EXAMINATION OF THE DISTRIBUTION AND BIOLOGICAL ACTION OF A PARATHYROID HORMONE-LIKE PEPTIDE (PLP) ASSOCIATED WITH THE HYPERCALCEMIA OF MALIGNANCY

by

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ABSTRACT

The role played by a parathyroid hormone-like peptide (PLP) in the pathogenesis of hypercalcemia associated with malignancy has been examined. Elevated circulating levels of PLP were found more frequently in hypercalcemic cancer patients. A reduction of tumor burden in two of these patients resulted in concomitant decreases in both plasma calcium and plasma PLP. Immunoneutralization of endogenous PLP in a rat model of malignancy associated hypercalcemia resulted in a rapid and sustained reversal of the biochemical abnormalities manifest in the disease state. These studies, therefore, provided strong evidence for a role for PLP in the pathogenesis of hypercalcemia associated with neoplasia. The frequent association of squamous cell carcinoma with hypercalcemia and elevated circulating PLP levels prompted examination of a keratinocyte model of tumor progression for evidence of dysregulated PLP expression. Increased constitutive production of PLP, accompanied by resistance to previously identified regulatory agents, was demonstrated in the progression to the malignant phenotype. Finally, a potential autocrine or paracrine role for PLP in keratinocyte cell growth was suggested by the demonstration of functional adenylate cyclase-linked PLP receptors on an established keratinocyte cell line. These studies have therefore provided important insights into the role played by PLP as both an endocrine factor involved in the pathogenesis of malignancy associated hypercalcemia and as a potential autocrine/paracrine factor in keratinocyte homeostasis.

RESUME

Le rôle joué par le "parathyroid hormone-like peptide" (PLP) dans la pathogénèse de l'hypercalcémie lieé au cancer a été examine. Des concentrations élevées de PLP sont présentes plus fréquemment chez les patients hypercalcémiques atteints de cancer. La réduction de la masse tumorale chez deux de ces patients a entrainé une diminution parallèle de la calcémie et du PLP circulant. La neutralisation du PLP endogène par immunisation passive dans un modèle d'hypercalcémie associee au cancer chez le rat a amené une normalisation rapide des anomalies biochimiques. Ces études supportent fortement un rôle endocrinien du PLP dans la pathogénèse de l'hypercalcémie associée au cancer. L'association fréquente des cancers épithéliaux avec l'hypercalcémie et l'augmentation des taux circulants de PLP nous a amenés à étudier la régulation de l'expression du PLP dans un modèle de progression tumorale impliquant le keratinocyte. Une augmentation intrinsèque de la production de PLP accompagnée d'une résistance aux agents régulateurs dejà identifiés a été démontrée en cours de progression vers un phénotype malin. Enfin, la démonstration de la présence de récepteurs fonctionnels pour le PLP sur une lignée établie de kératinocyte suggère un rôle potentiel autocrine du PLP dans la régulation de la croissance de ces cellules. Ces études ont donc contribué à renforcer le rôle endocrinien du PLP dans la pathogénèse de l'hypercalcémie liée au cancer et à suggérer un rôle autocrine/paracrine potentiel dans l'homéostase du kératinocyte.

PREFACE

The Guidelines Concerning Thesis Preparation issued by the Faculty of Graduate Studies and Research at McGill university reads as follows:

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> While the inclusion of manuscripts co-authored by the candidate and others is acceptable, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent and supervisors must attest to the accuracy of the claims at the PhD. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear."

I have chosen to write my thesis according to the above quoted option with two papers published and two papers In Press. The thesis is organised in seven chapters. Chapter I is a general introduction and literature review. Chapters II-V contain the four manuscripts, each with its own abstract, introduction, methods, results, discussion and references. Chapter VI is a general discussion of all four manuscripts and Chapter VII contains the claims to original research.

PUBLICATIONS ARISING FROM THE WORK OF THIS THESIS AND CONTRIBUTIONS MADE BY CO-AUTHORS

1. **Henderson J.E, Shustik C, Kremer R, Rabbani S.A, Hendy G,N and Goltzman D. 1990 Circulating concentrations of parathyroid hormone-like peptide in malignancy and in hyperparathyroidism. J Bone Min Res 5 105-113.**

The candidate was responsible for all aspects of the development of the PLP radioimmunoassay and for all analyses except for those using automated techniques which were performed by the clinical biochemistry laboratory at the Royal Victoria Hospital Gel filtration analyses were performed by the candidate with guidance from **Drs. G.N Hendy and S.A.Rabbani** and plasma samples were kindly supplied by **Drs. C.Shustik, R.Kremer**, R.Benoit and L.Panasci. The candidate received help from **Dr. D.Goltzman** with the interpretation of medical data and with preparation and revision of the manuscript for publication.

2. **Henderson J.E, Bernier S, D'Amour P and Goltzman D. 1990 Effects of passive immunization against parathyroid hormone (PTH)-like peptide and PTH in hypercalcemic tumor-bearing rats and normocalcemic controls. Endocrinology 127 1310-1318.**

PLP antiserum was raised and characterized by the candidate whereas the PTH antiserum was a kind gift from **Dr. P.D'Amour of the Centre de Recherche Clinique Andre-Viallet, Montreal, Canada.** UMR 106 cells were cultured and plated by **Ms S.M. Bernier** and the Leydig cell tumor used for implantation was a kind gift of Dr. S.A Rabbani. The candidate performed all experiments and analyses except for those requiring automated techniques which were performed by clinical

biochemistry The manuscript was prepared and revised for publication by the candidate in consultation with Dr. D.Goltzman.

