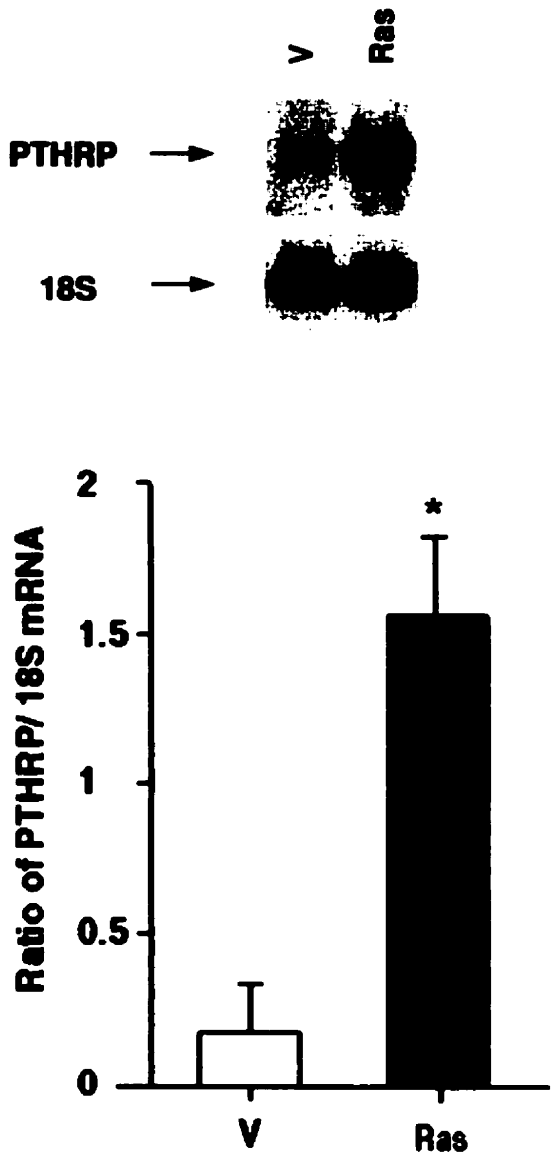
Figure 3. 1. Effect of Ras expression on PTHRP production.

Panel A: Northern blot analysis of Fr 3T3 cells infected with empty vector alone (V) or with experimental vector (Ras). Cells were grown in the absence of serum and twenty μg of total cellular RNA was extracted and electrophoresed on a 1.1% agarose formaldehyde gel. Filters were probed with a ^{32}P labelled PTHRP cDNA or with a ^{32}P labelled 18 S RNA probe as described in "Materials and Methods". All blots were quantified by densitometric scanning (lower panel).

Panel B: Conditioned culture medium from Fr 3T3 cells infected with empty vector alone (V) or with experimental vector (Ras) was collected after 48 hrs and analyzed for PTHRP immunoreactivity (iPTHRP) as described in "Materials and Methods". Results are expressed as ng equivalents of PTHRP (1-34) (ng eq 1-34).

Significant differences from control cells are represented by asterisks * ($^{*}p<0.05$).

A.


V
Ras
PTHRP
18S
Ratio of PTHRP/ 18S mRNA
2
1.5
1
0.5
0
*
V
Ras

B.

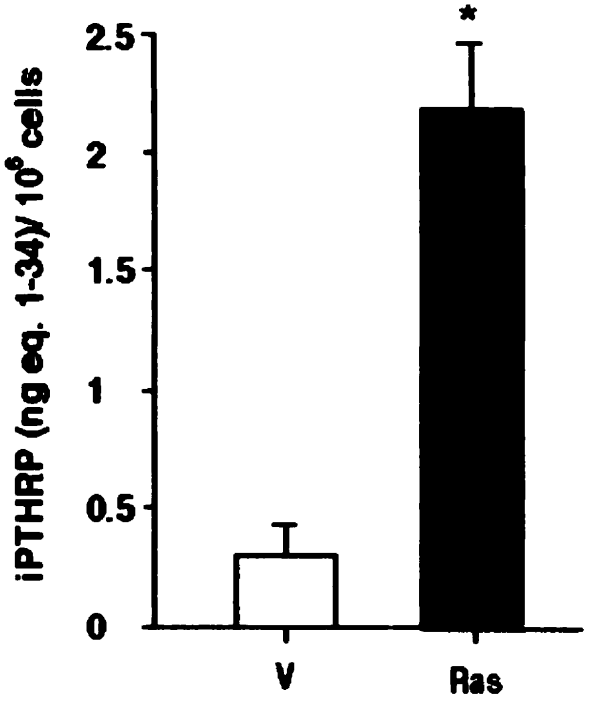

iPTHRP (ng eq. 1-34)/ 10^6 cells
2.5
2
1.5
1
0.5
0
*
V
Ras

Figure 3.2. Treatment with B-1086 inhibits Ras protein processing in Ras-3T3 cells *in vitro*.

Ras-3T3 cells were grown to 70% confluence and treated with either vehicle, 10 mg/ml of B-1086 or 50 mg/ml of B-1086 for 72 hours. The cell lysates were electrophoresed and immunoblotted with anti-Ras antibody and analyzed for Ras processing as described in "Materials and Methods". "U" represents unprocessed and "P" processed forms of Ras.

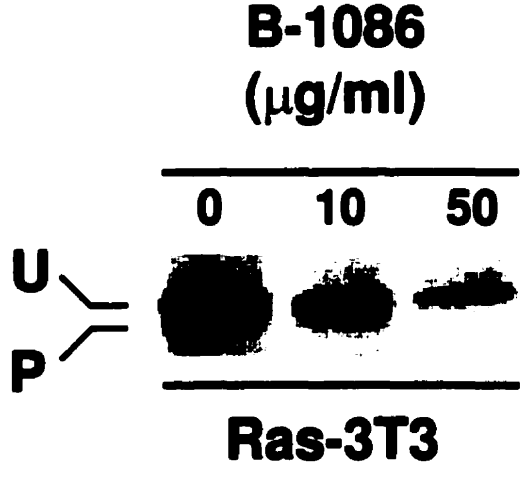

B-1086
(μg/ml)
0
10
50
U
P
Ras-3T3

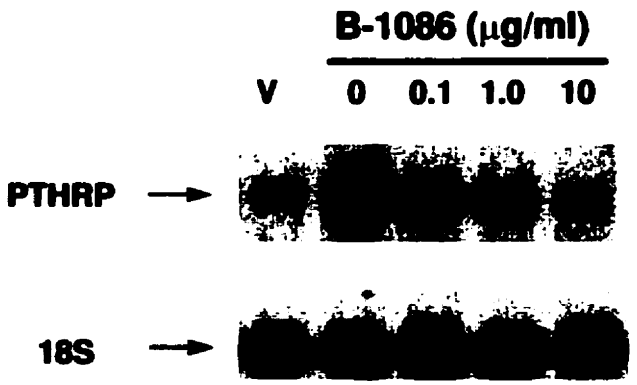
Figure 3.3. Effect of B-1086 on PTHRP production in Ras-3T3 cells.

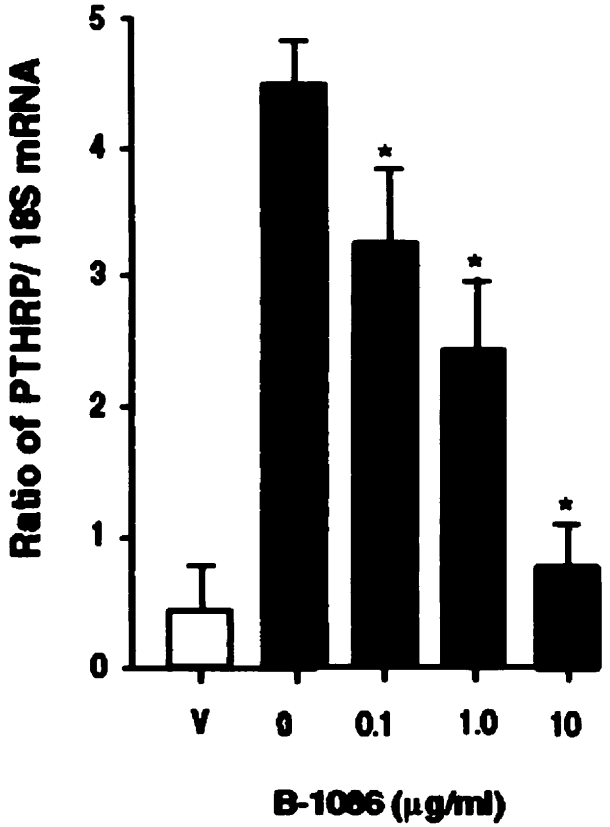
Panel A: Fr 3T3 cells transfected with the empty vector (V) or with the experimental plasmid (Ras), were grown to 70% confluence. Ras transfected cells were incubated with 0, 0.1, or 10 μg/ml B-1086. Twenty μg of total cellular RNA was extracted from control and experimental cells and electrophoresed on a 1.1% agarose formaldehyde gel. Filters were probed with a ^{32}P labelled PTHRP cDNA or with a ^{32}P labelled 18 S RNA probe as described in "Materials and Methods". All blots were quantified by densitometric scanning (lower panel).

Panel B: Conditioned culture medium from control Fr 3T3 cells (V) and from Ras-transferred Fr 3T3 cells treated with graded concentrations of B-1086 was collected after 48 hrs and analyzed for PTHRP immunoreactivity as described in "Materials and Methods".

Significant differences from control cells are represented by asterisks * ($^{*}p<0.05$).

A.


B-1086 (μg/ml)
V
0
0.1
1.0
10
PTHRP
18S


Ratio of PTHRP/ 18S mRNA
5
4
3
2
1
0
V
0
0.1
1.0
10
B-1086 (μg/ml)

B.

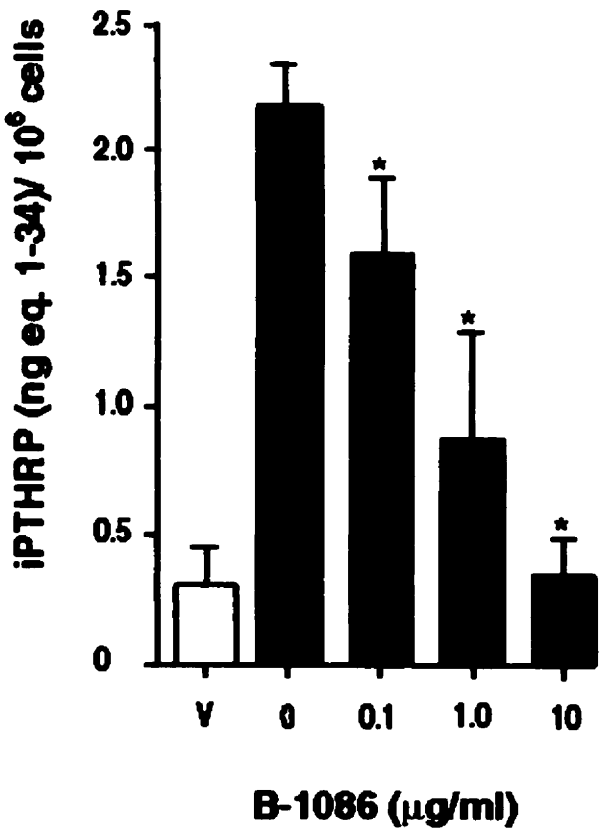

iPTHRP (ng eq. 1-34)/ 10^6 cells
2.5
2.0
1.5
1.0
0.5
0
V
0
0.1
1.0
10
B-1086 (μg/ml)

Figure 3.4. Effect of B-1086 on growth curves of Ras-3T3 cells .

Growth curves of wild type cells (Δ) or Ras transfected 3T3 cells either untreated (○) or treated (●) with 10 μg/ml B 1086. Cultures were seeded at a density of 5.0×10^4 cells per dish and incubated for the indicated periods of time in standard culture medium. Cells from triplicate dishes were trypsinized and counted as described in "Materials and Methods". Each point represents the mean ± SEM of four different experiments. Significant differences from Ras-3T3 cells treated with vehicle alone are represented by asterisks * ($^*p<0.05$).

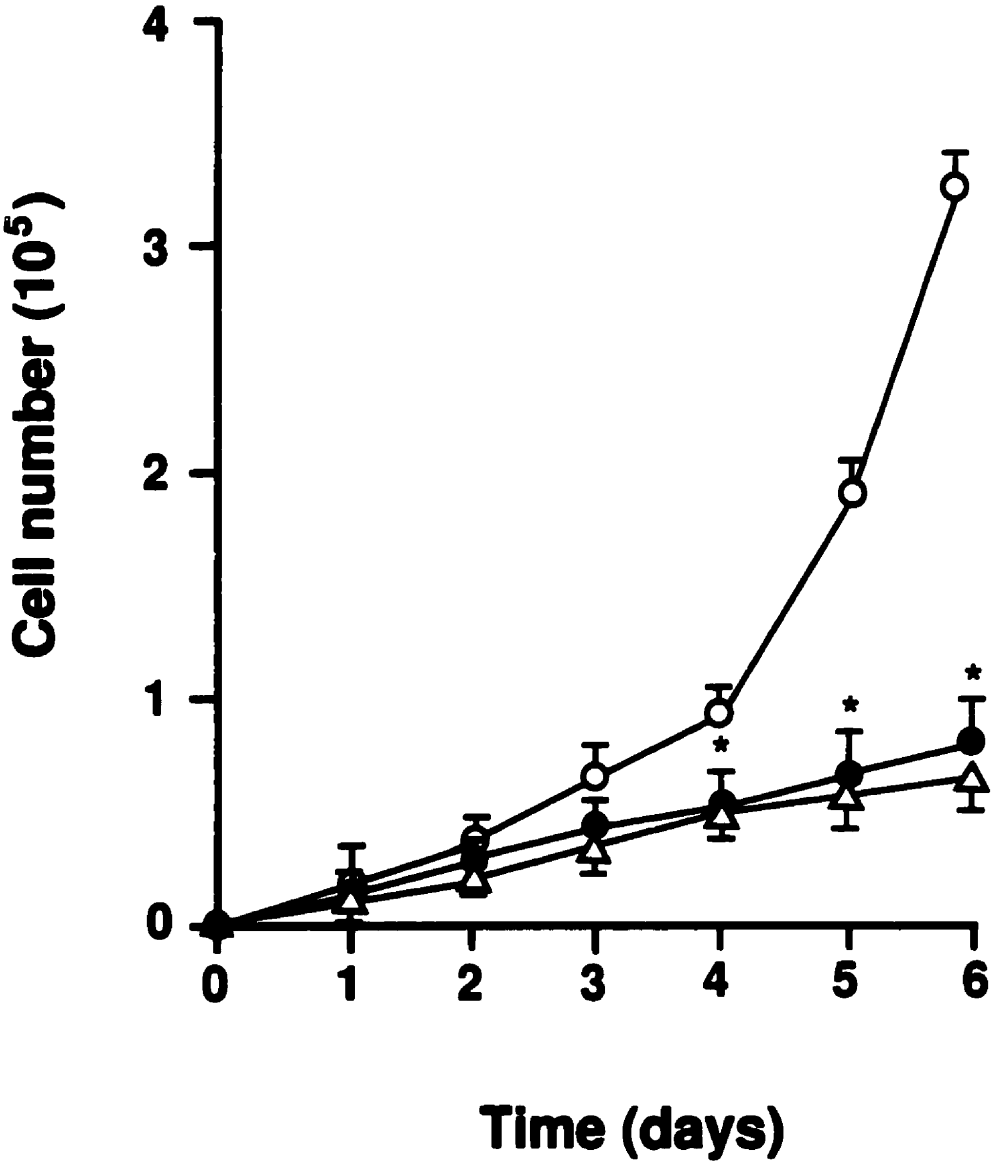

Cell number (10^5)
4
3
2
1
0
0
1
2
3
4
5
6
*
*
*
Time (days)

Figure 3.5. Effect of B-1086 on the tumor volume of Ras-3T3 tumor bearing animals.

Tumor bearing animals were injected daily with 50 mg/kg (●) or 100 mg/kg (▲) of B-1086 and tumor volume was determined at daily intervals (↓) as described in "Materials and Methods". Comparison was made with tumor volume of animals receiving vehicle alone (untreated) (Δ). Results represent the mean ± SEM of four starting animals in each group in three different experiments. Significant differences from control tumor bearing animals at each time point are represented by single .asterisks * ($^{*}p<0.05$). Double asterisks represent significant difference from values with single asterisks ($^{**}p<0.05$).

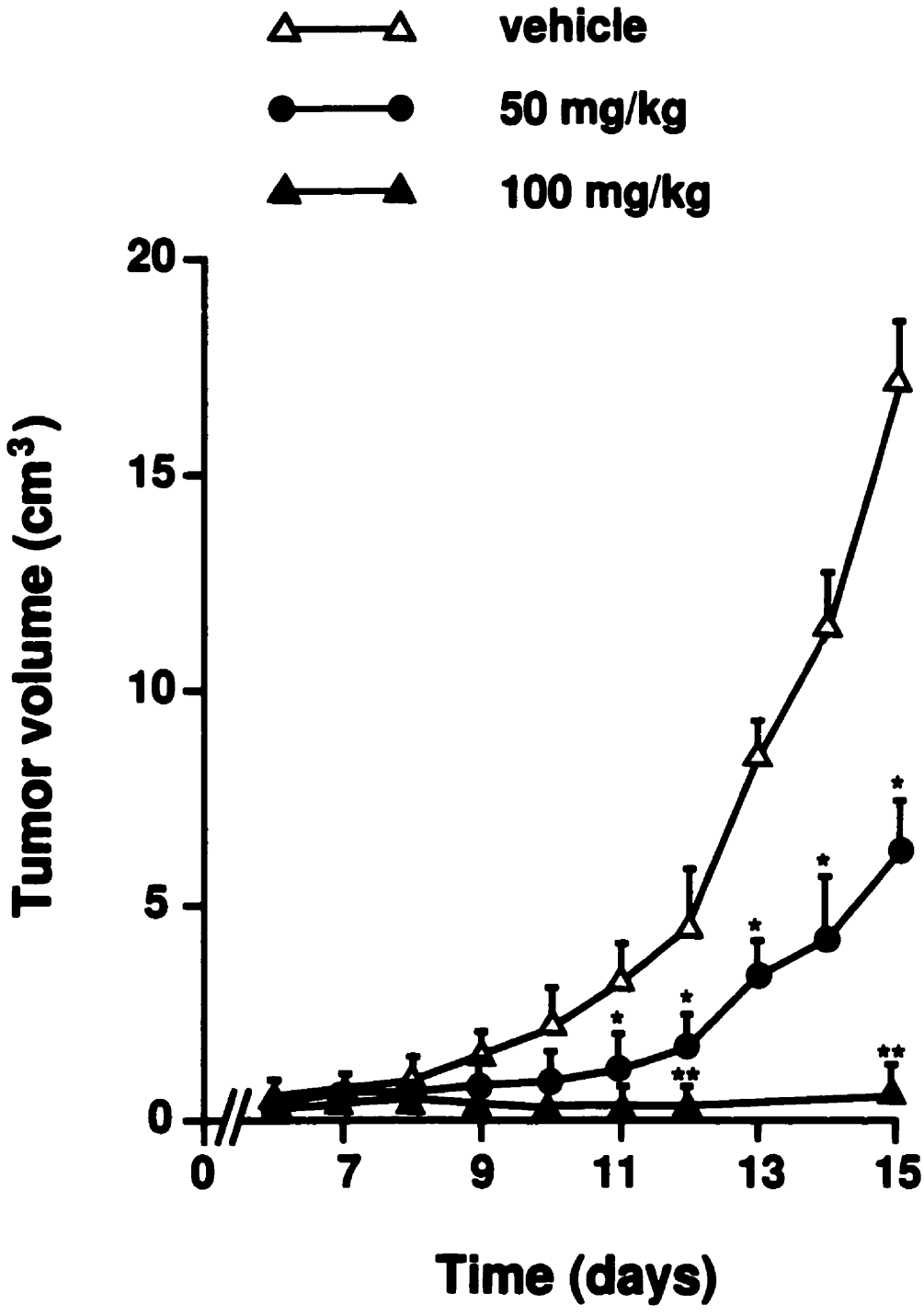

vehicle
50 mg/kg
100 mg/kg
Tumor volume (cm³)
20
15
10
5
0
0
7
9
11
13
15
Time (days)
*
*
*
*
*
**
**

Figure 3.6. Effect of B-1086 on serum calcium of Ras-3T3 tumor bearing animals.

Tumor bearing animals were infused with 50 or 100 mg/kg of B-1086 or vehicle (CTL) and were sacrificed at the end of the experiment on day 15. Their serum calcium was then determined as described in "Materials and Methods". Serum calcium in non-tumor bearing animals is also shown (N). Results represent the mean ± SEM of at least three animals in each group in three different experiments. Significant differences in serum calcium from normal animals (N) are represented by one and from tumor bearing control (CTL) by two asterisks ($p<0.05$).

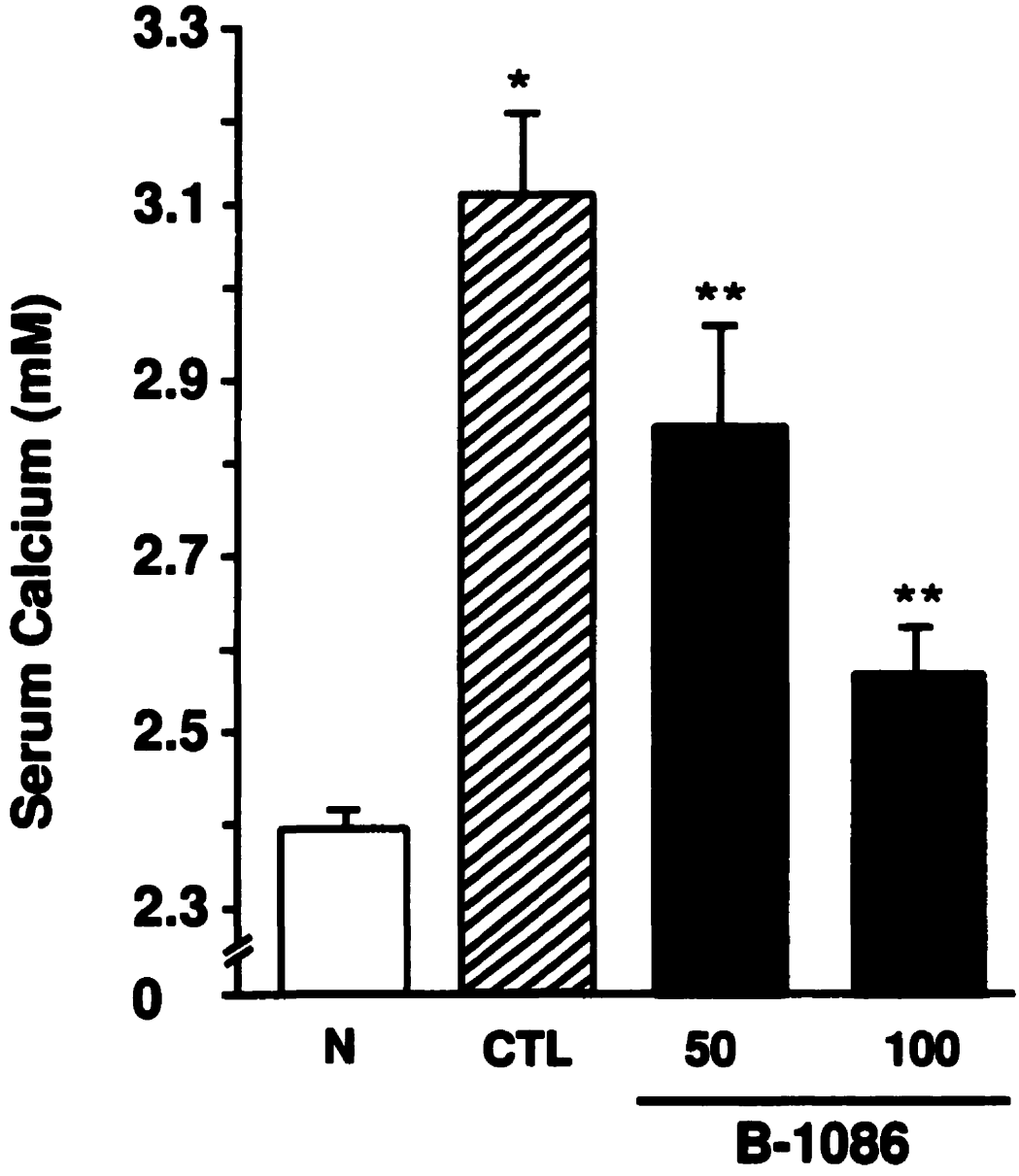
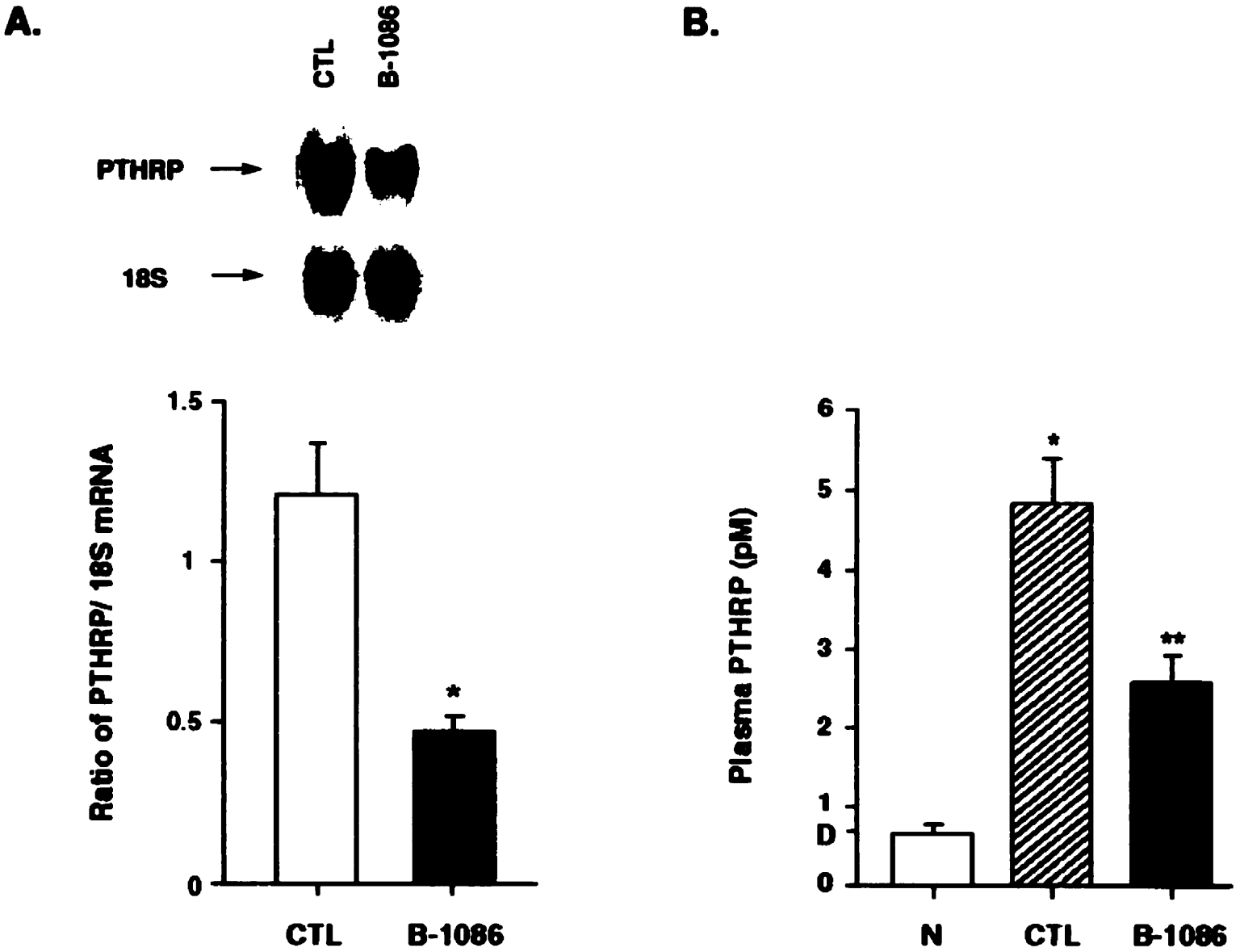

Serum Calcium (mM)
3.3
3.1
2.9
2.7
2.5
2.3
0
*
**
**
N
CTL
50
100
B-1086

Figure 3.7. Effect of B-1086 on tumoral PTHRP production in tumor bearing animals.

Panel A: PTHRP mRNA expression was determined in tumor extracts as described in "Materials and Methods". PTHRP mRNA expression after treatment with B-1086 (100mg/kg) was compared with PTHRP mRNA levels in control animals (CTL) receiving vehicle alone (top panel). All blots were probed with an 18S RNA cDNA as a control for RNA loading and the densitometric ratios of PTHRP/18S mRNA are shown (lower panel).

Panel B: PTHRP concentration was determined as described in "Materials and Methods" and is expressed as pM PTHRP. Plasma PTHRP levels were examined in Ras-3T3 tumor bearing animals receiving vehicle alone (CTL) or 100 mg/kg of B-1086. Plasma PTHRP in non- tumor bearing animals is also shown (N). Results represent the mean ± SEM of three animals in each group in 3 different experiments. D represents the detection limit of the assay. Significant difference from control tumor bearing animals is represented by an asterisk ($^{*}p<0.05$). Double asterisks represent significant difference from values with single asterisks ($^{**}p<0.05$).


A.
CTL
B-1086
PTHRP
18S
Ratio of PTHRP/ 18S mRNA
1.5
1
0.5
0
*
CTL
B-1086
B.
Plasma PTHRP (pM)
6
5
4
3
2
1
D
0
*
**
N
CTL
B-1086

3.2.4 Results

Effect of the Ras oncogene on PTHRP production.

In order to examine the effect of Ras oncogene transfection on PTHRP production, total cellular RNA was extracted from control and Ras transfected cells and analyzed by Northern blot analysis. Both control and experimental cell lines were maintained in culture for at least 24 hr in the absence of serum to rule out any change in PTHRP expression by serum derived growth factors. Transfection of the Ras oncogene caused a marked increase (5-10 fold) in PTHRP mRNA expression (Fig.1A). The effect of transfection of this oncogene on PTHRP secretion into the cell conditioned culture medium was determined by RIA and comparison was made with the amount of PTHRP released into the wild type and vector transfected 3T3 cells. Secretion of immunoreactive PTHRP was significantly greater in 3T3 cells transfected with Ras as compared to control cells (Fig.1B). These results demonstrated that Ras itself was a potent inducer of PTHRP production, independent of any growth factors present in the cell culture medium.

Effect of B-1086 on Ras processing and PTHRP production by Ras-3T3 cells, *in vitro*.

In order to examine the capacity of B-1086 to inhibit Ras processing , Ras 3T3 cells were treated with B-1086 (10 or 50 mg/ml). B-1086 produced a dose dependent

reduction in Ras protein processing in Ras-3T3 cells (Fig.2). Treatment with vehicle alone (0.1% DMSO) revealed no inhibitory effect on Ras processing.

The ability of B-1086 to influence PTHRP production was examined in experimental Ras-3T3 cells *in vitro*. Ras-3T3 cells were treated with increasing concentrations (0.01-10μg/ml) of B-1086 for 48 hr. At the end of this treatment, cells were isolated and total cellular RNA extracted and analyzed for PTHRP mRNA expression by Northern blot analysis. Conditioned medium from control and experimental cells was assayed for immunoreactive PTHRP by an NH_2 terminal PTHRP RIA. B-1086 caused a dose dependent decrease in PTHRP mRNA expression (Fig. 3A) and secretion of PTHRP protein into conditioned culture medium (Fig. 3B). Morphological analysis of Ras-3T3 cells following treatment with graded doses of B-1086 and cell viability assay using trypan blue, failed to show any cytotoxic effects of B-1086 in concentrations used in this study (data not shown).

Effect of B-1086 on the growth of Ras-3T3 cells.

The effect of cell growth following transfection with the Ras oncogene was examined for 6 days *in vitro*, and comparison was made with growth of wild type cells and with Ras-3T3 cells treated with B-1086 (10μg/ml). Transfection of the Ras oncogene caused a significant increase in cell growth as compared to wild type cells or cells transfected with vector alone (data not shown). Treatment of Ras-3T3 cells with B-1086

caused a marked decrease in cell doubling time and growth was reduced to a rate comparable with that seen in untransfected 3T3 cells (Fig.4).

Effect of infusion of B-1086 on tumor volume, *in vivo*.

BALB/c/*nu/nu* mice inoculated with experimental Ras-3T3 cells developed palpable tumors by 5 days post tumor cell inoculation. These tumors continued to increase in volume for the next 10 days. Experimental animals were inoculated with Ras-3T3 and beginning on day 5 post tumor inoculation, animals were injected daily with either vehicle alone or with different concentrations (50 or 100 mg/kg) of B-1086. Animals infused with B-1086 showed a marked decrease in tumor growth as compared to animals infused with vehicle alone and the effects of B-1086 on decreasing tumor volume was dose dependent (Fig. 5).

Effect of B-1086 on serum Ca^{++} of Ras-3T3 tumor-bearing mice.

Ras-3T3 cells were inoculated into the right flank of nude mice, and animals were monitored for the development of hypercalcemia. Inoculation of Ras-3T3 cells resulted in the development of palpable tumors by 6 days and hypercalcemia by day 15 post tumor cell inoculation. Treatment of these experimental animals with B-1086 (50 or 100 mg/kg) for 10 days (day 6-15) resulted in a significant reduction in their serum Ca^{++} levels which appeared dose dependent (Fig.6)

Effect B-1086 on tumoral PTHRP production *in vivo*.

On day 15 post tumor cell inoculation, all control and experimental animals were sacrificed and their tumors removed to analyze the level of tumoral PTHRP mRNA expression. Treatment of experimental animals with B-1086 (100 mg/kg) caused a significant decrease in PTHRP mRNA expression (Fig.7A). Also at the time of sacrifice, on day 15, blood was collected and analyzed for plasma PTHRP. Treatment of tumor bearing mice with B-1086 caused a significant decrease in their plasma PTHRP levels (Fig.7B).