3. **Henderson J,E, Sebag M, Rhim J, Goltzman D and Kremer R. 1991 Dysregulation of parathyroid hormone-like peptide expression and secretion in a keratinocyte model of tumor progression. Cancer Research In Press.**

The candidate was responsible for all aspects of cell culture and performed all experiments except those involved in the analysis of mRNA which were performed by **M.Sebag**. Both cell lines were developed and kindly donated by **Dr. J.Rhim** at the **National Institute of Health, Bethesda, Maryland** in collaboration with **Dr. R.Kremer**. The manuscript was prepared and revised for publication in consultation with **Drs. R.Kremer and D.Goltzman**.

4. **Henderson J.E, Kremer R, Rhim J,S and Goltzman D. 1991 Identification and functional characterization of adenylate cyclase-linked receptors for parathyroid hormone-like peptides on immortalized human keratinocytes. Endocrinol In Press**

The candidate was responsible for all aspects of cell culture and performed all binding, adenylate cyclase and cell growth assays. EGF tracer for binding assays was kindly prepared by Ms. S.M.Bernier. Cell lines were developed and kindly donated by **Dr. J.Rhim** in collaboration with **Dr. R.Kremer**. The manuscript was prepared and revised for publication by the candidate in consultation with **Dr. D.Goltzman**.

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ABBREVIATIONS

AC	adenylate cyclase
ACTH	adrenocorticotrophic hormone
AD12-SV40	adenovirus 12-simian virus 40 hybrid virus
AP	alkaline phosphate
ATLL	acute T-cell leukemia lymphoma
ATP	adenosine triphosphate
bp	base pairs
Ca	total plasma calcium
Ca^{++}	ionised calcium
iCa^{++}	intracellular calcium
Bu_2cAMP	dibutyryl cyclic adenosine monophosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBA	cytochemical bioassay
cDNA	complimentary deoxyribonucleic acid
CEA	carcinoembryonic antigen
CM	conditioned medium
Creat	creatinine
CT	human calcitonin
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
E_2	17ß-estradiol
EC_{50}	effective concentration for half maximal
EGF	epidermal growth factor

ELISA	enzyme linked immunosorbent assay
EM	electron microscope
FBS	fetal bovine serum
G_i	inhibitory guanyl nucleotide binding protein
G_s	stimulatory guanyl nucleotide binding protein
GF	growth factor
GGT	gamma glutamyl transpeptidase
G6PD	glucose-6-phosphate dehydrogenase
GTC	guanidine thiocyanate
HBSS	Hanks balanced salt solution
HHM	humoral hypercalcemia of malignancy
HPK1A	HPV-16 transfected keratinocytes
HPK1A-ras	HPV-16 and H-ras transformed keratinocytes
HPV-16	human papilloma virus type 16
H-ras	Harvey-ras oncogene
HTLV-1	human T-cell lymphotrophic virus type-1
HPT	hyperparathyroidism
IL-1	interleukin-1
IL-6	interleukin-6
IRMA	immunoradiometric assay
Kb	kilobase
KBM	keratinocyte basal medium
Kd	kilodalton
KGM	keratinocyte growth medium
MEM	minimal essential medium
Mg	magnesium

mRNA	messenger ribonucleic acid
NH_2	amino
NHK	normal human keratinocytes
NPX	nephrectomized
NRS	normal rabbit serum
OBBP	outdated blood bank plasma
OAF	osteoclast activating factor
$1,25(OH)_2D_3$	1,25 dihydroxyvitamin D_3
OK	opposum kidney cell line
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PG	prostaglandin
PGE2	prostaglandin type E2
bioPLP	bioactive parathyroid hormone-like peptide
hPLP	human parathyroid hormone-like peptide
iPLP	immunoreactive parathyroid hormone-like peptide
rPLP	rat parathyroid hormone-like peptide
PMA	phorbol myristate acetate
PO_4	phosphate
bPTH	bovine parathyroid hormone
hPTH	human parathyroid hormone
rPTH	rat parathyroid hormone
(r)	recombinant
RHEK	AD12-SV40 transformed keratinocytes
RHEK-ras	AD12-SV40 and H-ras transformed keratinocytes
RHEK-fos	AD12-SV40 and fos transformed keratinocytes

RIA	radioimmunoassay
ROS17/2.8	rat osteosarcoma cell line
RP-HPLC	reverse phase high pressure liquid chromatography
SAOS-2	human osteosarcoma cell line
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TGF	transforming growth factor
TNF	tumor necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TPTX	thyroparathyroidectomy
UMR 106	rat osteosarcoma cell line

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CHAPTER I GENERAL INTRODUCTION

Preface

The hypothesis that a malignant tumor could secrete a systemically active factor resulting in hypercalcemia was formulated by Fuller Albright in 1941 (1). A patient presenting with hypercalcemia and hypophosphatemia, the biochemical hallmarks of hyperparathyroidism (HPT) at that time, was shown to have no parathyroid dysfunction. However, blood calcium and phosphorous levels normalized temporarily following irradiation of a renal carcinoma metastatic to bone. When gradually the calcium rose and phosphate fell as the tumor resumed it's growth Dr. Albright reasoned that since hyperphosphatemia had not accompanied hypercalcemia, despite evidence of osteolysis, perhaps a phosphaturic agent might be produced by the tumor. Albright therefore had it assayed for parathyroid hormone (PTH). Although no hormone was detected, an important and insightful observation had been made: namely that the hypercalcemia associated with malignancy could have a humoral basis rather than resulting from local osteolysis by tumor cells and that the humoral factor might be PTH-like. This observation was subsequently corroborated by others and the terms pseudohyperparathyroidism, later also known as the humoral hypercalcemia of malignancy (HHM), were coined to describe the syndrome. In 1987 a parathyroid hormone-like peptide (PLP) was finally isolated from tumors associated with HHM and shown to share significant amino-terminal sequence homology with PTH accounting for its affinity for the PTH receptor.