3.2.5 Discussion

In previous studies, we demonstrated that PTHRP secretion by a tumor cell model is largely constitutive (Rabbani et al. 1993). Consequently, in contrast to PTH, whose secretion is tightly regulated by concentrations of the extracellular calcium ion such that calcimimetics acting on the calcium receptor can block PTH secretion, it appeared unlikely that attempts to reduce PTHRP release by inhibition of secretion would be rewarding (Brown et al. 1987). Indeed, the rate of PTHRP secretion appears to be a direct function of its rate of synthesis and the capacity of a given tumor to release PTHRP has been found to correlate with its level of gene expression (Henderson et al. 1991; Wyslomerski et al. 1996). Consequently, we have employed alternative strategies to inhibit PTH release *in vitro* and to test these effects on *in vivo* models.

One approach we previously used emanated from our *in vitro* observations that PTHRP gene transcription could be inhibited by the active metabolite of vitamin D 1,25-dihydroxy vitamin D (vitamin D3) (Kremer et al. 1991; Kremer et al. 1996). Indeed, vitamin D3 and a non-hypercalcemic analog were then shown to diminish PTHRP production by tumors *in vivo* (Haq et al. 1993). A second approach we have used was to employ antisense technology to diminish PTHRP translation *in vitro* and this strategy also reduced PTHRP production by tumors *in vivo* (Liu et al. 1995). Finally, a third strategy was to inhibit the postranslational processing of PTHRP. PTHRP is synthesized as a prohormone which has low intrinsic bioactivity; when the prohormone sequence is removed, the mature form is a fully active entity. We demonstrated that this cleavage can be accomplished by a furin-like prohormone convertase (Liu et al. 1994) and then showed that inhibition of the action of furin in tumors which overproduce PTHRP

reduces levels of bioactive PTHRP *in vitro* and is also effective *in vivo* (Liu et al. 1995; Liu et al. 1994). Consequently, several approaches may be employed based on mechanisms of PTHRP regulation that have been determined *in vitro*, which can diminish PTHRP production by tumors implanted *in vivo*.

The use of Ras farnesylation inhibitors to inhibit PTHRP production was based on our initial observations that growth factors such as EGF or IGF-1 can be potent stimulators of PTHRP gene transcription *in vitro* (Kremer et al. 1991; Sebag et al. 1994; Henderson et al. 1991). Subsequently, we reported that an oncogenic, constitutively active, growth factor receptor, Tpr-Met, could also increase PTHRP production and that the Ras signalling pathway was involved in this stimulation. In the present study we have shown that the Ras oncogene itself is a potent stimulus for PTHRP production *in vitro* and that overexpression of Ras in tumors *in vivo* can also lead to augmented PTHRP release and to the development of hypercalcemia. Furthermore a potent inhibitor of Ras action can reverse these effects both *in vitro* and *in vivo*.

The cascade leading to increased PTHRP production involves binding of growth factors such as EGF or HGF to their receptors, activation of Ras proteins by exchange of bound GDP for GTP, and the triggering of a phosphorylation cascade which involves raf kinase, mitogen-activated protein (MAP) kinase, and MAP kinase; the end result is the activation of nuclear transcription factors which can apparently enhance PTHRP gene transcription and which can also regulate cell growth (Khosravi-Far et al. 1996; Cowley et al. 1994).

Signal transduction by Ras is dependent on its capacity to localize to the plasma membrane which, in turn, is dependent on farnesylation of the cysteine in the CAAX

consensus sequence at the COOH terminus of the Ras protein (Sepp-Lorenzino et al. 1995). Inhibition of farnesylation is therefore being explored by several laboratories as a potential mechanism for inhibiting tumor growth. In the present study, we employed a small organic molecule that mimics the tetrapeptide farnesylation site of Ras protein but is a methyl ester prodrug. It is more potent than the free acid form of the drug (B956) *in vitro*, because of its higher membrane permeability, but *in vivo* is rapidly hydrolyzed to the acid within the circulation (Nagasu et al. 1995). This inhibitor, in common with other farnesyl transferase inhibitors (Nagasu et al. 1995; Gibbs et al. 1993; Sepp-Lorezino et al. 1995), reduced Ras posttranslational processing and also decreased cell growth. Furthermore, infusion of different concentrations of B-1086 used in this study failed to show any evidence of toxicity or change in the body weight of experimental animals as compared to control animals receiving vehicle alone (Nagasu et al. 1995).

The Ras oncogene is the site of one of the most prevalent genetic alterations in human cancer and can occur in a broad spectrum of cancers, many of which have been demonstrated to overproduce PTHRP (Burtis et al. 1990; Stewart et al. 1980). Consequently, inhibition of Ras action appeared to be a particularly effective site to target in order to inhibit PTHRP production and the hypercalcemic syndrome as well as to reduce tumor growth. Nevertheless, human cancers evolve as a result of multiple genetic alterations and inhibition of more than one disordered function may be needed to retard tumor progression. Therefore, with increased knowledge of the control of PTHRP production, targeting the multiple sites of aberrant regulation may be the most effective means of reversing increased secretion of this protein. Inhibition of Ras action should nevertheless be an important component of this approach.

3.2.6 Acknowledgements

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CHAPTER 4

THE ROLE OF MITOGEN ACTIVATED PROTEIN KINASE PATHWAYS IN SERUM INDUCED EXPRESSION OF PARATHYROID HORMONE-RELATED PROTEIN (PTHRP)

4.1 PREFACE

The studies presented in Chapters 2 and 3 demonstrate the critical role of Ras in the regulation of PTHRP gene expression, and suggest that this pathway may be critical for PTHRP overexpression leading to hypercalcemia of malignancy. In continuation of these studies, the work in Chapter 4 is aimed at investigating the molecular mechanisms that act downstream of Ras to mediate the induction of PTHRP gene expression. In intracellular signaling cascades, Ras is located upstream of many cellular signaling events. Among the targets of Ras, the MAP kinase pathways involving ERK and JNK have been well characterized, and are known to relay the message from Ras to the nucleus, to modulate gene transcription. This study explores the potential role of these kinases in mediating increases in PTHRP expression. For this study we selected the Fisher rat Leydig tumor cell line H-500, first described by Sica and colleagues (Sica et al. 1983) to cause hypercalcemia and increased urine cAMP in rats, features that are similar to the clinical syndrome. In culture, H-500 cells express PTHRP mRNA and secrete PTHRP into conditioned medium, under basal conditions. Expression of PTHRP in these cells is further augmented in response to serum. The high level of PTHRP gene expression found in H-500 cells thus provided an ideal model with which to study the regulatory mechanisms that lead to PTHRP overexpression in cancer. The results from this study are included in this chapter in the form of an original paper submitted for publication. I would like to thank Dr. Morag Park, McGill University, Montreal, Canada, for the generous gifts of the cDNA constructs used throughout this study. I was responsible for the experimental work presented in this chapter.

4.2 ORIGINAL ARTICLE

4.2.1 Abstract

Tumor production of parathyroid hormone related protein (PTHRP) is responsible for most cases of hypercalcemia of malignancy. The transplantable rat Leydig tumor H-500 is known to cause hypercalcemia in rats, by the release of abundant PTHRP, and to closely reproduce the human syndrome. We have recently demonstrated that Ras oncogene can stimulate PTHRP gene expression in Fr3T3 fibroblasts *in vitro*, and cause hypercalcemia *in vivo*. Using rat Leydig tumor H-500 cells, we have investigated the role of effector pathways downstream of Ras in serum induced PTHRP expression. Lovastatin and B-1086, which interfere with Ras processing, completely blocked induction of PTHRP mRNA in H-500 cells. In contrast, wortmannin, which acts on PI 3-kinase, had no effect on PTHRP expression. An increase in the basal expression of PTHRP mRNA was seen after transient expression of an activated Raf-1 (Raf BXB). Conversely, a decrease in serum stimulated PTHRP expression was seen with a dominant negative Raf (Raf C4B), and, an inhibitor of MEK (PD 098059), implicating the involvement of Ras-Raf-MEK pathway. In addition, stimulation with UV light, or expression of an activated form of Rac (RacV12) was sufficient to increase PTHRP mRNA. Moreover, a dominant negative Rac (RacN17) blocked serum-induced PTHRP gene expression. These data suggest that the stress-activated pathways are an alternative mechanism for the regulation of PTHRP expression. We demonstrate that serum induced PTHRP gene expression is mediated through Ras, and depends on activation of the two parallel MAP kinase pathways.

4.2.2 Introduction

PTHRP is the major pathogenic factor in Hypercalcemia of Malignancy. In addition to its endocrine effects, several studies have demonstrated that increased expression of PTHRP in cancer is associated with accelerated tumor growth and a more malignant phenotype (Liu et al. 1995; Alipov et al. 1997; Ito et al. 1997; Nagataki et al. 1995), suggesting that PTHRP may play a critical role in promoting tumor progression. Studies on gene regulation have shown that PTHRP is inducible by an array of substances including serum, growth factors and cycloheximide (Liu et al. 1992; Sebag et al. 1992; Allinson and Drucker 1993; Ikeda et al. 1990). Additionally, transfection of oncogenes into fibroblasts was shown to induce PTHRP gene expression through a Ras-dependent mechanism (Li and Drucker 1994a; Chapters 2 and 3).

The family of Ras proteins are GDP/GTP-regulated molecular switches. They relay their signals from the cell surface receptor to the nucleus through activation of various downstream signal transduction pathways (Barbacid 1987). Among the targets of Ras, the best characterized are mitogen activated protein (MAP) kinases. MAP kinases are a family of serine/threonine kinases that are involved in transduction of cellular signals to the nucleus, and in regulating a diverse range of biological processes including cell proliferation, differentiation, malignant transformation, inflammation, apoptosis and cytoskeletal rearrangement (Tanaka et al. 1997; Troppmair et al. 1994; Whitmarsh and Davis 1996). Upon activation, MAP kinases translocate to the nucleus, where they activate transcription of genes that mediate the cellular response. Activation of Ras leads to the sequential activation of the serine/threonine kinase Raf-1, MAP kinase kinases (MEK1 and MEK2), and extracellular signal regulated kinases (ERK1 and

ERK2) (van Aelst et al. 1993; Vojtek et al. 1993; Warne et al. 1993; Zhang et al. 1993). In addition to the ERK cascade, activated Ras has also been shown to activate the c-Jun N-terminal kinases (JNKs). JNKs, also known as stress-activated protein kinases (SAPK), represent a group of MAP kinases that are activated by cytokines and exposure of cells to environmental stresses such as UV light (Derijard et al. 1994a; Whitmarsh and Davis 1996). Ras, by activating Rac1 and CDC42, can generate the successive activation of MEK kinase (MEKK-1), JNK kinase (JNKK) and ultimately JNK (Coso et al. 1995; Minden et al. 1995).

We have previously shown data suggesting that the mechanism of PTHRP expression in cancer may be Ras-dependent. In this study, we have investigated the role of MAP kinase pathways leading to ERK and JNK activation in serum stimulated induction of PTHRP expression. For these studies we have chosen the rat hypercalcemic Leydig tumor cell line H-500, which produces large quantities of PTHRP and, upon inoculation into host animals, is an *in vivo* model that closely mimics the clinical syndrome of hypercalcemia of malignancy.

4.2.3 Materials and Materials

Cells and Tissue Culture

Rice H-500 Leydig tumor cells were maintained *in vitro* in RPMI 1640 supplemented with 2 mM L-glutamine (BRL/GIBCO Canada, Burlington, Ontario, Canada), 10% F BS, 100 units/ml of penicillin-streptomycin sulphate (BRL/GIBCO) and 0.002% (w/v) gentamycin (Sigma, St. Louis, MO). For transfection studies, cells were plated at 1 x 10^5 cells per 60-mm dish 3 days before transfection and growth in 5% CO_2 in RPMI 1640. At 70% confluence the cells were transfected with lipofectin, 10 μg/ml (BRL/GIBCO) containing 0.1, 1 or 10 μg of plasmid DNA and cultured from 6 h to overnight in serum free RPMI culture medium. Then fresh culture media containing 10% fetal bovine serum (FBS) was added. Transient transfection assays were performed 48-h post transfection. For stable transfections in IPTG inducible Raf C4B studies, the cells were selected in G418 (400 μg/ml) for 15 days and resistant colonies were pooled as polyclonal cell populations. Polyclonal populations with low basal and high expression of Raf C4B were determined by Northern blot analysis. The plasmids encoding N17Ras, N17Rac were described previously (Feig and Cooper 1988; Ridley et al. 1992). The plasmids encoding Raf BXB, Raf C4B,V12Rac were the gifts of Dr. M. Park, McGill University, Montreal, Canada. The inducible Raf C4B was made using a mammalian IPTG inducible expression system (Clonetec). PD 098059 (Biomol), wortmannin (Sigma Canada) and B-1086 (Eisai Research Institute, Andover, MA) were dissolved in dimethyl sulfoxide and stored at –80 ^{0}C at stock concentrations of 50, 10 and 10 mM, respectively, and diluted to the desired concentrations immediately prior to use.

ERK and JNK Activity Assays

Control and experimental H-500 cells were washed twice with ice-cold phosphate-buffered saline, scraped off plates and harvested in lysis buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml PMSF, 30 μg/ml aprotinin, 10 μg/ml sodium orthovanadate). The kinases were immunoprecipitated in lysis buffer using anti-JNK and -ERK antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates were washed twice in lysis buffer and twice in kinase buffer (50 mM Tris-HCL, pH 8.0, 25, mM MgCl2, 0.5 mM EGTA, 100 mM sodium orthovanadate, 2 mM DTT, 10% glycerol). Kinase assay was performed by incubating the immunoprecipitates in 30 μl kinase buffer containing 20 μM ATP, 1 μCi [γ-^{32}P] ATP (3000 Ci/mmol) (Amersham), and 0.5 μg/ml myelin basic protein (MBP) as ERK kinase substrate, or 2 μg/ml of jun-glutathion S-transferase fusion protein (GST-Jun) as JNK substrate. After 30 min at 30 degrees Celsius, the reaction was stopped by adding 10 μl of 0.6 % HCL containing 1 mM ATP and 1% bovine serum albumin. 30 μl of the samples were then spotted on phosphocellulose paper (P81, Whatman), washed five times in 180 mM phosphoric acid, and the amount of radioactivity incorporated into the respective substrates was determined by liquid scintillation spectrometry. In some experiments, the reaction was stopped by adding 10 ml of 4 x SDS sample buffer and the incorporation of ^{32}P into MBP or GST-Jun was determined by SDS-PAGE, and autoradiography of the died gel. The ERK antibody used in these experiments recognised both 41- and 44 kDa species and phosphorylation of MBP was the sum of these two isoforms of ERK.

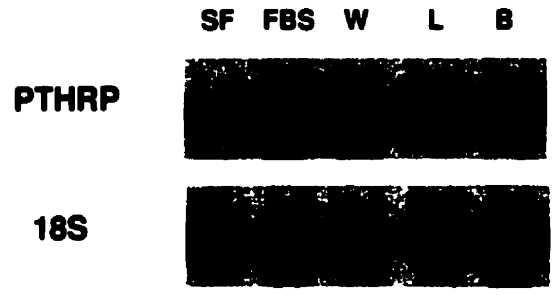
Northern Blot Analysis

Total cellular RNA was isolated from the control and experimental cells, following treatment with vehicle alone or increasing concentrations of B-1086, by acid guanidium thiocyanate-phenol-chloroform extraction. Twenty μg of total cellular RNA were electrophoresed on a 1.1% agarose-formaldehyde gel, transferred to a nylon membrane (Nytran, S&S, Keene, NH) by capillary blotting, fixed by air drying and cross-linked with UV for 10 min. The integrity of the RNA was assessed by ethidium bromide staining. Hybridization was carried out with ^{32}P-labelled PTHRP cDNA and with 18S RNA probe using a^{32}P dCTP as previously described (Thomas 1980). After a 24 h incubation at 42°C, filters were washed twice under low stringency conditions (1xSSC and 1% SDS; at RT for 40 min) and twice under high stringency conditions (0.1xSSC, 0.1% SDS; at 55^{0}C for 40 min). Autoradiography of filters was carried out at -70°C using XAR film (Eastman Kodak Co., Rochester, NY) with two intensifying screens or by using a phosphorimager screen (Eastman Kodak Co., Rochester, NY). The level of PTHRP expression was quantified by densitometric scanning using the Mac BAS V1.01 alias program.

Statistical Analysis. Results are expressed as the mean ± S.E. of at least triplicate determinations and statistical comparisons are based on the Student's *t* test or analysis of variance. A probability value of <0.05 was considered to be significant.

Figure 4.1: Effects of wortmannin, lovastatin and B-1086 on serum induced PTHRP gene expression.

H-500 Leydig cells were grown to 70% confluence, and incubated in serum free cuture medium as described in "Materials and Methods" for 24 h. During the serum free period, the cells were pretreated with wortmannin (100 nM) for 6 h, and lovastatin (3 μg/ml) and B-1086 (10 μg/ml) for 12 h. Following pretreatment the cells were stimulated with 10% FBS for 2 h and lysed. Fifteen μg of total cellular RNA was extracted from control and experimental cells and electrophoresed on a 1.1% agarose formaldehyde gel. Nylon filters with immobilized RNA were probed with a ^{32}P labelled PTHRP cDNA or with a ^{32}P labelled 18 S RNA probe as described in "Materials and Methods". All blots were quantified by densitometric scanning (lower panel). Error bars represent the standard deviation of triplicate determinations. Significant differences from control cells is represented by an asterisk ($^{*}p < 0.05$). Values with two asterisks represent significant differences from the values with a single asterisk ($^{**}p<0.05$). SF = serum-free control cell cultures.


SF
FBS
W
L
B
PTHRP
18S

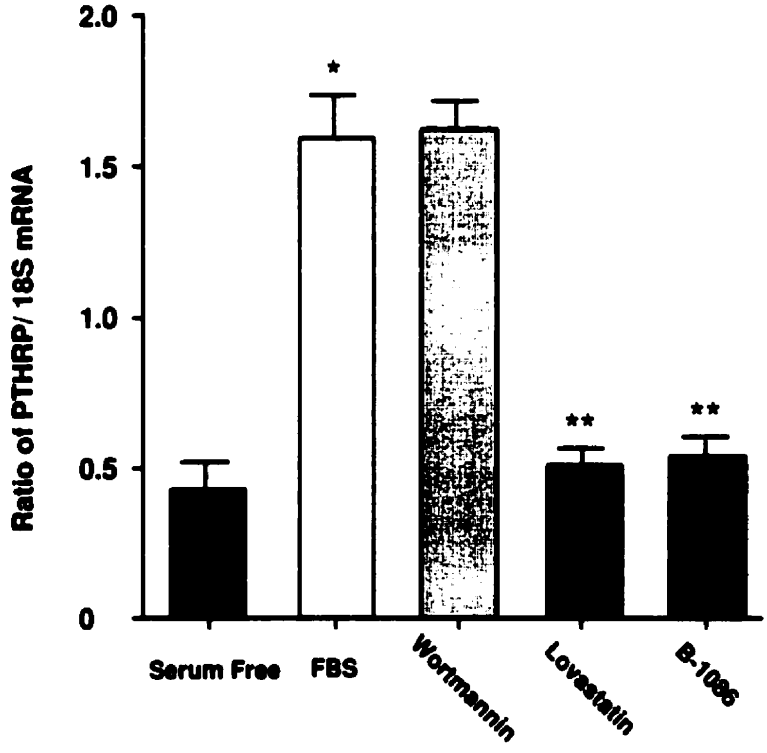

2.0
1.5
1.0
0.5
0
Ratio of PTHRP/ 18S mRNA
*
**
**
Serum Free
FBS
Wortmannin
Lovastatin
B-1066

Figure 4.2: Effect of transiently expressing Ras N17 on PTHRP gene expression and ERK kinase.

H-500 cells were grown to 70% confluence and transiently transfected with 0.1, 1 and 10 μg of Ras N17 plasmid as described in "Materials and Methods". Untransfected and transfected cells were stimulated with 10% FBS.

Panel A: After stimulation for 2 h, 15 μg of total cellular RNA extracted from control and experimental cells was electrophoresed on a 1.1% agarose formaldehyde gel. Nylon filters with immobilized RNA were probed with a ^{32}P labelled PTHRP cDNA or with a ^{32}P labelled 18 S RNA probe as described in "Materials and Methods". All blots were quantified by densitometric scanning (lower panel). Error bars represent the standard deviation of triplicate determinations. Significant difference from controls is represented by an asterisk ($^{*}p<0.05$). Values with two asterisks represent significant differences from values with a single asterisk ($^{**}p<0.05$).

Panel B: Following 10 mins of 10% serum stimulation, the cells were lysed and ERK 1 and 2 proteins were immunoprecipitated. Enzymatic activity was determined by immune complex kinase assays using myelin basic protein (MBP) as substrate, as described in "Materials and Methods". The results are representative of three determinations and are plotted as ratios over unstimulated control cells. SF = serum-free control cell cultures.

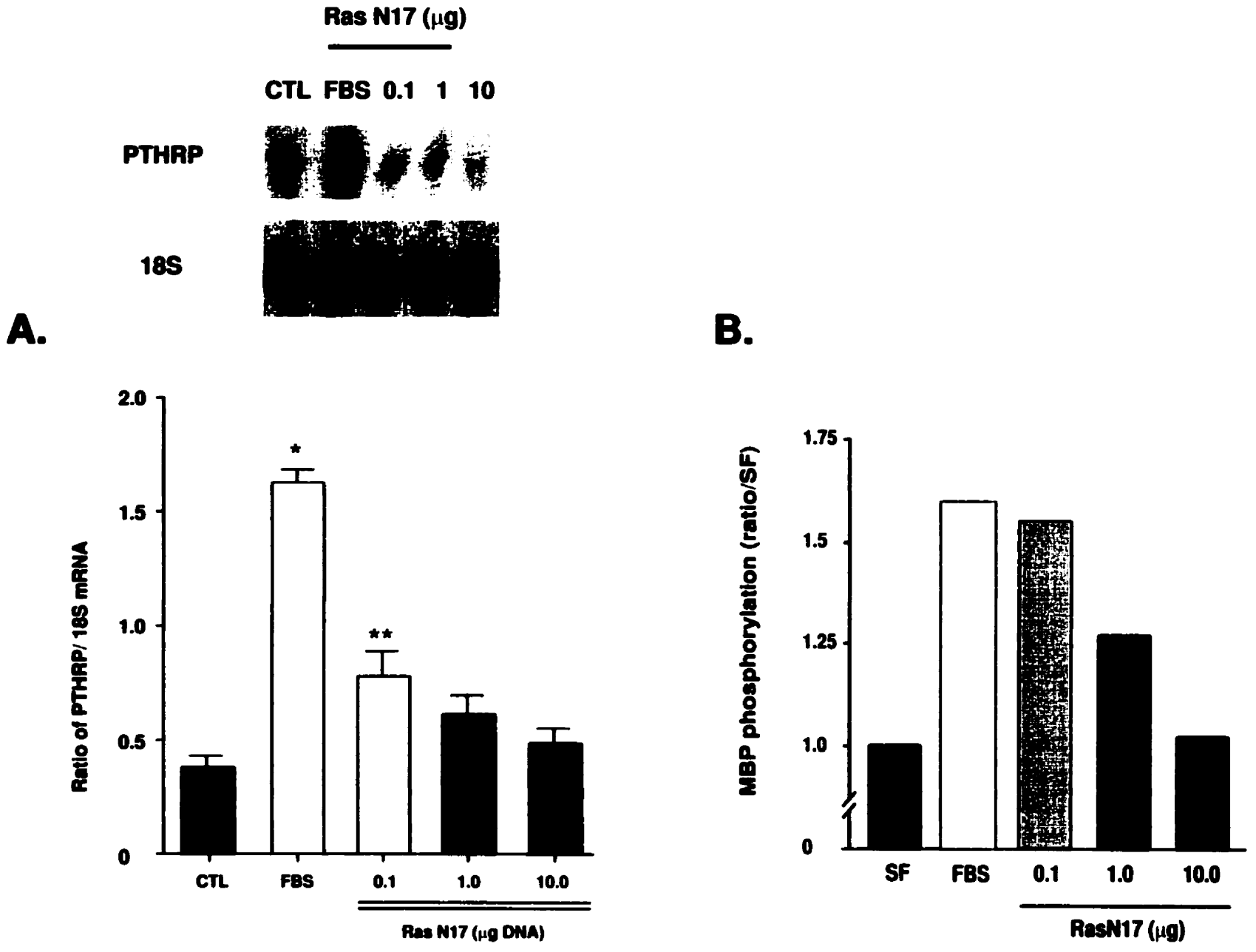

Ras N17 (μg)
CTL FBS 0.1 1 10
PTHRP
18S
A.
Ratio of PTHRP/ 18S mRNA
2.0
1.5
1.0
0.5
0
*
**
CTL
FBS
0.1
1.0
10.0
Ras N17 (μg DNA)
B.
MBP phosphorylation (ratio/SF)
1.75
1.5
1.25
1.0
0
SF
FBS
0.1
1.0
10.0
RasN17 (μg)

Figure 4.3: Effect of a truncation-activated form of Raf-1, Raf BXB, on PTHRP mRNA expression and ERK activity.

Semi-confluent H-500 cells were transiently transfected with 0.1, 1 and 10 μg of Raf BXB construct as described in "Materials and Methods". 48 h after transfection cells were starved in serum free media for 24 h and analyzed as follows:

Panel A: Control and transfected cells were lysed, 15 μg of total cellular RNA was extracted from the cells and electrophoresed on a 1.1% agarose formaldehyde gel. Following immobilization of RNA, nylon filters were probed with a ^{32}P labelled PTHRP cDNA or with a ^{32}P labelled 18 S RNA probe as described in "Materials and Methods". All blots were quantified by densitometric scanning (lower panel). Error bars represent the standard deviation of triplicate determinations. A significant difference from controls is represented by an asterisk ($^{*}p<0.05$). Values with two asterisks represent significant differences from the values with a single asterisk ($^{**}p<0.05$).

Panel B: The cells were lysed and ERK 1 and 2 proteins immunoprecipitated. Enzymatic activity was determined by immune complex kinase assays using myelin basic protein (MBP) as substrate, as described in "Materials and Methods". The results are representative of three determinations and are plotted ratios of non-stimulated control cells. SF = serum-free control cell cultures.

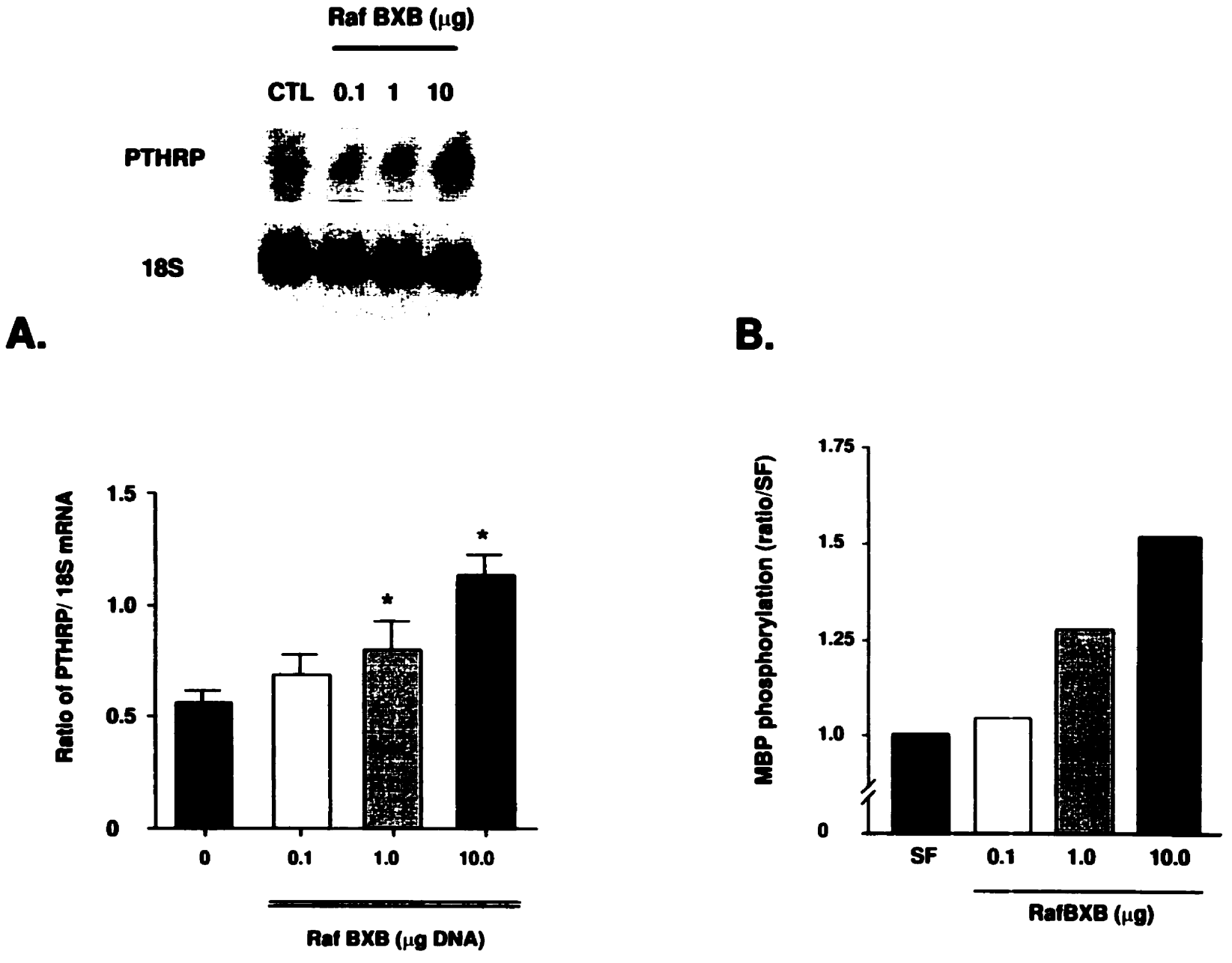

Raf BXB (μg)
CTL 0.1 1 10
PTHRP
18S
A.
Ratio of PTHRP/ 18S mRNA
1.5
1.0
0.5
0
0 0.1 1.0 10.0
Raf BXB (μg DNA)
B.
MBP phosphorylation (ratio/SF)
1.75
1.5
1.25
1.0
0
SF 0.1 1.0 10.0
RafBXB (μg)

Figure 4.4: Effect of inducing a dominant negative mutant of Raf-1, Raf C4B, on PTHRP gene expression and ERK activity.

H-500 cells, stably transfected with an IPTG inducible Raf C4B construct, were starved for 24 h in serum free media prior to induction of Raf C4B expression with 100 μM IPTG for 2, 4 and 8 h. Following stimulation with 10% serum for 2 h, 15 μg of total cellular RNA was extracted from control and experimental cells and electrophoresed on a 1.1% agarose formaldehyde gel. Following transfer of RNA, the filters were probed with a ^{32}P labelled PTHRP cDNA or with a ^{32}P labelled 18 S RNA probe as described in "Materials and Methods". All blots were quantified by densitometric scanning (lower panel). Error bars represent the standard deviation of triplicate determinations. Significant difference from controls is represented by an asterisk ($^{*}p<0.05$). Values with two asterisks represent significant differences from the values with a single asterisk ($^{**}p<0.05$).

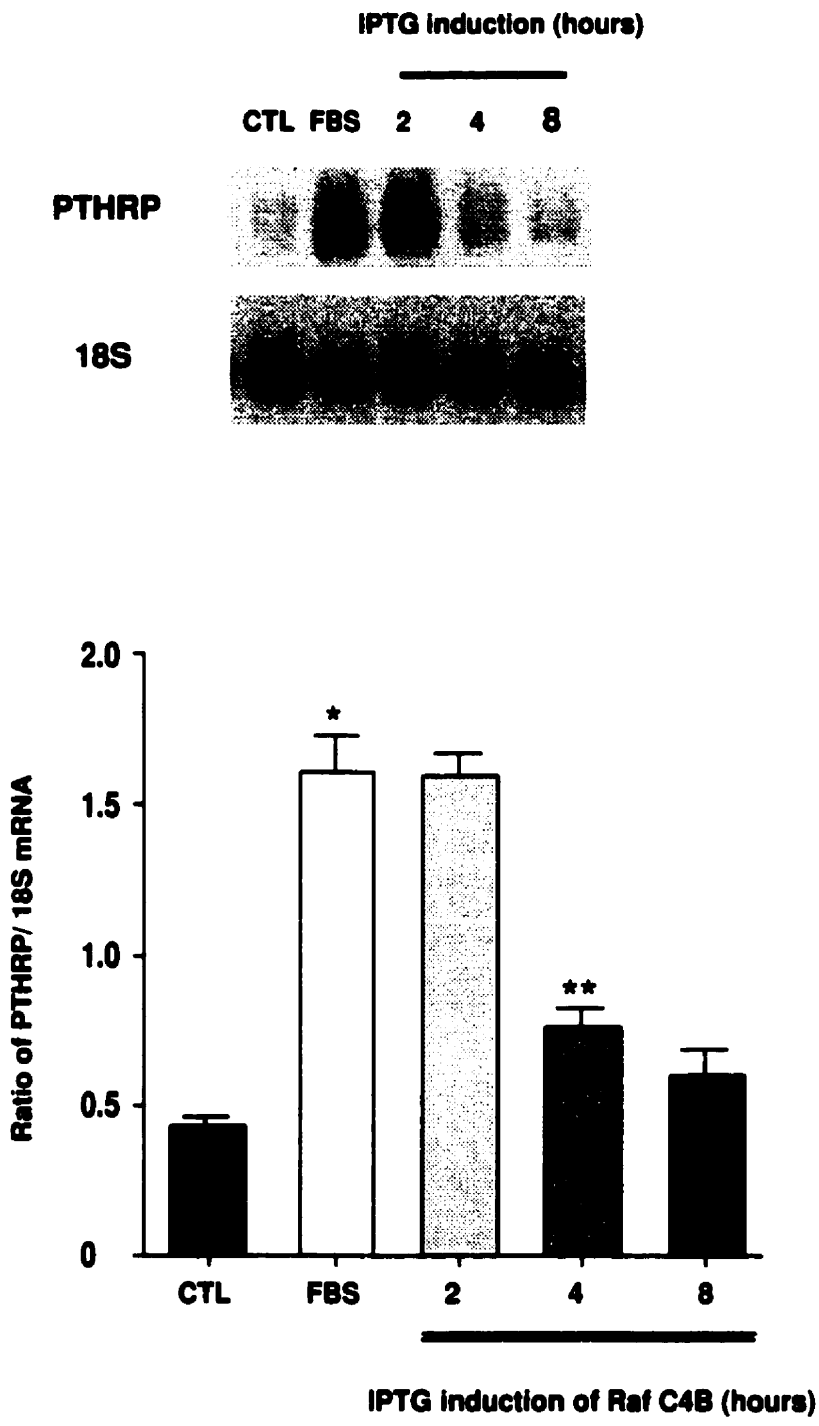
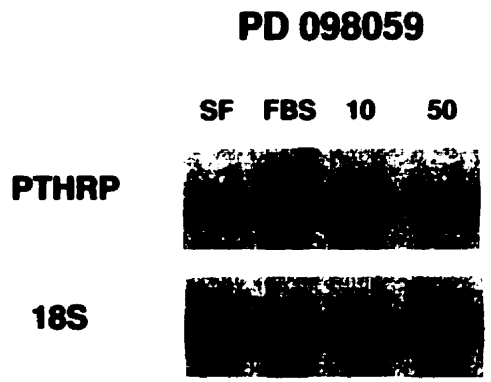