2.Calcium Homeostasis: overview. Under normal circumstances calcium homeostasis is maintained by the direct action of PTH on it's target tissues of bone and kidney and by its indirect actions in the gut (2) (Fig 1). The parathyroid gland responds to an acute decrease in ambient calcium with an increased secretion of bioactive hormone. In bone PTH acts to increase the efflux of calcium from an exchangeable pool presumably via the osteocyte/lining cell complex and increases the turnover of the non-exchangeable pool by stimulating osteoclastic resorption (3). In the kidney sodium, phosphate and bicarbonate reabsorption are inhibited by PTH in the proximal tubule while that of calcium is stimulated in the distal tubule via cyclic adenosine monophosphate (cAMP) mediated mechanisms. Activity of the 25 hydroxyvitamin D, 1 alpha hydroxylase enzyme in the proximal tubule is stimulated directly by PTH and also by the PTH-induced decrease in phosphate concentration resulting in increased 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) production. This active metabolite of vitamin D then increases calcium absorption in the gut and works synergistically with PTH in bone to activate existing cells and to stimulate the replication of osteolytic and osteotropic precursor cells. The rise in blood calcium resulting from these increases in gut absorption, renal reabsorption and calcium release from bone, as well as the increased circulating $1,25(OH)_2D_3$ level, eliminate the signal for further increases in synthesis and secretion of PTH.

3.Hyperparathyroidism vs hypercalcemia of malignancy. Excessive amounts of circulating PTH, in cases of parathyroid adenoma or hyperplasia for instance, result in hypercalcemia, hypophosphatemia, renal phosphate

Calcium Homeostasis

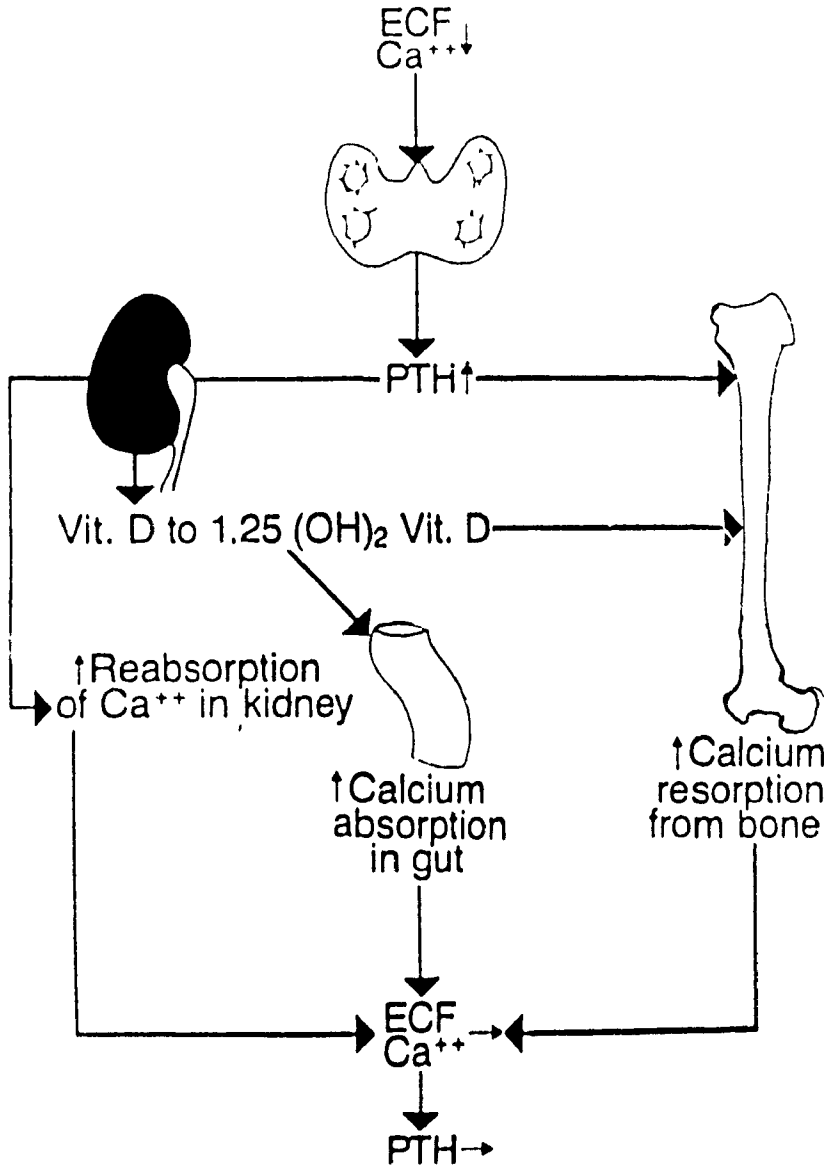

Figure 1 Calcium Homeostasis: A decrease in extracellular fluid calcium (ECF Ca^{++}) results in an increase in parathyroid hormone (PTH) secretion. PTH then acts in its target tissues of kidney, gut and bone to increase calcium reabsorption, absorption and resorption respectively. The subsequent rise in ECF Ca^{++} eliminates the stimulus for increased PTH production and the status quo is resumed

wasting associated with increased nephrogenous cAMP, elevated circulating $1,25(OH)_2D_3$ and an increase in bone turnover. Although many of these characteristics are shared with the hypercalcemia of malignancy syndrome there are a number of important differences (4,5). Whereas PTH and $1,25(OH)_2D_3$ levels are increased in HPT they are decreased in HHM. Osteoblastic activity is increased along with osteoclastic activity in HPT whereas osteoblastic activity is decreased in HHM resulting in an uncoupling of bone turnover and net bone loss. In addition, hyperparathyroid disease is associated with metabolic acidosis whereas patients with malignancy associated hypercalcemia often exhibit a mild hypokalemic alkalosis. Taken together these characteristics suggest biologically related yet immunologically distinct mediators of the two syndromes.

4. Putative Mediators of malignancy associated hypercalcemia. As increased bone resorption stimulated by a systemically active agent was considered to be the primary cause of elevated blood calcium levels much attention focused on agents capable of stimulating bone resorption, both IN VIVO and IN VITRO.