IPTG induction (hours)
CTL FBS 2 4 8
PTHRP
18S
2.0
1.5
1.0
0.5
0
Ratio of PTHRP/ 18S mRNA
*
**
CTL
FBS
2
4
8
IPTG induction of Raf C4B (hours)

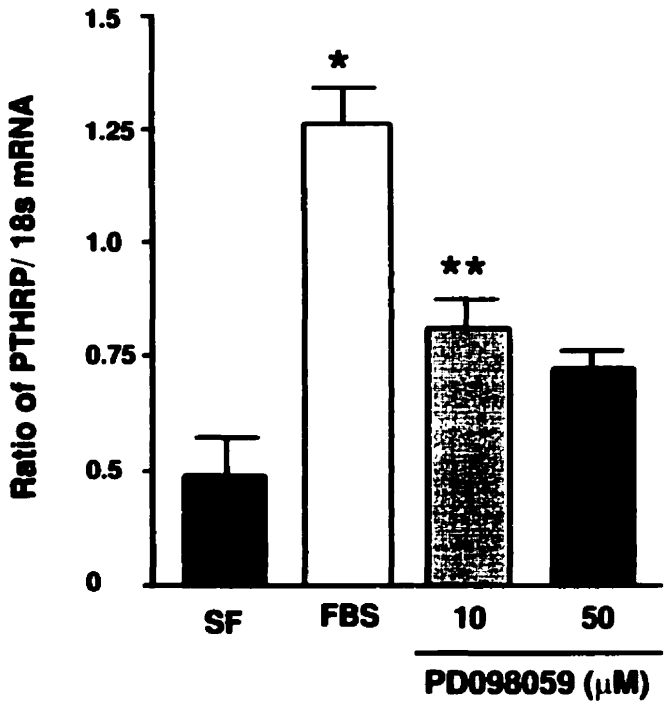
Figure 4.5: Effect of MEK inhibitor, PD 098059, on expression of PTHRP gene and efficacy of inhibiting ERK activation.

Panel A: Following 24 h preincubation with 10 or 50 μg PD 098059 in serum free media, H-500 cells were stimulated with 10% serum for 2 h. Fifteen μg of total cellular RNA was extracted from control and experimental cells and electrophoresed on a 1.1% agarose formaldehyde gel. Following transfer of RNA to nylon membranes, the filters were probed with a ^{32}P labelled PTHRP cDNA or with a ^{32}P labelled 18 S RNA probe as described in "Materials and Methods". All blots were quantified by densitometric scanning (lower panel). Error bars represent the standard deviation of triplicate determinations. Significant difference from controls is represented by an asterisk ($^{*}p<0.05$). Values with two asterisks represent significant differences from the values with a single asterisk ($^{**}p<0.05$).

Panel B: Following 10 mins of 10% serum stimulation, the cells were lysed, ERK 1 and 2 proteins were immunoprecipitated. Enzymatic activity was determined by immune complex kinase assays using myelin basic protein (MBP) as substrate as described in "Materials and Methods". The results are representative of three such determinations and are plotted as fold increase over unstimulated activity. SF= serum-free control cell cultures.


PD 098059
SF
FBS
10
50
PTHRP
18S

A.


Ratio of PTHRP/ 18s mRNA
1.5
1.25
1.0
0.75
0.5
0
*
**
SF
FBS
10
50
PD098059 (μM)

B.

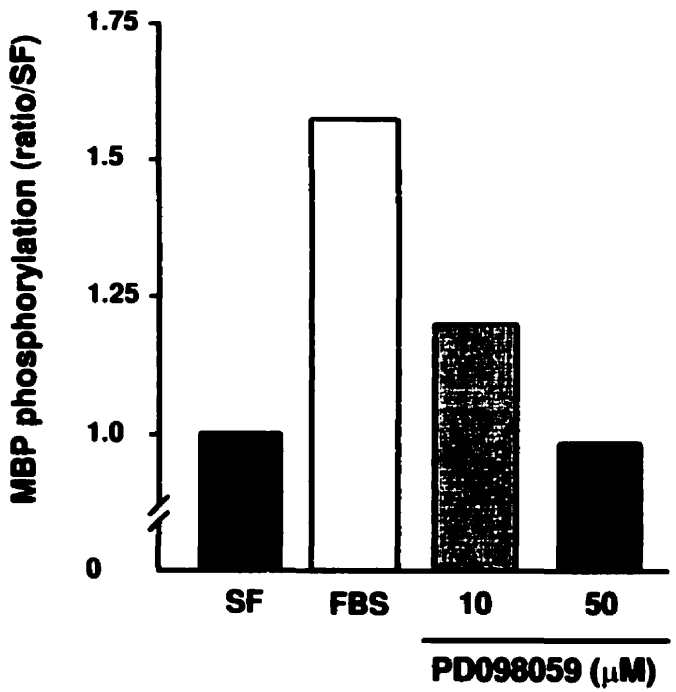

MBP phosphorylation (ratio/SF)
1.75
1.5
1.25
1.0
0
SF
FBS
10
50
PD098059 (μM)

Figure 4.6: Effect of UV irradiation on PTHRP gene expression.

H-500 cells were serum starved for 24 h and were either left untreated (0) or exposed to 40 J/m^2 of UV-C irradiation. The cells were lysed at 0.5, 1 and 3 h following stimulation. Fifteen μg of total cellular RNA was extracted from unstimulated and experimental cells and electrophoresed on a 1.1% agarose formaldehyde gel. Following RNA transfer to nylon membranes, the filters were probed with a ^{32}P labelled PTHRP cDNA or with a ^{32}P labelled 18 S RNA probe as described in "Materials and Methods". All blots were quantified by densitometric scanning (lower panel). Error bars represent the standard deviation of triplicate determinations. Significant difference from controls is represented by an asterisk (*$p<0.05$).

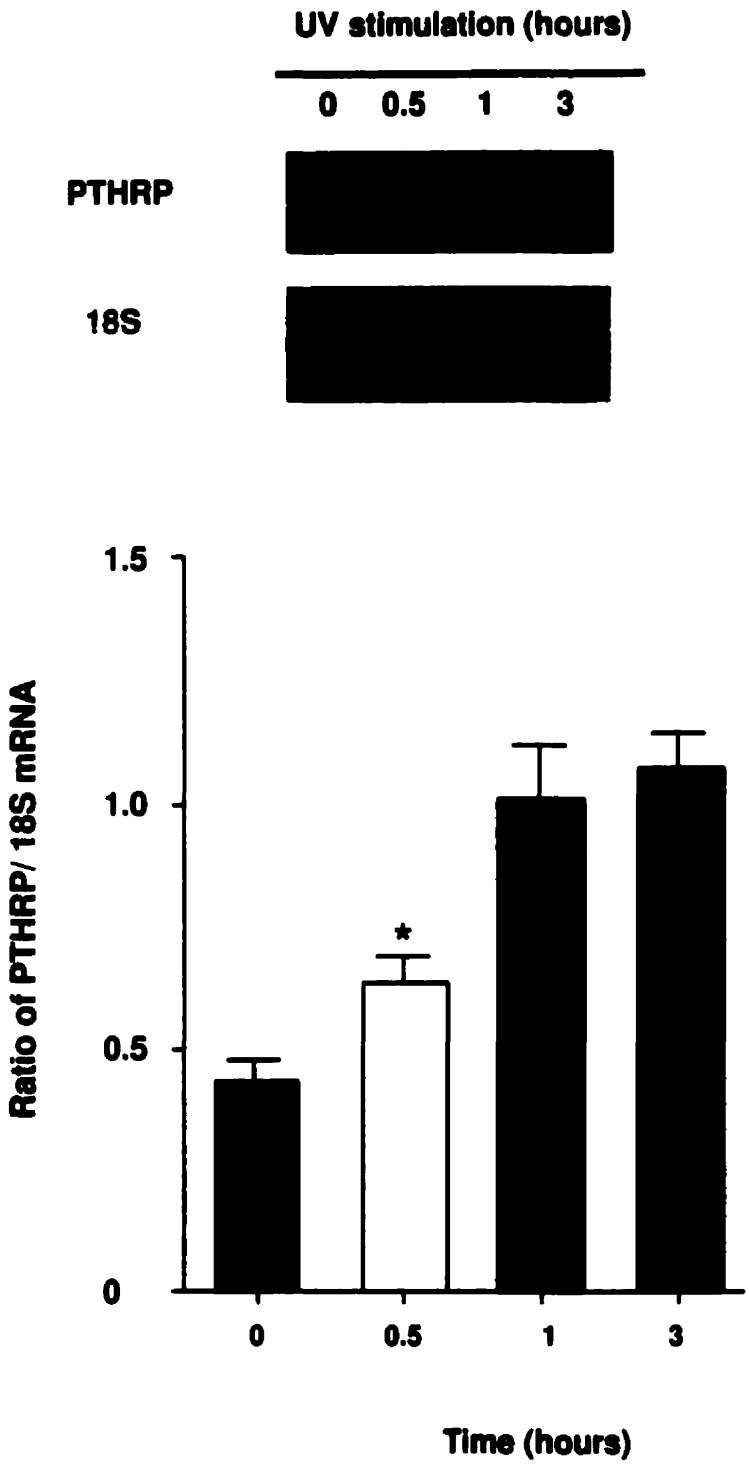
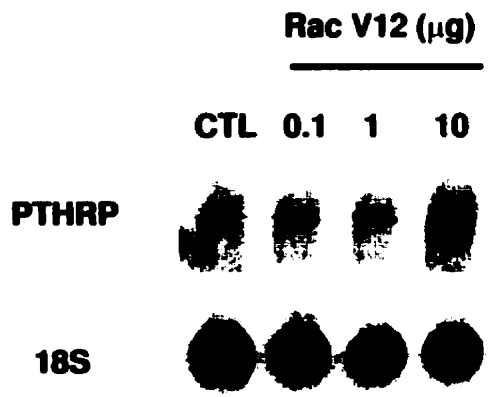

UV stimulation (hours)
0
0.5
1
3
PTHRP
18S
1.5
1.0
0.5
0
Ratio of PTHRP/ 18S mRNA
*
0
0.5
1
3
Time (hours)

Figure 4.7: Effect of expressing an activated mutant Rac V12 on PTHRP gene expression levels and on JNK activation.

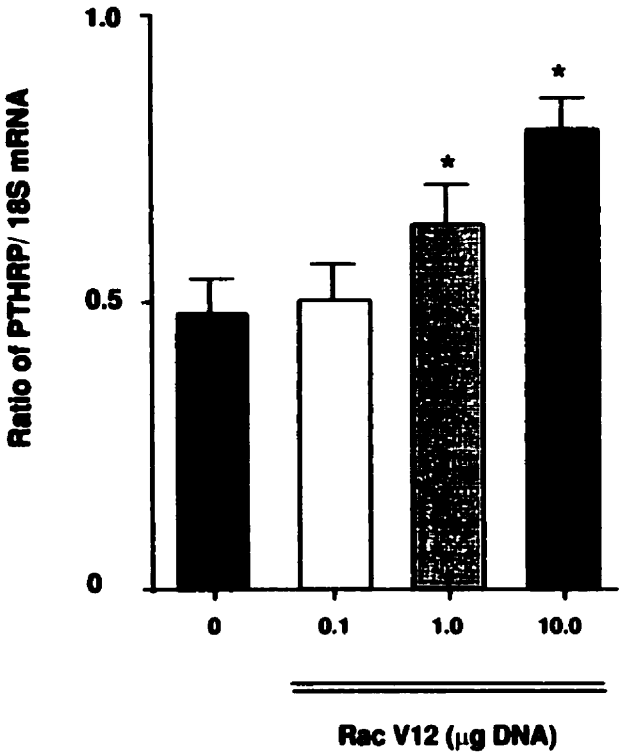
H-500 cells were grown to semi-confluence and transiently transfected with 0.1, 1 and 10 μg of Rac V12 construct as described in "Materials and Methods". Following transfection cells were starved for 24 h in serum free media.

Panel A: Fifteen μg of total cellular RNA was extracted from control and transfected cells and electrophoresed on a 1.1% agarose formaldehyde gel. Following RNA transfer to nylon membranes, the filters were probed with a ^{32}P labelled PTHRP cDNA or with a ^{32}P labelled 18 S RNA probe as described in "Materials and Methods". All blots were quantified by densitometric scanning (lower panel). Error bars represent the standard deviation of triplicate determinations. Significant difference from controls is represented by an asterisk (*$p<0.05$).

Panel B: The cells were lysed and JNK-1 protein was immunoprecipitated. Enzymatic activity was determined by immune complex kinase assays using GST-jun fusion protein as substrate as described in "Materials and Methods". The results are representative of three such determinations and are plotted as fold increase over unstimulated activity. SF = serum-free control cell cultures.


Rac V12 (μg)
CTL 0.1 1 10
PTHRP
18S

A.


1.0
0.5
0
Ratio of PTHRP/ 18S mRNA
*
*
0 0.1 1.0 10.0
Rac V12 (μg DNA)

B.

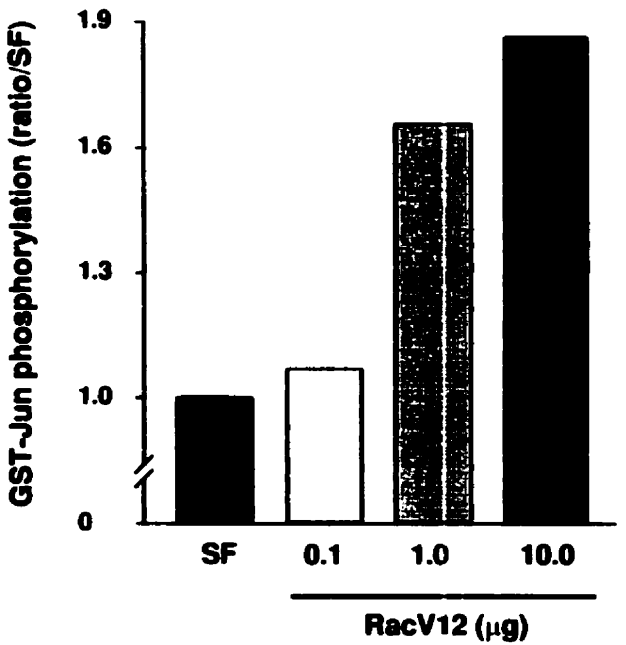

1.9
1.6
1.3
1.0
0
GST-Jun phosphorylation (ratio/SF)
SF 0.1 1.0 10.0
RacV12 (μg)

Figure 4.8: Effect of a dominant negative mutant of Rac-1, Rac N17, on PTHRP gene expression and JNK activity.

Semi-confluent H-500 cells were transiently transfected with 0.1, 1 and 10 μg of Rac N17 construct, as described in "Materials and Methods". 48 h after transfection cells were starved in serum free media for 24 h and analysed as indicated below.

Panel A: Control and transfected cells were lysed and 15 μg of total cellular RNA extracted from the cells was electrophoresed on a 1.1% agarose formaldehyde gel. RNA was transferred to nylon membranes which were probed with a ^{32}P labelled PTHRP cDNA or with a ^{32}P labelled 18 S RNA probe as described in "Materials and Methods". All blots were quantified by densitometric scanning (lower panel). Error bars represent the standard deviation of triplicate determinations. Significant difference from controls is represented by an asterisk ($^{*}p<0.05$). Values with two asterisks represent significant differences from the values with a single asterisk ($^{**}p<0.05$).

Panel B: After 10 mins of serum stimulation, the cells were lysed and JNK-1 protein was immunoprecipitated. Enzymatic activity was determined by immune complex kinase assays using GST-jun fusion protein as substrate as described in "Materials and Methods". The results are representative of three such determinations and are plotted as fold increase over unstimulated activity.