4.1 Parathyroid hormone. Because of its striking similarity to hyperparathyroid disease there were many early attempts to identify ectopic PTH as the pathogenetic agent in malignancy associated hypercalcemia (6-9). Although immunoassayable PTH was detected in both tumor tissue and in the peripheral circulation of patients having malignancy and hypercalcemia the levels appeared to be inappropriately low

for the degree of hypercalcemia and for the values of cAMP achieved. Later attempts to identify PTH messenger ribonucleic acid (mRNA) in tumors associated with HHM were unsuccessful suggesting that the immunoassayable material demonstrated earlier was most likely an artifact (10). Only two authentic cases of ectopic PTH production associated with tumoral elaboration of PTH mRNA have been reported (11,12) and remain isolated examples amongst the vast majority demonstrating PTH-like bioactivity without PTH production

4.2 Prostaglandins. Several clinical studies (13,14) have demonstrated modest increases in circulating concentrations of metabolites of prostaglandins (PGs) of the E series in some patients with malignancy associated hypercalcemia yet inhibitors of PG synthesis have been largely ineffective in the treatment of the disease (15). Tumor cells were shown to secrete high levels of PGE into culture medium IN VITRO but the rapid and efficient degradation of eicosanoids IN VIVO make it unlikely that the levels required for effective bone resorption would ever be attained in the circulation Attempts to induce hypercalcemia by infusion of PGE_2 into rats were successful only with pharmacological doses lending support to this observation (16). The necessity to proceed with caution when extrapolating IN VITRO data to explain IN VIVO findings was well demonstrated in the case of the PGE_2 producing VX2 carcinoma (17). Rabbits bearing this tumor become severely hypercalcemic 3-4 weeks following tumor transplantation. Elevated PGE_2 levels were demonstrated in venous effluent from the tumor as well as in the systemic circulation. When tumor cells in culture were shown to produce bone resorbing activity and PGE_2, both

inhibitable with indomethacin, it was suggested that the hypercalcemia in this model resulted from PGE_2 stimulated bone resorption. Following a report that PGE_2 stimulated 25 hydroxyvitamin D, 1 alpha hydroxylase activity in chick kidney cells (18) the VX2 model was re-examined (19). Hypercalcemia in tumor-bearing rabbits was shown to be reversible when the animals were maintained on a calcium-free diet. Taken together with data showing that an inhibitor of bone resorption had no significant effect on plasma calcium these findings suggested a gut-mediated, $1,25(OH)_2D_3$ dependent hypercalcemia.

4.3 Transforming Growth Factors . In the 1980's the focus shifted from prostaglandins to transforming growth factors (TGF) as mediators of osteolysis. Although not found in the circulation under normal circumstances, many solid tumors commonly associated with HHM produce TGFα, a 50 amino acid peptide which mediates its biologic effects through the epidermal growth factor (EGF) receptor (20). Both EGF and TGFα have been shown to stimulate bone resorption in organ culture, the release of previously incorporated ^{45}Ca being used as an index of activity. In neonatal mouse calvaria TGFα enhanced bone resorption indirectly via a PGE_2 mediated mechanism (21). On the other hand, ^{45}Ca release stimulated by TGFα in cultures of fetal rat long bones was indomethacin insensitive suggesting multiple mechanisms by which this peptide influenced bone resorption. Additional data supporting a role for TGFα in osteolysis came from experiments in which addition of antiserum to the EGF receptor inhibited the resorptive activity of conditioned medium harvested from tumor cells known to cause hypercalcemia and bone resorption IN VIVO (22).

However, co-purification of TGFß-like and PTH-like bioactivity from these same tumor cells suggested a multifactorial mechanism underlying the hypercalcemia associated with this tumor (23). Unlike TGFα, TGFß is found in large quantities in bone in an inactive, protein-bound complex. When dissociated from its binding protein the activated peptide is capable of inducing osteolysis although its major role appears to be osteotropic rather than osteolytic (24).

4.4 Cytokines. Interleukin-I (IL-1), lymphotoxin and tumor necrosis factor (TNF) were collectively known as osteoclast activating factor (OAF) when first isolated from the conditioned medium of mitogen stimulated peripheral blood leukocytes (25). Subsequent IN VITRO studies demonstrated a complicated interdependence of these factors on one another as well as a dependence on osteoblasts as mediators of their effects on osteoclasts (26). Chinese hamster ovary cells transfected with the human TNF gene generated TNF producing tumors when transplanted in nude mice (27). Peripheral TNF levels were 100 fold higher in mice with tumors containing the TNF gene compared with those with only vector. Blood calcium levels on the other hand were only modestly increased at 2.96mM compared with 2.51mM in controls suggesting a relatively minor contribution to the hypercalcemic syndrome being made by this cytokine in the absence of other factors.

4.5 1,25 dihydroxyvitamin D_3 Some hematologic malignancies such as Hodgkin's disease and acute T cell leukemia-lymphoma (ATLL) have been associated with hypercalcemia and increased circulating levels of 1,25

$(OH)_2D_3$, often in the presence of renal impairment, indicating an extra-renal source of this steroid (28,29). Lymphoma cells in culture can convert 25 hydroxyvitamin D to 1,25 $(OH)_2D_3$ but also release numerous cytokines which could be involved in the pathogenesis of hypercalcemia in these malignancies. Several studies have identified PLP mRNA and PTH-like bioactivity in conditioned medium from human T cell lymphotrophic virus type 1 (HTLV-1) infected cells derived from patients with ATLL implicating yet another hypercalcemic factor in this disease (30-32).

This list of putative mediators of the hypercalcemic syndrome associated with malignancy demonstrates the diversity and interdependence of factors which could be involved either directly or indirectly in the pathogenesis of the disease state.

5.Rodent models of malignancy associated hypercalcemia. PTH-like bioactivity in the absence of skeletal metastases has been documented in a number of cases of mouse and rat tumors over the years.

5.1 Carcinogen induced squamous carcinoma. Mice bearing a cutaneous squamous carcinoma, induced by painting their skin with the carcinogen dimethylbenzanthracene, exhibited many features of the HHM syndrome (33). However a 6 month lag time in tumor induction made this an impractical model for studying aspects of malignancy associated hypercalcemia.

5.2 Walker rat. The Walker 256 breast carcinoma which arises spontaneously in rats is of interest in that there are both hypercalcemic and normocalcemic variants (34). This model however remains largely

uncharacterized.

5.3 Athymic mouse bearing human tumor xenografts. Another approach used by numerous investigators has been the passage of human tumor xenografts in athymic mice (27,35,36). Extensive characterization of one of these models in a series of papers strongly suggested a multifactorial basis to the hypercalcemic syndrome (37-41). Tumor-bearing mice developed severe hypercalcemia and hypophosphatemia associated with elevated urinary cAMP and increased calcium reabsorption, although renal phosphate clearance remained unchanged. In bone, resorption was increased and formation decreased. Passive immunization of tumor-bearing mice with repeated doses of antisera raised against PLP resulted in modest and transient reversal of the biochemical and histomorphometric indices of disease compared with animals in which tumors were resected. In addition, tumor bearing mice developed cachexia and lost weight prior to the development of renal failure suggesting the presence of TNF (cachectin). Using a cytochemical bioassay others hav found no correlation between hypercalcemia and PTH-like bioactivity in the peripheral blood of cachectic mice bearing a human renal carcinoma supporting the hypothesis of multiple factorial involvement (42). The immunocompromised nature of the host animal should also be kept in mind when interpreting data from this model. Mice which are deficient in T-lymphocytes could perhaps have compensatory changes in the B-lymphocyte and monocyte branches of their immune system. As osteoclasts are thought to be derived from the monocyte lineage (43,44) this may not be the best model in which to study the effects of systemic factors on osteoclastic bone resorption.

5.4 Fischer rat bearing the Rice 500 Leydig cell tumor. Perhaps the best characterized and most extensively used rodent model of malignancy associated hypercalcemia is the male Fischer rat bearing the transplantable Rice 500 Leydig cell tumor This non-metastatic, testicular tumor was shown to arise spontaneously in aged Fischer rats and when passaged sub-cutaneously in younger male animals produced the hypercalcemic syndrome associated with malignancy (45) Hypercalcemia developed in tumor-bearing rats despite thyroparathyroidectomy (TPTX) demonstrating an independence from the parathyroid gland and was rapidly reversed following tumor excision suggesting a dependence on the presence of the neoplasm. In view of the steroid-secreting nature of Leydig cells, initial studies attempted to identify a novel steroid in this tumor which could be involved in calcium metabolism (46). Phytosterols, which had been associated elsewhere with hypercalcemia, were identified in tumor tissue removed from hypercalcemic rats. However, they were also identified in other tumor tissue unassociated with hypercalcemia and shown to be a minor synthetic product of Rice 500 leydig cells. Inhibitors of steroid biosynthesis had little effect in lowering serum calcium in tumor bearing rats and electron microscopic (EM) analysis of tumor tissue indicated less differentiation toward steroid secretory cells. Therefore, although the hypercalcemia associated with this tumor was exacerbated by administration of gonadotropins it seemed unlikely to be caused by a steroid. Biochemical, histomorphometric and renal characterization of the model during the 1980's consistently demonstrated a syndrome identical to that found in humans except for an elevated circulating level of 1,25 $(OH)_2D_3$ compared with a decreased level in the human syndrome (47-51). In this

respect the rat syndrome more closely resembles HPT. Differences in the severity and longevity of hypercalcemia and hypophosphatemia as well as species specific receptor and/or post receptor activation of 25 hydroxyvitamin D, 1 alpha hydroxylase have been advanced as possible explanations for this phenomenom (52). Despite elevated $1,25(OH)_2D_3$ levels, failure of a low calcium diet to significantly alter the blood calcium levels in tumor-bearing rats makes it unlikely that the syndrome represents an example of absorptive hypercalcemia as was the case in the VX2 rabbit carcinoma.

6.Bioassay of PTH/PLP. Prior to the development of specific radioimmunoassays (RIAs) for PLP, a number of bioassays, which had been developed for PTH were used to identify PTH-like bioactivity in tumors and peripheral plasma of patients with HHM.

6.1 The cytochemical bioassay (CBA) of glucose-6-phosphate dehydrogenase (G6PD) in guinea pig renal tubular cells (53,54). Following a short incubation with test substances segments of kidney cortex were sectioned and reacted for G6PD and the intensity of a colored product (formazan) was measured on a microdensitometer as the index of bioactivity. Incubation with cAMP, PTH or plasma from some hypercalcemic cancer patients resulted in a significant increase in G6PD activity compared with control. Following chromatography of plasma on Bio-gel P100 profiles of activity were shown to differ for patients with HPT and HHM suggesting different circulating forms of the bioactive substance. The same assay was used to identify PTH-like bioactivity in acid urea extracts of tumors associated

with hypercalcemia and malignancy (55). Four of 5 tumor extracts showed marked stimulation of G6PD activity which was inhibited by co-incubation with the competitive PTH analogue $Nle^{8,18}Tyr^{34}$bPTH 3-34. Extracts from tumors not associated with hypercalcemia showed no activity in this assay

6.2 Adenylate cyclase assay (renal, osseous). Adenylate cyclase (AC) activity in purified canine renal cortical membranes was measured as the conversion of ^{32}P ATP to ^{32}P cAMP using bovine (b)PTH 1-34 as a standard (56). The ability of tumor extracts from hypercalcemic patients to stimulate this conversion was inhibited by PTH antagonists but not by pre-incubation with antiserum raised against PTH. Therefore, although the active agent appeared to be interacting with the PTH receptor it was not recognised as PTH per se. Gel filtration studies once again demonstrated bioactive material with an estimated molecular weight in excess of that of PTH or its precursors. A variation of this assay was developed to measure the conversion of ^{3}H adenine which was incorporated into the cellular pool of adenosine triphosphate (ATP) of rat osteosarcoma cells, into ^{3}cAMP (57).