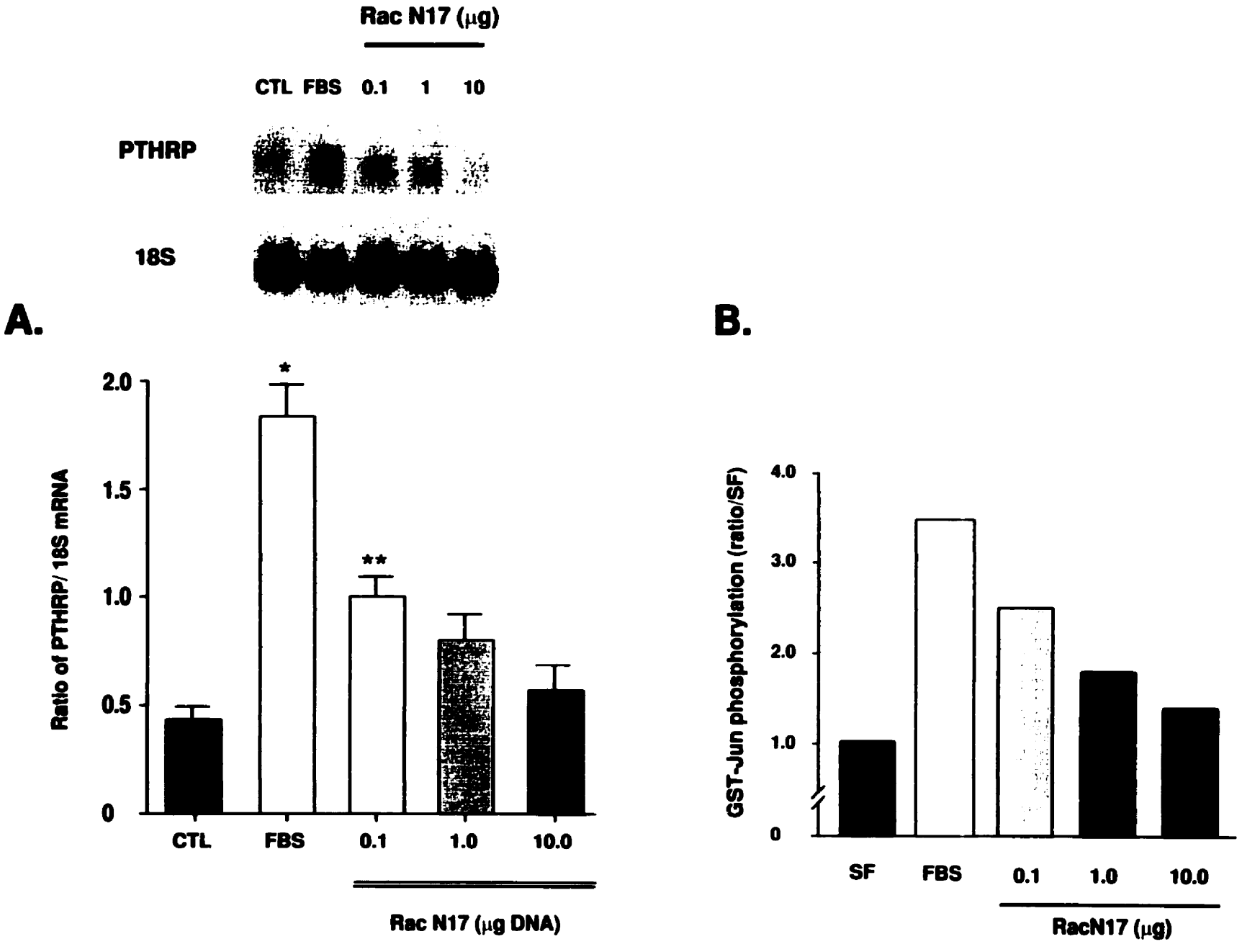

Rac N17 (μg)
CTL FBS 0.1 1 10
PTHRP
18S
A.
Ratio of PTHRP/ 18S mRNA
2.0
1.5
1.0
0.5
0
*
**
CTL
FBS
0.1
1.0
10.0
Rac N17 (μg DNA)
B.
GST-Jun phosphorylation (ratio/SF)
4.0
3.0
2.0
1.0
0
SF
FBS
0.1
1.0
10.0
RacN17 (μg)

Figure 4.9: Effect of dominant negative Rac N17 on cycloheximide induced PTHRP gene expression.

Control cells and cells transiently transfected with 1 μg Rac N17 were serum starved for 24h, stimulated with 10 μg/ml cycloheximide (CHX) for 2h and lysed. Fifteen μg of total cellular RNA extracted from the cells was electrophoresed on a 1.1% agarose formaldehyde gel. RNA was transferred to nylon membranes which were probed with a ^{32}P labelled PTHRP cDNA or with a ^{32}P labelled 18 S RNA probe as described in "Materials and Methods". All blots were quantified by densitometric scanning (lower panel). Error bars represent the standard deviation of triplicate determinations. Significant difference from controls is represented by an asterisk (*$p<0.05$).

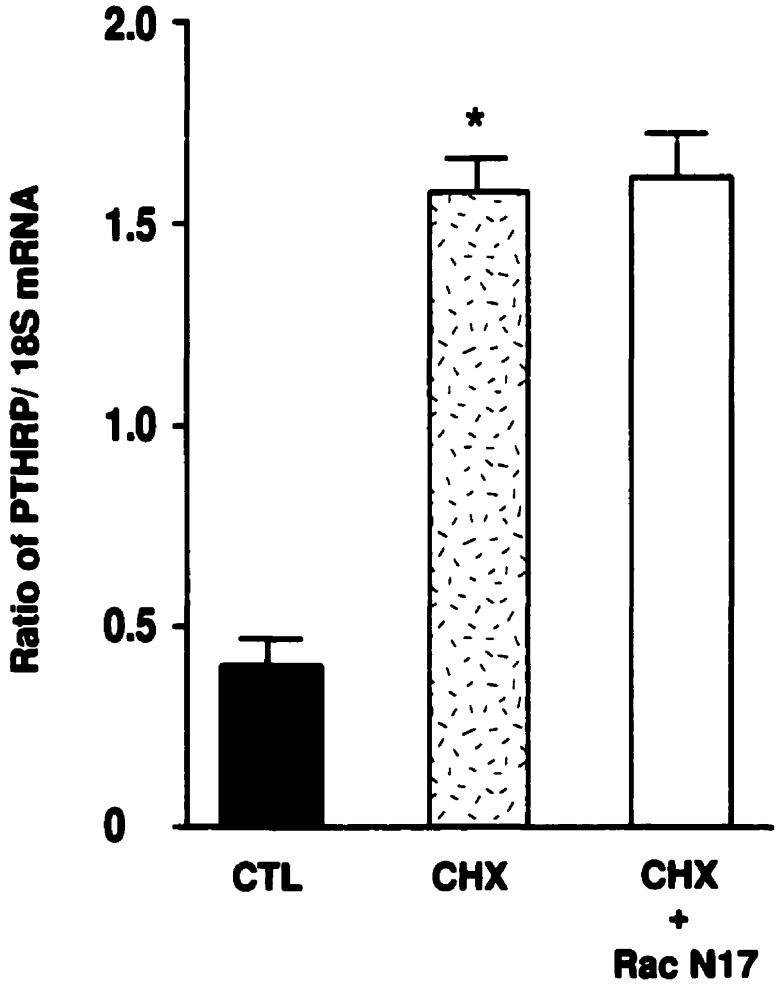
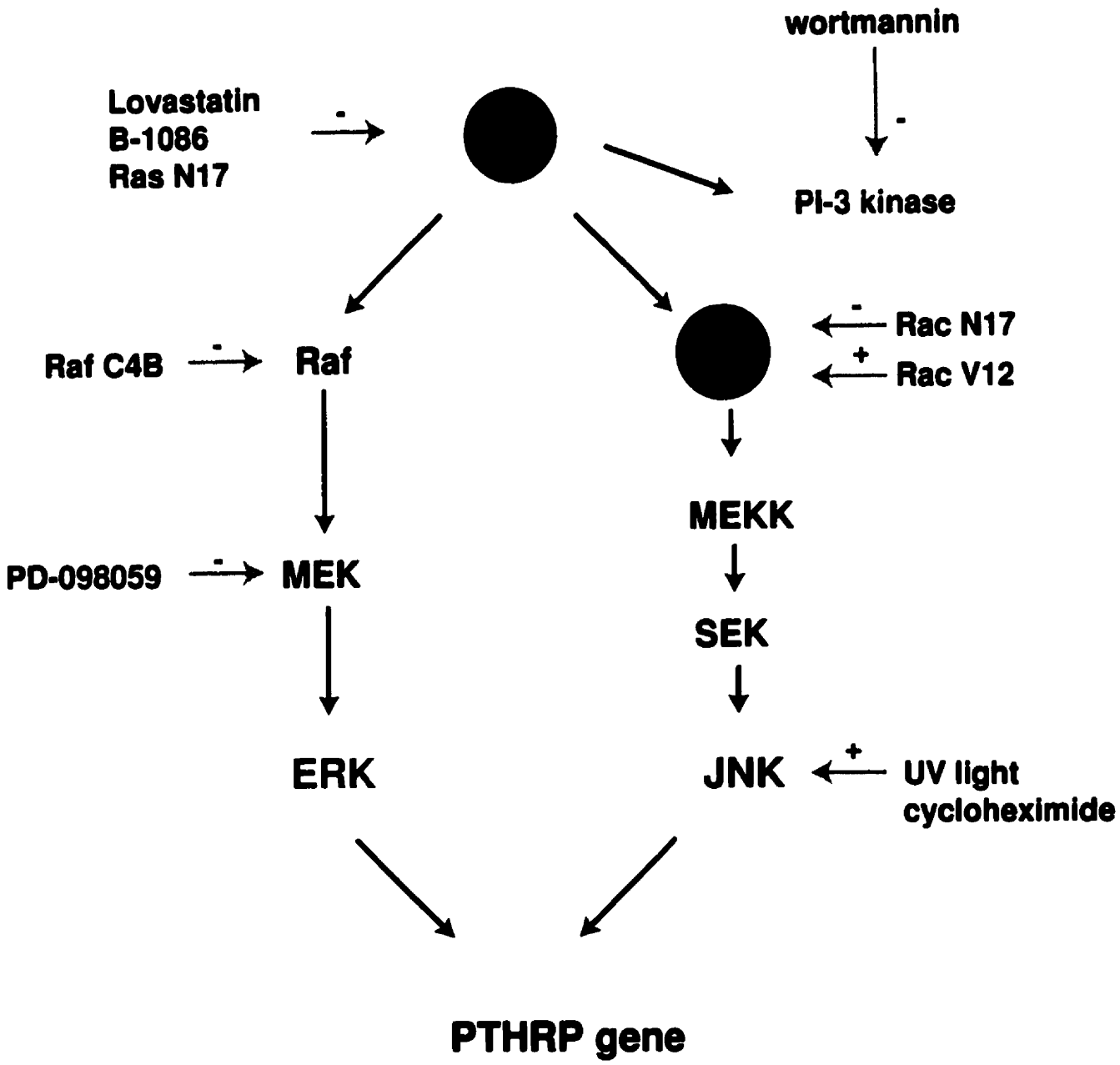

2.0
1.5
1.0
0.5
0
Ratio of PTHRP/ 18S mRNA
*
CTL
CHX
CHX
+
Rac N17

Figure 4.10: A schematic diagram of MAP kinase signaling pathways involved in regulating the PTHRP gene.

Our findings suggest that parallel MAP kinase pathways mediate serum induced expression of the PTHRP gene. Classically, ERK- and JNK- dependent pathways regulate the expression of genes involved in growth, differentiation and apoptosis.


wortmannin
Lovastatin
B-1086
Ras N17
PI-3 kinase
Raf C4B
Raf
Rac N17
Rac V12
MEKK
PD-098059
MEK
SEK
ERK
JNK
UV light
cycloheximide
PTHRP gene

4.2.4 Results

Effects of wortmannin, lovastatin and B-1086 on serum induced PTHRP gene expression.

We have previously reported that PTHRP expression is increased more than 10-fold in a RasV12 transformed fisher rat fibroblast cell line (Chapter 3). To determine whether activation of Ras is necessary for overexpression of PTHRP, we used H-500 cells, which express high levels of PTHRP in response to 10% FBS (serum). Serum induced PTHRP expression in H-500 cells was used as our model system to investigate the role of the Ras activated pathways in the regulation of PTHRP expression. As shown in Fig. 1, there was a 4-fold increase of PTHRP mRNA expression after a 2h incubation with serum, as compared to PTHRP expression in serum-free cells. Pretreatment of H-500 cells with the PI 3-kinase inhibitor wortmannin (100nM) (Powis et al. 1994), for 6 h, had no effect on the expression of PTHRP mRNA. A marked decrease in PTHRP mRNA expression was seen after pretreatment of H-500 cells for 24 h with lovastatin (3 μg/ml) and B-1086 (10 μg/ml), which are potent inhibitors of Ras processing (Sinensky et al. 1990; Nagasu et al. 1995; Gibbs et al. 1993). As determined by trypan blue staining, the inhibitors used at these concentrations, were not cytotoxic in H-500 cells (data not shown).

Effect of transiently expressing Ras N17 on PTHRP gene expression and ERK kinase.

To further investigate the role of Ras in the regulation of PTHRP expression, H-500 cells were transiently tranfected with 0.1, 1.0 and 10 μg of Ras N17 and 0.1 μg of

control LTR-GH cDNA. As shown in Fig. 2A, transient expression of Ras N17 led to a dose-dependent inhibition of serum induced PTHRP expression with greater than 50% inhibition occurring at 0.1 μg, and complete inhibition at 10 μg of Ras N17. Inhibition of PTHRP mRNA expression was correlated with a pronounced suppression of MAP kinase activity as determined by MBP phosphorylation in *in vitro* kinase assay (Fig. 2B). Transfection of similar concentrations of control LTR-GH cDNA failed to inhibit PTHRP mRNA expression or to suppress MAP kinase activity (data not shown).

Effect of an activated form of Raf-1, Raf BXB, on PTHRP mRNA expression and ERK activity.

Serum and Ras induced gene expression involves a signal cascade that leads to activation of multiple signaling pathways including the Raf-MEK-ERK pathway. Raf-1 was shown to be required for serum- and Ras-induced promoter activation (Bruder et al. 1992). To examine whether activation of Raf alone could increase expression of PTHRP gene, we transiently overexpressed an activated mutant Raf BXB in H-500 cells. Cells expressing Raf BXB were serum starved for 24 h and expression of PTHRP mRNA was analyzed. As shown in Fig 3A, expression of Raf BXB enhanced basal PTHRP expression in a dose dependent manner. Addition of 10 μg of Raf BXB caused a fold increase, whereas 0.1 μg of the cDNA increased PTHRP mRNA by 50%. The induction of PTHRP mRNA also coincided with stimulation of MAP kinase activity as shown in Fig. 3B.

Effect of a dominant negative form of Raf-1, Raf C4B, on PTHRP mRNA expression and ERK activity.

We next investigated whether Raf-1 might also play a role in serum induced expression of PTHRP in H-500 cells. To determine whether activation of Raf-1 was required, we used H-500 cells stably transfected with an inducible Raf C4B expression construct. We found that addition of IPTG into the culture medium rapidly induced expression of the dominant negative Raf C4B reaching peak level of expression at 8 h (data not shown). We used these cells to determine whether Raf-1 activity was required for serum increased PTHRP expression. As shown in Fig. 4A, PTHRP was highly induced 2 h after serum stimulation, and this was attenuated after Raf C4B was induced. The effect of Raf C4B on the expression of PTHRP was detected 4 h after addition of IPTG, and PTHRP expression was inhibited to near basal levels after 8 h of incubation. These data are consistent with previous findings demonstrating that Raf-1 is a direct target of Ras, and expression of many Ras-induced genes are dependent on Raf activity.

Effect of MEK inhibitor, PD 098059, on expression of PTHRP gene and efficacy of inhibiting ERK activation.

PD 098059 is a specific inhibitor of mitogen activated protein kinase kinase (MEK), and has been shown at (50 μM) concentration, to completely inhibit the activation of MEK *in vitro* and *in vivo* (Alessi et al. 1995). Serum has been shown to stimulate gene expression by activating the MEK-ERK pathway (Bruder et al. 1992; Troppmair et al. 1994). To determine whether MEK activation was required for serum induced PTHRP expression, H-500 cells were first pretreated with 10 μM or 50μM PD

098059 and then stimulated with serum. Pretreatment with PD 098059 inhibited expression of PTHRP mRNA, which was detectable at 10 μM concentration (Fig. 5A). The effect of PD 098059 on PTHRP expression was also associated with inhibition of ERK activity at the same concentrations (Fig. 5B)

Effect of UV irradiation on PTHRP gene expression.

We next explored a role for stress activated JNK pathway in stimulation of PTHRP expression by serum. Recent studies have demonstrated that JNKs can be phosphorylated and activated in response to enviromental stress, UV light and serum (Kyriakis et al. 1994; Minden et al. 1994; Derijard et al. 1994). To approach this question we first examined the effect of UV stimulation on PTHRP expression. Exposure to 40 J/m^2 and 80 J/m^2 UVC induced a rapid increase in JNK activation within 10 minutes in H-500 cells (data not shown). Serum starved H-500 cells were stimulated with 40 J/m^2 of UVC and PTHRP expression was measured 0.5, 1 and 2 h following stimulation. As shown in Fig. 6, UVC irradiation led to increased PTHRP mRNA expression in a time dependent manner. After 1h, the level of expression remained relatively constant.

Effect of expressing an activated mutant Rac V12 on PTHRP mRNA levels and on JNK activation.

Activation of Rac1 leads to the sequential activation of MEKK1, JNKK and JNK (Bagrodia et al. 1995; Zhang et al. 1995; Frost et al. 1996). Consequently, we determined the effect of an activated mutant of Rac1, Rac V12, on PTHRP expression.

Serum starved H-500 cells were transfected with varying amounts of Rac V12. Expression of Rac V12 increased PTHRP expression by approximately 50% when compared to untransfected cells (Fig. 7). A ten-fold μg excess of the construct was slightly more active in this regard. Similarly, 1 and 10 μg of the Rac V12 expression vector were found to increase JNK activation, as measured by GST-Jun substrate phosphorylation in *in vitro* immunocomplex kinase assays (Fig. 7B).

Effect of a dominant negative mutant of Rac-1, Rac N17, on PTHRP gene expression and JNK activity.

The signaling intermediates between Ras and JNK are not well understood. Recently, several groups showed that Rac1 and CDC42 are involved in mediating the signals from Ras to JNK (Coso et al. 1995; Minden et al. 1995). We tested the possibility that Rac1 may be mediating the Ras-dependent, serum induction of PTHRP expression. To determine the role of Rac1, we made use of a dominant negative Rac N17 construct transiently transfected at 0.1, 1 and 10 μg, into H-500 cells. Expression of dominant negative Rac N17 potently blocked the effects of serum on PTHRP expression, and almost 100% inhibition was achieved at 10 μg DNA (Fig. 8A). This is mirrored by a similar dose dependent inhibition of JNK activity as determined by *in vitro* kinase assay (Fig. 8B). These data indicates that serum stimulated PTHRP gene expression requires Rac1 activation.

Effect of dominant negative Rac N17 on cycloheximide induced PTHRP gene expression.

As in the case of many immediate early genes, PTHRP was previously shown to be induced by cycloheximide. Cycloheximide has been shown to induce gene expression through a JNK dependent mechanism (Raingeaud et al. 1995; Chen et al. 1996). To determine if Rac1 was involved in cycloheximide induced PTHRP expression, the effect of cycloheximide on H-500 cells transiently expressing Rac N17 was assessed. As shown in Fig. 9, cycloheximide potently increased PTHRP expression by 4-fold. We found that expression of Rac N17 does not interfere with cycloheximide induced PTHRP expression.

4.2.5 Discussion

PTHRP plays a critical role in disease and in normal physiology. Studies by us and other investigators have identified numerous exogenous agents including EGF, serum (Sebag et al. 1992; Heath et al. 1995; Ferrari et al. 1994), phorbol esters (Rodan et al. 1989), and various oncogenes (Chapters 2 and 3; Motokura et al. 1995; Li and Drucker 1994a) which induce the synthesis of this protein. We have also previously demonstrated that tyrosine kinases mediate their effect on this gene via Ras-dependent pathways (Chapter 2). Indeed, many stimulators of PTHRP gene expression are also activators of Ras. However, the mechanisms that connect Ras to nuclear events leading to induction of PTHRP gene expression are not known. To our knowledge, this is the first demonstration of a role for ERK- and JNK-dependent signaling in the regulation of PTHRP expression.

We have previously shown that PTHRP is induced in response to serum in H-500 cells (Liu et al. 1992). Serum is known to potently activate the MAP kinases ERKs and JNKs (Troppmair et al. 1994; Minden et al. 1995), which convey the intracellular signal to the nucleus, to promoters of the hundreds of serum-regulated genes. Our findings suggest that ERK and JNK pathways are both required for serum induced PTHRP expression, since inhibition of either pathway completely attenuates PTHRP expression. Furthermore, as compared to activation of both pathways in response to serum, independent activation either JNK or ERK cascades results in a detectable but relatively small increase in PTHRP expression. This suggests that these pathways may co-operate to regulate the transcription of target genes. Supporting this notion, cross-talk between these pathways has been shown to occur in activation of nuclear transcription factors

affecting AP-1 activity. While ERKs phosphorylate TCF/Elk-1, they are unable to activate c-Jun or ATF2 (Hipskind et al. 1994). JNKs, on the other hand, can phosphorylate and activate both c-Jun and ATF2 (Derijard et al. 1994; Gupta et al. 1995).

We were surprised to find a requirement for JNK in the regulation of PTHRP expression by serum, since JNK, which was initially identified as a stress-induced kinase activated in response to UV light and changes in osmolarity (Derijard et al. 1994; Kyriakis et al. 1994), is widely perceived as a stress-activated kinase. Although these stress stimuli are indeed the most potent activators of JNK, serum has been shown to produce a three-fold increase of JNK activation (Minden et al. 1994).

Previously, PTHRP expression was shown to be induced by cycloheximide (Ikeda et al. 1990). Subsequently, it has been shown that cycloheximide as well as other protein synthesis inhibitors can activate JNK (Newton et al. 1997; Iordanov et al. 1997). However, induction of JNK activity by protein synthesis inhibitors has been suggested to involve mechanisms alternative to activation of Rac1/CDC42 (Newton et al. 1997; Iordanov et al. 1997). In agreement with these studies, we report that RacN17 does not inhibit cyclohexmide induced PTHRP expression. Whether cycloheximide induces PTHRP via the JNK pathway, has yet to be directly demonstrated.

The current study also provides a possible mechanism through which a diverse set of stimuli can induce PTHRP gene expression. Indeed, many factors that induce PTHRP gene expression can also activate these pathways. These include G-protein coupled receptors (Coso et al. 1995b) phorbol esters (Yamaguchi et al. 1995), cytokines (Chen et

al. 1996; Nikolakaki et al. 1994; Bird et al. 1994), and mechanical stretch (Hamada et al. 1998; Tumminia et al. 1998).

Regulation of genes by the ERK- and JNK- dependent pathways is in part, through activation AP-1 transcription factors (Fos and Jun proteins), the serum response factor/ tertiary complex factor (SRF/TCF) and the c-*sis* inducible factor (SIF) (Lewis et al. 1998) The PTHRP promoter contains binding elements for TCF transcription factors, which have been shown to mediate retinoic acid induced PTHRP expression (Karperien et al. 1997). However, the exact nature of the transcription factors involved in activation of the PTHRP promoter in response to serum and growth factors requires further investigation.

In conclusion, the results from this study show that PTHRP is regulated by MAP kinase signaling pathways, involving ERK and JNK activation. MAP kinases are molecular cross-roads where multiple signals converge, thus providing exciting possibilities for drug targeting to reduce PTHRP expression in hypercalcemia of malignancy.

4.2.6 Acknowledgements

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CHAPTER 5

GENERAL DISCUSSION

5.1 PREFACE

The genesis of cancer is a multi-step process and involves the expression of multiple oncogenes which disrupt the control of ordinary pathways that regulate cell proliferation and differentiation (Land et al. 1983). Growth factors are a family of polypeptides that can act on a broad range of target cells to modulate cell function. Under appropriate conditions, growth factors can either stimulate or inhibit cell proliferation, or exert other cellular effects unrelated to proliferation (Sporn and Roberts 1988). By binding cell surface tyrosine kinase receptors, they generate specific intracellular signaling cascades that result in the expression of target genes involved in mediating the biological response (Heschman 1991). The discovery that many oncogenes were once genes for growth factors, their receptors, or intracellular transducers of growth factor activated signals, which have undergone genetic alterations leading to their uncontrolled activation, brought to light the inextricable link between growth factors and oncogenes. During oncogenesis, many genes normally regulated by growth factors are aberrantly expressed in cancers and some, including many polypeptide hormones, growth factors, interleukins and cytokines, can lead to development of paraneoplastic syndromes (Ascensao et al. 1987; Hocking et al. 1983; Ellis et al. 1987; Beutler et al. 1985). Among the intracellular messenger proteins, Ras GTPases are key intermediates in growth factor signal transduction and are ubiquitously activated by virtually all known growth factors (Khosravi-Far and Der 1994). Signaling proteins collectively known as MAP kinases transduce the Ras signal to the nucleus.

The PTHRP gene is overexpressed in a significant subset of cancers leading to HHM. The mechanism whereby this occurs is not known. However, regulation of the

PTHRP gene by growth factors has been demonstrated on various occasions (Liu et al. 1993; Sebag et al. 1994; Rizzoli et al. 1994). I hypothesized that deregulated PTHRP gene expression in cancer may be the direct consequence of the abnormal function of growth factor signal transduction pathways. In support of this, this thesis describes the regulation of the PTHRP gene in relation to growth factor signaling pathways.

5.2 REGULATION OF PTHRP BY A GROWTH FACTOR RECEPTOR DERIVED ONCOGENE.

Under physiological conditions, ligand binding activates the enzymatic activity of a receptor tyrosine kinase. This event promotes receptor dimerization and the receptor becomes trans-phosphorylated on tyrosine residues. The activated receptor then acts as a center for the assembly of a multi-protein complex that transmits the intracellular signals (Schlessinger and Ullrich 1992). The hepatocyte growth factor (HGF), also known as *scatter factor* is a mesenchymal cytokine which triggers a unique integrated biological program of proliferation, dissociation, migration and invasive growth, or scattering, that is an essential part of organogenesis during embryonic development (Weidner et al. 1990; Medico et al. 1996; Bardelli et al. 1996). HGF induced invasive growth is mediated by the Met receptor. Tpr-Met oncogene, derived from the Met receptor, was originally identified in a human osteosarcoma cell line treated *in vitro* with a chemical carcinogen N-methyl-N-nitro-N-nitrosoguanidine (MNNG) (Cooper et al. 1984; Park et al. 1986). Oncogenic activation of the Met receptor involves a genomic rearrangement that deletes the regulatory transmembrane sequences and replaces them with sequences from the Tpr gene. As a consequence of this rearrangement the Tpr-Met fusion oncoprotein is localized to the cytoplasm, and a leucine zipper motif within the Tpr sequences mediates dimerization and constitutive activation (Rodrigues and Park 1993). A rearranged Tpr-Met oncogene was also recently identified in a MNNG transformed human xeroderma pigmentosum cell line (Wicker et al. 1995). A preliminary survey of cell lines derived from a variety of human tumors have, in addition, detected expression of a rearranged Tpr-Met gene at low levels in 50 % of the sampled cell lines (Soman et

al. 1990). This indicates that rearrangements generating Tpr-Met are likely to represent a mechanism of oncogenic transformation in a significant portion of human tumors.

In the study presented in Chapter 2, an *in vitro* model of carcinogenesis was developed by stable transfection of a Tpr-Met oncogene in Fr3T3 cells. A major component of increased PTHRP gene expression in response to serum and growth factors involves prolongation of mRNA half-life (Heath et al. 1995; Falzon 1996). Subsequently, we found that although Tpr-Met increases PTHRP gene expression by four-fold, we were unable to detect an increase in mRNA half-life. The molecular mechanisms that control gene transcription and those involved in mRNA stabilization, are distinct and regulated independently (Ross 1995; Karin 1995). On the one hand, mRNA stability is regulated by growth factors through complex interactions that are not clearly understood. However, many factors that bind and protect mRNAs from degradation have been identified including poly (A)-binding proteins (Bernstein et al. 1989), AU-rich region binding proteins (Stephens et al. 1992), ribonucleotide (Ohh and Takei 1994), and *trans*-acting regulatory factors that do not directly bind to RNA, such as β-tubulin, histones and heat shock proteins (Ross 1995). On the other hand, the transcriptional mechanism of gene regulation involves phosphorylation and activation of multiple transcription factors, including AP-1, CREB, TCF, NFκB, ATF-2 and many more (Karin 1995), which regulate the expression of genes by interacting with specific binding elements within the promoter region. It is possible that differences may exist in the control of these mechanisms by Tpr-Met as compared to EGF and other growth factors found in serum that might explain the lack of PTHRP mRNA stabilization by Tpr-Met. The differences that exist between exogenous addition of growth factors as

compared to stable transfection of an oncogene may also explain the observed disparity between the two mechanisms of induced PTHRP gene expression.

Deregulation of PTHRP production by Tpr-Met may have important implications for the role of receptor tyrosine kinases in mediating the aberrant production of this protein in malignancy. Many of the tumors that contain oncogenic tyrosine kinase receptors are indeed derived from the same tissues as tumors that frequently overexpress PTHRP. Although Tpr-Met was originally identified in a human osteosarcoma cell line treated *in vitro* with a chemical carcinogen, a number of reports have indicated a role for oncogenic Met/HGF receptor in an increasing number of human tumors . The Met receptor has been documented to be overexpressed in thyroid carcinomas (Di Renzo et al. 1994; Di Renzo et al. 1992), in ovarian and colorectal carcinomas (Ponzetto et al. 1991; Di Renzo et al. 1995), and in papillary renal carcinoma (Schmidt et al. 1997), and in subset of human leukemia and lymphoma (Jucker et al. 1994). Other receptor tyrosine kinases such as the EGF receptor family, and PDGF, FGF and *ret* receptors have also been associated with human cancers. EGF receptors are frequently overexpressed in squamous cell carcinomas and glioblastomas (Libermann et al. 1985; Yamamoto et al. 1986). Other members of the EGF receptor family, such as HER-2/neu, ErbB-2 and ErbB–3 are found often in adenocarcinomas of the breast, stomach and ovary (Slamon et al. 1989; Kraus et al. 1987; Kraus et al. 1989). Expression of the PDGF receptor has been documented in a high fraction of sarcomas and glial derived neoplasms (Heldin and Westermark 1989; Maxwell et al. 1990), oncogenes of the FGF receptor family have been isolated from stomach carcinoma cell lines (Soman et al. 1991; Ponzetto et al. 1991; Hattori et al. 1990), and the receptor oncogene *ret* is activated by gene

rearrangements in a large fraction of human thyroid carcinomas (Grieco et al. 1990; Fusco et al. 1987). Many cancers of these tissues are also frequently associated with aberrant PTHRP expression. Elevated levels of PTHRP have been reported in patients with squamous cell carcinoma (Danks et al. 1989, 1995; Dunn et al. 1993), and renal cell carcinoma (Vargas et al. 1992), breast cancers (Powell et al. 1991), colon (Sidler et al. 1996), endometrial cancers (Sachmechi et al. 1995), thyroid cancers (Nakashima et al. 1995), leukemia and lymphomas (Burtis et al. 1990; Ikeda et al. 1994). Although other mechanisms most likely exist, oncogenic receptor tyrosine kinases may contribute significantly to the events leading to abnormal expression of PTHRP in malignancy.

The downstream targets of Tpr-Met oncogene include PI 3-kinase and Ras. In Chapter 2, we demonstrated that induction of PTHRP expression by Tpr-Met was dependent on the Ras pathway, and independent of PI 3-kinase. A role for growth factor activated PI 3-kinase has been delineated in a number of cellular responses, including regulation of protein turnover through 70K S6 kinase activation (Dardevet et al. 1996), and PDGF induced actin reorganization and chemotaxis (Arieumerlou et al. 1998; Hooshmand-Rad et al. 1997). A number of these studies have also suggested that the lipid kinase may be acting up-stream of Ras-like Rho family of proteins (Rodriguez-Viciana et al. 1994; Arieumerlou et al. 1998; Hooshmand-Rad et al. 1997). Our findings indicate, however, that these cellular events mediated by PI 3-kinase do not lead to transcriptional activation of the PTHRP gene.

As discussed above, PTHRP expression is significantly induced following the expression of Tpr-Met oncogene in Fr3T3 cells. In Chapter 3, we report that expressing an oncogenic Ras in the same cells produces an even greater fold increase in PTHRP

production. Endogenous Ras protein is normally tightly regulated with many of its target proteins, such as rasGAPs and NF1, providing negative feedback (Bollag and McCormick 1991). Thus, Ras represents a major rate limiting step for signal transduction generated by oncogenic receptors such as Tpr-Met. This phenomenon might explain why expression of an oncogenic Ras can induce the expression of PTHRP more effectively than the expression of Tpr-Met.

5.3 INHIBITION OF RAS AS POTENTIAL THERAPEUTIC STRATEGY FOR HYPERCALCEMIA OF MALIGNANCY

Ras is the most frequently detected oncogene in human cancer (Aaronson 1992). The importance of Ras in cancer has inspired many attempts to intervene with its function, and these have led to a number of successful approaches.

One group of Ras inhibitors that were identified by high throughput random screening were blockers of hydroxymethylglutaryl (HMG) CoA reductase and mevalonate kinase, enzymes involved in the production of isoprenoid in the cholesterol biosynthetic pathway. These include the drugs compactin, and the 'statins' (including lovastatin, mevastatin and simvastatin) (Cox and Der 1997). By blocking isoprenoid synthesis, these compounds prevent the production of precursors of farnesyl and geranylgeranyl groups (Cox and Der 1997). However, since many cellular proteins are prenylated including nuclear lamins, G-protein α and γ subunits, and other small G-proteins in addition to Ras, it is clear that this mechanism of Ras inhibition lacks specificity (Zhang and Casey 1996).

Another approach to interfere with Ras function is at the level of the enzyme farnesyl protein transferase , which catalyzes the bond between farnesyl pyrophosphate (FPP) and the CAAX motif of Ras (Zhang and Casey 1996). These two substrates have been used to develop rationally designed inhibitors of Ras farnesylation (Kohl et al. 1993; James et al. 1993; Garcia et al. 1993). The most successful of these are a variety of CAAX based peptidomimetic compounds, which competitively inhibit farnesylation by being substrate to the farnesyl transferase enzyme (Garcia et al. 1993; Sun et al. 1995). It has been suggested that the accumulation of unprocessed Ras in the cytoplasm

leading to the formation of inactive Ras-target protein complexes may also contribute to inhibition of the Ras pathway (Lerner et al. 1995). The short mimetic peptide proved to be highly specific for farnesyl transferases (FPTases) without affecting the activity of a related protein geranylgeranyltransferase (GGTase) involved in modifying other membrane anchored small G-proteins and the Gγ subunit (Cox et al. 1994). FPTase inhibitors were shown to inhibit Ras mediated transformation *in vitro* in NIH3T3 fibroblast cells, and *in vivo* tumor models using Ras transformed cell lines and human xenografted tumors in nude mice (Garcia et al. 1993; Sun et al. 1995). In addition, inhibitors of FPTases were reported to have a comparatively greater effect on the growth of transformed cells as compared to normal cells, and were shown to cause tumor reduction without apparent toxicity in nude mice (Cox and Der 1997).