6.3 Fetal rat bone resorption assay (^{45}Ca release). Pregnant rats injected with radioactive $CaCl_2$ had their pups removed 2 days later. Fetal long bones, dissected free of soft tissues, were then incubated along with test substances over a 48h period and ^{45}Ca released into the medium was measured (58). Conditioned medium and tumor extracts from several human and animal tumors associated with hypercalcemia and malignancy were shown to increase AC activity in rat osteosarcoma cells as well as to stimulate

the release of ^{45}Ca in this assay (57).

6.4 IN VIVO infusion in thyroparathyroidectomised rats. Partially purified extracts of tissues derived from rat and human tumors associated with hypercalcemia and malignancy were infused into acutely TPTX'd rats (59). Tumor extracts which were shown to stimulate AC and G6PD activity IN VITRO were also capable of stabilizing plasma Ca and PO_4 and increasing the fractional excretion of PO_4 and cAMP, in a manner analagous to PTH, when infused into TPTX'd rats. These studies once again demonstrated that extracts of tumors differing in both cell type and species of origin, but associated with the same hypercalcemic syndrome IN VIVO, were capable of eliciting the same bioactivity IN VITRO and IN VIVO.

The bioassays which had been developed for PTH were therefore successful in demonstrating PTH-like bioactivity, in the absence of PTH immunoreactivity, in both tumors and plasma taken from patients with HHM and were subsequently used in various combinations for identification of this activity during purification procedures.

7. **Purification and cloning of the PLP peptide.** A peptide sharing 65% amino (NH_2)-terminal sequence homology with PTH was purified from 4 different human tumors and a rat tumor associated with hypercalcemia by 4 independent groups within months of one another.

7.1 Human PLP. An 18 kilodalton (kD) protein was isolated by T.J.Martin's group from conditioned medium harvested from the BEN cell line which had been established from a squamous carcinoma of the

bronchus.(60) Using limited NH_2 terminal sequence data, oligonucleotide probes were constructed and used to screen a complementary deoxyribonucleic acid (cDNA) library prepared from BEN cell RNA.(61). The sequence of positive clones predicted a mature, full length protein of 141 amino acids with a molecular weight of 16 kD which was in close agreement with that of 18kD estimated by sodium dodecyl sulfate (SDS) polyacrylimide gel electrophoresis (PAGE) and amino acid analysis of the purified protein. In addition, the mRNA encodes a 36 amino acid pre-pro sequence characteristic of secretory proteins . Sequence homology with PTH was restricted to the last two amino acids of the pro sequence representing the Lys-Arg cleavage site and 8 of the first 13 amino acids of the mature peptide, accounting for both its affinity for the PTH receptor and also its lack of cross-reactivity with antisera raised against PTH. All positive clones had identical DNA sequence in the coding and 3'noncoding regions but different 5'noncoding regions were evident. Transient transfection of COS-7 monkey kidney cells with an expression vector containing the full-length cDNA resulted in the expected PTH-like bioactivity in conditioned medium verifying the authenticity of the cloning procedure.

Using similar purification techniques, a second group headed by A.E.Broadus isolated a 17kD protein from a breast tumor associated with hypercalcemia.(62) Poly(A)$^+$RNA from a renal carcinoma was used by this group to construct the cDNA library which was then screened with oligonucleotide probes based on sequence data obtained from the breast tumor purification.(63) One of two positive clones identified the same 531 base pair (bp) open reading frame coding for the 177 amino acid pre-pro

peptide which had been reported earlier. Deduced (from renal carcinoma cDNA) and determined (from breast carcinoma purification) amino acid sequences corresponded well and indicated a predominance of basic residues confirming the results obtained from isoelectric focussing conducted on the purified protein. No methionine or cysteine residues and no potential N-glycosylation sites were evident. However, a potential site for post-translational amidation in the form of a PGKKKK run at positions 86-91 was identified. The open reading frame of this clone was flanked by 938 bp of 5' and 472 bp of 3' untranslated sequence together with a 60 base poly(A) tail and was judged to be representative of a near complete message. When probed with this cDNA, RNA prepared from tumors associated with hypercalcemia demonstrated complex hybridization patterns on Northern analysis whereas RNA from tumors unassociated with hypercalcemia failed to hybridize. In addition, a hybridization pattern similar to that obtained from the original renal carcinoma, though much reduced in signal intensity, was obtained from normal human keratinocytes. Identification of PLP mRNA in normal cells not only verified earlier experiments which demonstrated PTH-like bioactivity in conditioned medium from those cells (64) but also predicted the potential biological importance of PLP in normal physiology. Southern blotting of mammalian DNA, IN SITU hybridization and somatic cell hybrid analysis revealed a single copy gene which was mapped to the short arm of chromosome 12. Multiple transcripts evident on RNA analysis were therefore most likely a function of alternative splicing of the single copy gene rather than products of multiple related genes. This hypothesis was later confirmed when analysis of the second positive clone revealed a longer reading frame, coding for

a 173 amino acid peptide with the same 36 amino acid pre-pro sequence, a shorter 5' untranslated region and a unique 3'untranslated region.(65)

The third group, led by G.Strewler, to successfully clone the PTH-like peptide used sequence data obtained from a peptide purified from another renal carcinoma. (66) In addition to activity in bone and renal bioassays the peptide was shown to bind competitively to the PTH receptor in canine renal cortical membranes. Screening of the cDNA library prepared from the renal carcinoma uncovered yet another peptide isoform of 139 amino acids with a different 3' noncoding region (67). Alternative splicing of a single copy gene generating mature peptide isoforms of 139, 141 and 173 amino acids with variable 5' and 3' flanking sequences was therefore demonstrated by 3 independent laboratories. Taken together with evidence for the existence of this peptide in normal tissues these findings suggested a potential mechanism for developmental and/or tissue specific gene regulation.

7.2 Rat PLP. Rat PLP was cloned and characterized using a library prepared from a rat Leydig cell tumor. (68,69) A 1200bp positive clone contained the same open reading frame encoding the original 177 amino acid prepro peptide identified in a human tumor. Comparison of rat and human sequences revealed only 4 substitutions in the prepro sequence and 2 between positions 1-111 verifying an earlier report of extensive amino acid homology between purified rat and human peptides (59). The strict conservation of residues 35-111, beyond the region required for PTH-like bioactivity, suggests this domain is of biological relevance to mammalian physiology. Unlike the complex hybridization pattern obtained from human

RNA, Northern analysis of rat RNA prepared from both normal and malignant tissue revealed a single band at 1.4 kb predicting a much less complex transcriptional unit in the rat compared with man.

8. Cloning and Characterization of the PLP gene

8.1 Human PLP gene. The single copy human PLP gene spans at least 15 kilobases (Kb) of genomic DNA, contains a minimum of 7 exons and although far more complex, shares a functional organization of introns and exons with PTH suggesting a common ancestral origin.(70-72) In both genes the same 3 functional domains i.e. 5'noncoding region, prepro coding region and lys-arg cleavage site plus mature peptide, are encoded by single exons (Fig 2). Exons I and II of the PLP gene encode alternate 5' non-coding regions each associated with its own promoter. However, the presence of the 5' sequence encoded by exon II in the majority of cDNAs identified to date indicates preferential use of the downstream promoter lying between Exons I and II. The 5' non-coding region contained in Exon I is unusually long and some evidence exists that it may be alternatively spliced giving rise to additional 5' exons (73). Exon III encodes the pre-pro sequence of PLP and exon IV the lys-arg cleavage site and one isoform (PLP 1-139) of the mature peptide. Exons V, VI and VII of the PLP gene encode alternative 3' non-coding regions and in the case of exons VI and VII, different carboxy termini of PLP composed of 34 and 2 additional amino acids respectively. Alternative use of the two promoters, together with alternative splicing of 3' exons therefore accounts for the heterogeneity of PLP mRNA identified on Northern analysis and for the existence of three peptide isoforms with different 5' and 3' flanking sequences (Fig 3).

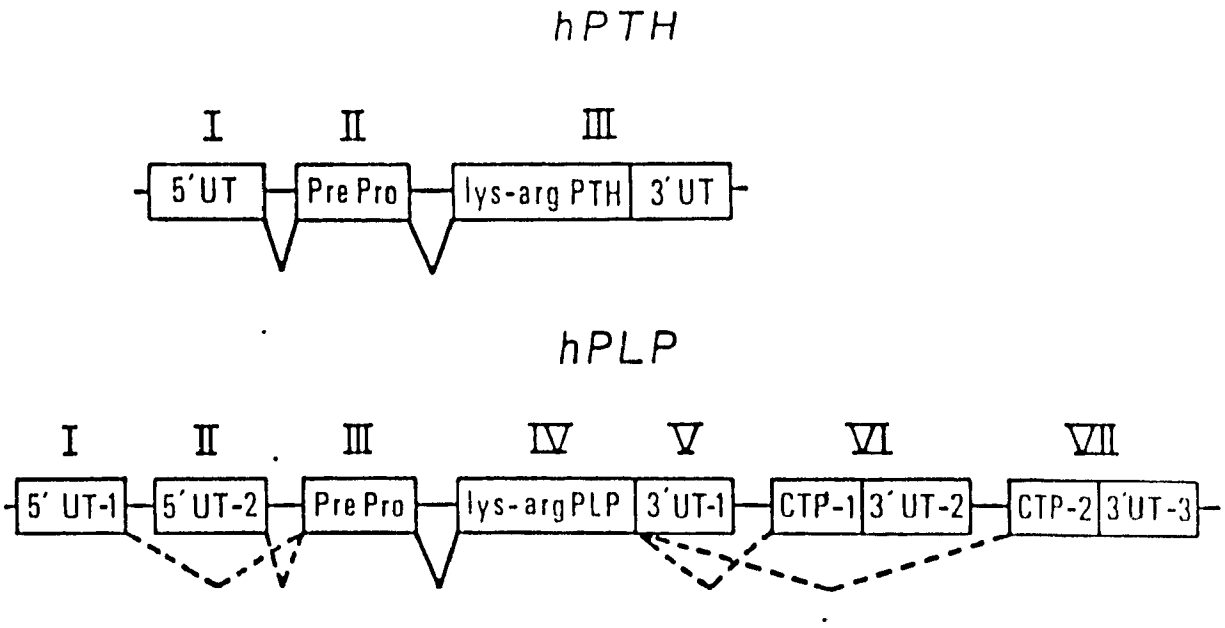

Figure 2 **Functional organization of hPTH and hPLP genes:** Constitutively used exons are joined by solid lines and alternatively used exons are joined by dashed lines. Single exons encode the 5' untranslated region, the pre-pro peptide and the lys-arg cleavage site with the mature peptide in both hPTH and hPLP genes. The PLP gene makes use of alternative 5' and 3' untranslated sequences whereas the PTH gene makes use of a single 5' and a single 3' sequence.