The study presented in Chapter 3 showed that inoculating cells transfected with an oncogenic Ras into nude mice resulted in the rapid development of biochemical signs characteristic of hypercalcemia, occurring within two weeks. Ras farnesylation inhibitor, B-1086, inhibited tumor growth and suppressed the onset of hypercalcemia. These findings demonstrate that Ras oncogene can directly lead to overproduction PTHRP, and suggest that Ras inhibitors may provide a potential strategy for treatment of HHM. Studies by other investigators have also shown that Ras can increase PTHRP production in keratinocytes (Yu et al. 1995), in human prostate cells (Kremer et al. 1997), and in human lung cancer xenografts (Oshika et al. 1998).

Oncogenic mutations in Ras are present in almost all pancreatic tumors, 40-50 % of colorectal carcinomas and 30% of lung adenocarcinomas (Almoguera et al. 1988; Bos et al. 1987; Rodenhuis and Slebos 1990), yet are seldom found in breast and renal

carcinomas (Dawkins et al. 1993; Rochlitz et al. 1992). In spite of our observations and those of other investigators that Ras oncogene leads to increased production of PTHRP (Chapter 3; Li and Drucker 1994a; Yu et al. 1995; Kremer et al. 1997; Oshika et al. 1998), it appears that cancers in which Ras mutations frequently occur such as pancreatic adenocarcinoma are less likely to overexpress PTHRP and cause HHM. However, in the absence of Ras mutations, activation of Ras can result from events upstream of Ras, such as growth factors, their receptors or cellular tyrosine kinases. Tumors that do not contain Ras mutations have been shown to be dependent on Ras activity including tumors that contain activated EGF receptors (Pazin and Williams 1992), rasGAP (Basu et al. 1992) and guanine nucleotide exchange factors (Egan and Weinberg 1993). In cases such as pancreatic tumors where Ras oncogene is present but does not lead to increased PTHRP gene expression, alternative mechanisms may interplay to regulate gene expression. DNA methylation was previously shown to correlate with levels of PTHRP gene transcription in renal carcinoma cells (Holt et al. 1993). The importance of this control mechanism was confirmed by the ability of the demethylating agent 5-azacytidine to induce PTHRP mRNA in previously non-expressing cells (Holt et al. 1993). It is possible that in tumors where activation of Ras does not lead to increased PTHRP expression, the gene may have been 'silenced' by epigenetic changes involving methylation.

Since the majority of HHM is caused by elevated levels of circulating PTHRP (Stewart et al. 1980; Burtis et al. 1991), the need for specifically targeting PTHRP secretion by tumors is evident. Before 1990, the treatment for HHM was nonspecific and involved the use of diuretics to increase urinary calcium excretion or toxic bone

inhibiting agents such as mithramycin or gallium nitrate to inhibit bone destruction (Perlia et al. 1970; Suki et al. 1970; Warrell et al. 1988; Thalassinos et al. 1970). These approaches had limited efficacy and often caused more harm by accelerating calcium loss and leading to further skeletal dysfunction (Mundy et al 1983). The recent advent of bisphosphonate therapy represents a major progress in the treatment of HM (Nussbaum et al. 1993). Among the bisphonates extensively used for treatment of HM, Pamidronate is reported to be highly efficacious and non-toxic within the therapeutic range (Nussbaum et al. 1993). Although bisphosphonates are very successful in HM associated with bone metastasis, the success rate of these agents is negatively correlated with increasing levels of plasma PTHRP (Nussbaum et al. 1993), presumably due to increased renal tubular reabsorption of calcium by PTHRP. Perhaps the apparent success of bisphosphonate therapy combined with other strategies, such as Ras inhibitors, which can directly disrupt tumor production of PTHRP, may provide a useful approach for treatment of HHM in the future.

5.4 MITOGEN ACTIVATED PROTEIN KINASE PATHWAYS LINK GROWTH FACTOR SIGNALS TO THE PTHRP GENE

In Chapter 4, we report that induction of PTHRP gene expression requires activation of ERK and JNK signaling cascades. This is the first demonstration of a role for ERK and JNK dependent signaling cascades in the regulation of PTHRP and has enormous implications for the regulation of this gene. ERK and JNK are parallel serine/threonine MAP kinases that mediate the signal from activated Ras to the nucleus and are essential for mediating a multitude of cellular responses elicited by growth factors (Fig 1.2). These pathways have also been shown to be required for the expression of numerous other growth factor-induced genes, including c-Fos, c-Jun, pip92, egr-1 and prostaglandin synthase-2 (Chung et al. 1998; Kato et al. 1997; Dieckegraefe et al. 1997; Yao et al. 1995; Jhun et al. 1995; Xie and Herschman 1996).

MAP kinase activation can be triggered by a diverse set of extracellular stimuli in addition to serum, including cytokines, G-protein coupled receptors and cellular stress (Sadoshima et al. 1995; Wang et al. 1992, 1993; Crespo et al. 1994; Rao et al. 1995; Coso et al. 1995b ; Derijard et al. 1994a). Since agonists of G-protein coupled receptors, cytokines and stress factors can also stimulate the production of PTHRP (Pirola et al. 1994; Pirola et al. 1993; Winquist et al. 1987; Massfelder et al. 1996; Ikeda et al. 1993; Mok et al. 1989; Chapter 4), it is possible that these diverse stimuli exert their effect on the PTHRP gene through the activation of MAP kinases.

Additional physiological stimuli exist that induce the activation of MAP kinases and also to lead to increased PTHRP production. A recent study showed that mechanical stretch in vascular smooth muscle cells activates the JNK pathway (Hamada et al.

1998). Mechanical stretch has been known for some time to increase PTHRP production in a variety of smooth muscle tissues including the stomach, uterus and vasculature (Ito et al. 1994; Diafotis et al. 1992; Pirola et al. 1994). It has been proposed that PTHRP acts on these tissues as a muscle relaxant (Shew et al. 1991; Barri et al. 1992; Yamamoto et al. 1992; Pirola et al. 1994; Mok et al. 1989a, 1989b). Consequently, it is possible that mechanical stretch induces PTHRP gene expression in smooth muscle through the activation of the JNK pathway.

Another instance where activation of MAP kinases coincides with induction of PTHRP production is in HTLV-1 associated adult T-cell leukemia. HTLV-1 associated adult T-cell leukemia is almost invariably associated with PTHRP overproduction and HHM (Watanabe et al. 1990). Studies by Dittmer et al. (1992, 1994) have shown the role of a viral Tax protein in transcriptional activation of the PTHRP gene in HTLV-1 associated leukemia. Recently, using the same model of human leukemia, Xu and colleagues demonstrated that the JNK pathway is constitutively activated in the HTLV-1 transformed cells (Xu et al. 1996). Taken together, it is possible that the JNK pathway, in addition to Tax proteins, may play a role in the mechanism that leads to increased PTHRP production in HTLV-1 associated leukemia.

MAP kinases may also play a role in mediating the autocrine secretion of PTHRP in malignancy. A fundamental concept in the growth-independence of cancer cells is the autocrine secretion of mitogenic factors (Cuttita et al. 1985). A multitude of growth promoting factors are secreted by cancerous cells, which by binding cell surface receptors, can act on their producer cells to promote accelerated tumor growth and malignant progression. This phenomenon has been documented for many growth factors

including TGFα (Todaro et al. 1986), PDGF (Johnsson et al. 1985); FGF (Gospodarowicz et al. 1986;) and TGFβ (McMahon et al. 1986). Increasing evidence suggests that PTHRP may likewise act as a cytokine on secreting cells, to promote cell proliferation and tumor progression (Liu et al. 1995; Alipov et al. 1997; Nagataki et al. 1995). PTHRP immunoreactivity has been detected in a large proportion of invasive and metastatic carcinoma including gastric adenomas (Alipov et al. 1997; Ito et al. 1997), and pituitary and thyroid tumors (Nagataki et al. 1995), which also report that PTHRP expression is higher in metastatic cells than in the primary tumors, possibly implicating accelerated PTHRP production with tumor progression. Furthermore, many PTHRP secreting tumors have been shown to co-express the membrane bound receptor (Iezzoni et al. 1998; Carron et al. 1997). ERK and JNK pathways are almost ubiquitously activated in proliferating cells and are required for cell growth and oncogenic transformation in cancer (Khosravi-Far et al. 1998; Denhardt 1996). Our observation that these pathways regulate PTHRP gene expression, opens up the possibility that ERK and JNK pathways may be an integral part of the mechanism leading to autocrine production of PTHRP in malignancy.

Extending the notion of autocrine secretion, PTHRP has in turn been shown to regulate its own expression (Walsh et al. 1997), as well as the expression of other growth-related genes including fos B, jun B, fra 1 and IGF-1 (Koe et al. 1997; Kameda et al. 1997; Hollnagel et al. 1996; Onyia et al. 1995). Furthermore, we have recently observed that PTHRP (1-34), acting through the PTH/PTHRP receptor, can activate both ERK and JNK kinases in the Leydig tumor H-500 cells (Appendix). PTHRP has been shown to promote the growth and malignant progression of H-500 tumors (Liu et al.

1995), and it is possible that this effect of PTHRP might require the activation of MAP kinases. Other investigators have also shown that activation of the PTH/PTHRP receptor can modulate the activity MAP kinases. Verheijin and Defize (1995) recently showed that stimulation of PTH/PTHRP receptor by PTH can either inhibit growth factor induced ERK activity, or PTH can activate the ERK pathway in different cell lines (Verheijen and Defize 1997). The effect of the activated PTH/PTHRP receptor on the growth of these cells was, however, not reported.

It appears that PTHRP, acting through the PTH/PTHRP receptor, belongs to a growing number of G-protein coupled receptors that can interact with MAP kinase pathways. The finding that PTHRP can activate these pathways might explain how PTHRP production in malignancy can lead to the growth and progression of tumors. By activating MAP kinases PTHRP can further modify its own expression, as well as the expression of other genes that are likewise under the control of MAP kinase pathways. Clearly our findings have important implications for the overexpression of PTHRP in disease and may provide the molecular basis for the autocrine action of this protein in cancer.

In summary, our studies clearly demonstrate that PTHRP is regulated by a tyrosine kinase receptor via the Ras pathway and that Ras itself potently induces PTHRP production. We next showed that Ras inhibitors could be used to specifically target the expression of PTHRP *in vitro*, and the development of hypercalcemia *in vivo*. Finally, our results establish that PTHRP expression is mediated by signal transduction involving the ERK and JNK cascades. PTHRP action in HHM has both a skeletal and renal component. Consequently, an approach to treatment that addresses both of these issues is

needed. PTHRP has also been implicated in promoting the tumor growth and progression of malignancy. It is evident that the benefits of an approach which can specifically target tumor production of PTHRP are multifold. The findings presented in this thesis are provocative and provide novel targets that can be used to interfere with PTHRP overproduction in malignancy, opening up exciting therapeutic possibilities for a better management of HHM.

5.5 SUGGESTIONS FOR FUTURE RESEARCH

The following are possible avenues that can be explored as a continuation of the work in this thesis.

1. Little is known of the transcriptional mechanisms by which regulatory factors affect their action on PTHRP gene expression. Hence, elucidation of transcription factors that bind to elements in the promoter regions and enhance the expression of PTHRP gene is required.

2. A comparative analysis of the efficacy of Ras inhibitors on suppressing the production of PTHRP by various hypercalcemic human tumor cell lines would be valuable.

3. Consequently, the development and evaluation of a combination therapy of bisphosphonates and Ras inhibitors in the treatment of HHM should be explored.

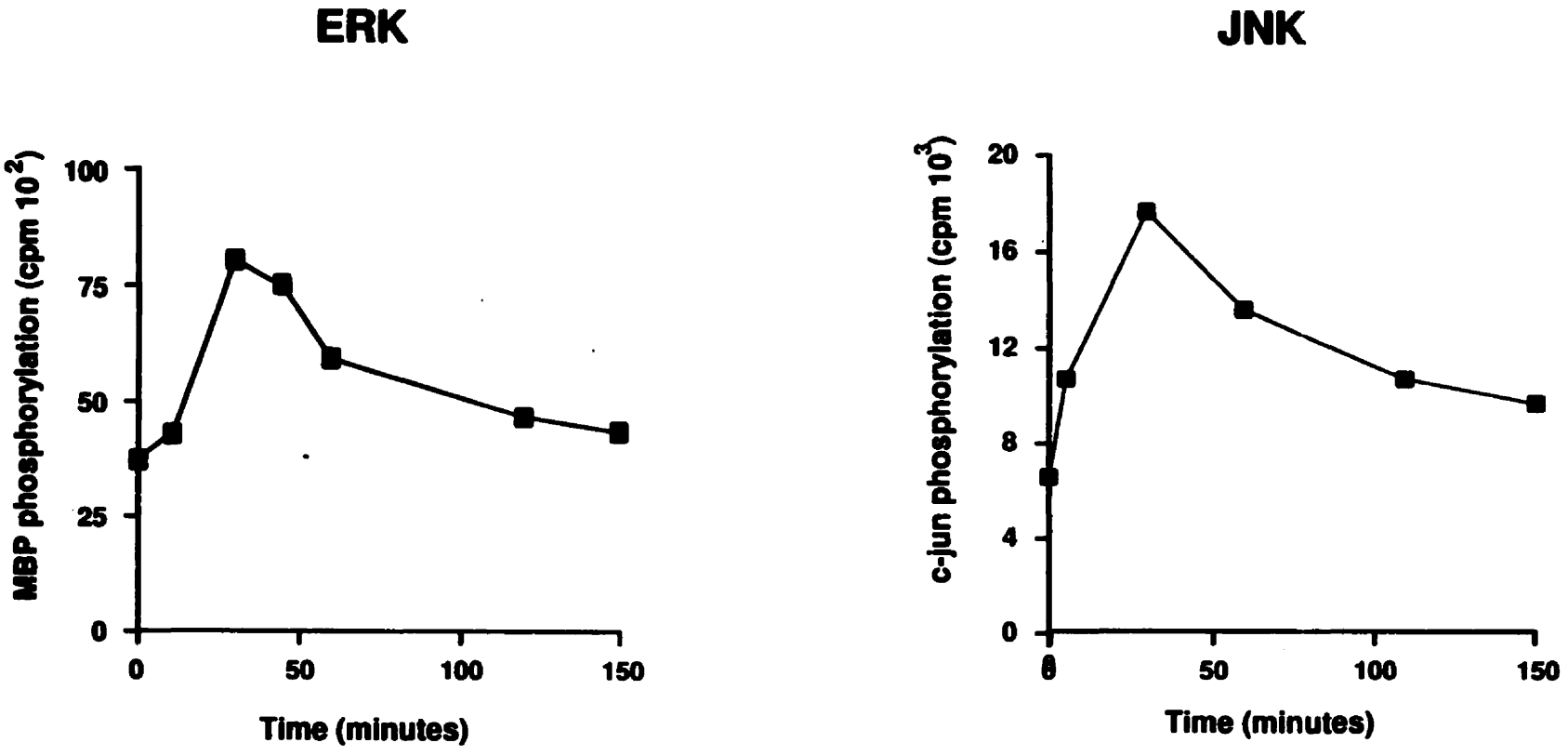
4. PTHRP has a broad range of biological and pathological actions mediated via the PTH/PTHRP receptor. Delineating the downstream signal transduction pathways that are generated by PTHRP binding to its receptor will enhance our understanding of the mechanism of these diverse effects, and will also provide additional targets to intervene with its action.

CHAPTER 6

APPENDIX

Figure 6.1: The effect of PTHRP (1-34) on ERK and JNK activation in H-500 cells.

H-500 Leydig tumor cells were stimulated with 100 nM PTHRP (1-34) and ERK and JNK were immunoprecipitated at the various time points and their kinase activity measured by their ability to phosphorylate their respective substrates in an *in vitro* kinase assay. As shown in Fig. 6.1, both ERK and JNK kinases were activated by PTHRP (1-34) with both of their maximal response occurring at 30 minutes, and returning to basal levels after 2 hours.



Appendix: PTHRP (1-34) stimulates ERK and JNK activity

CHAPTER 7

CLAIMS OF ORIGINAL CONTRIBUTIONS TO SCIENCE

The original contributions of the studies described in Chapters 2-4 are as follows.

1. Development of an *in vitro* model in which the mechanism of PTHRP overexpression could be assessed.

2. The demonstration that an oncogenic receptor tyrosine kinase, Tpr-Met, induces PTHRP expression and that this induction is mediated through the Ras pathway.

3. The direct demonstration that Ras oncogene leads to overexpression of PTHRP *in vitro* and causes hypercalcemia when inoculated into animals.

4. Demonstration that inhibiting Ras using a specific peptidomimetic inhibitor, B-1086, can block production of PTHRP *in vitro*, and suppresses the onset of hypercalcemia *in vivo*.

5. The first demonstration that increased expression of PTHRP is dependent on Ras and activation of ERK and JNK signaling pathways in a hypercalcemic tumor cell line.

CHAPTER 8

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