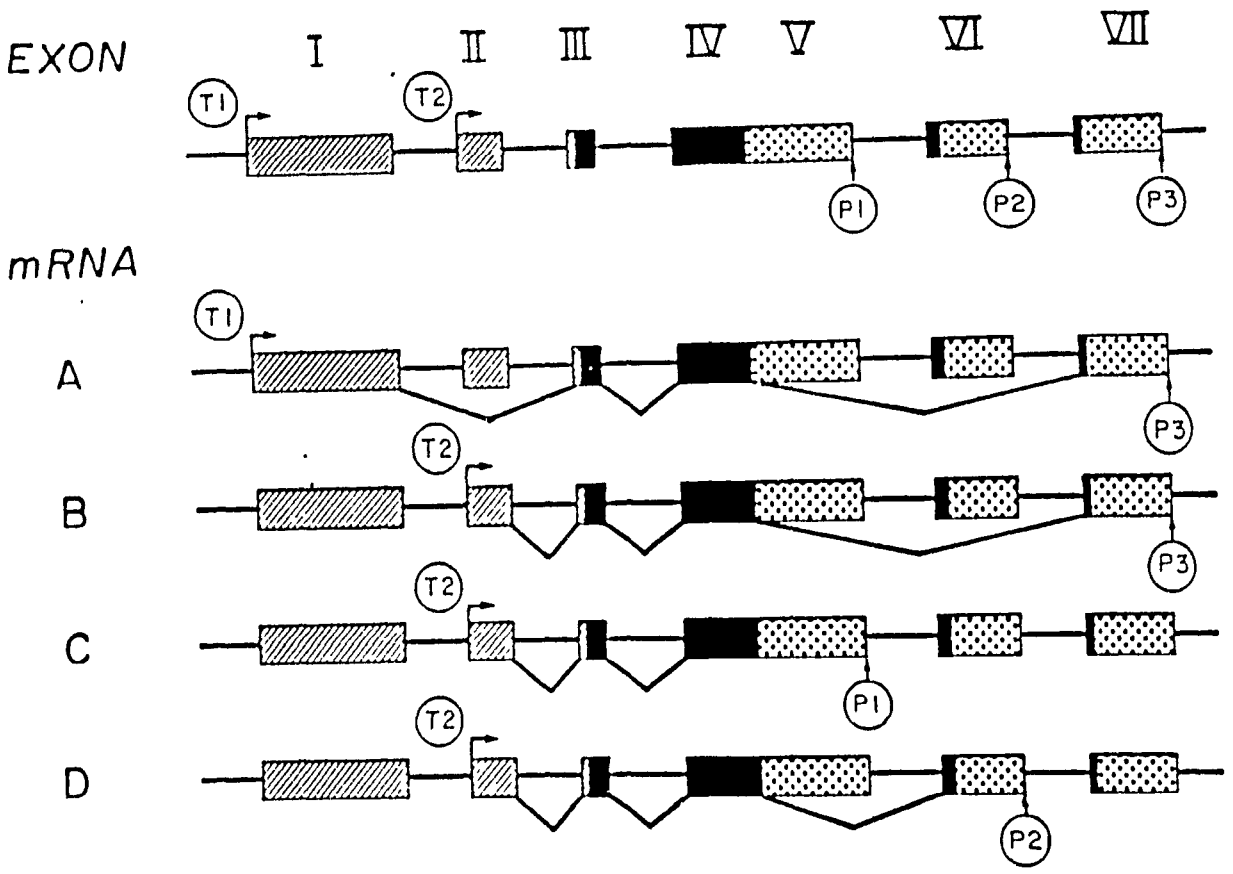

Figure 3 **Alternate splicing patterns for the hPLP gene:** Exons in the 5' untranslated regions are represented by the hatched rectangles, coding regions by the solid rectangles and 3' untranslated exons by the stippled rectangles. T1 and T2 represent alternative transcriptional start sites and P1, P2 and P3 alternative polyadenylation sites. A-D depict the 4 different mRNA species which have been identified and arise from the use of alternative promoters and different 3' splicing patterns. A and B code for 141 amino acid peptides and C and D encode peptides of 139 and 173 amino acids respectively.

8.2 Rat PLP gene. The single copy rat gene, isolated from a rat liver genomic DNA library, was shown to span 12 Kb and contain only 4 exons compared with the seven exon human gene.(74) Unlike the human PLP gene, that of the rat uses a single promoter and encodes a single species of mRNA confirming its less complex nature. As was the case with human PLP and PTH genes, those of the rat demonstrated similar functional organization i.e. single exons encoding 5′ noncoding (exon I), prepro sequence (exon II) and lys-arg cleavage site and mature peptide (exon III). In the case of rat PLP, exon IV encodes the 3′ non-coding sequence whereas the rat PTH gene has no separate exon for this purpose.

In addition to extensive sequence homology in the coding region and similar functional organization, the mRNA encoded by both rat and human PLP genes encode consensus motifs in 5′ (GC repeats) and 3′ (AU repeats) noncoding regions which are shared with a number of protooncogenes and cytokines (75). The presence of these motifs has been associated with superinduction of mRNA following inhibition of protein synthesis reflecting increased gene transcription and/or mRNA stabilization. Preliminary work exploring the regulation of rat and human PLP gene expression demonstrated superinduction of PLP mRNA following cycloheximide treatment and was attributed to inhibition of a suppressor protein as well as stabilization of mRNA by an unexplained mechanism.(76)

8.3 Evolutionary relatedness of PLP and PTH Apart from the striking similarity in functional organisation of the rat and human PLP and PTH genes, their localization to chromosomes bearing related functional genes is further evidence for a common ancestral origin (77). In the human

genome PLP has been assigned to the short arm of chromosome 12 which also bears the genes for lactate dehydrogenase B and the Kirsten-ras proto-oncogene. PTH on the other hand lies on the short arm of chromosome 11, co-localised with lactate dehydrogenase A and the Harvey-ras proto-oncogene (63). Assignment of the rat PLP gene to chromosome 4 (78) and that of the mouse to chromosome 6 (79), the known genetic loci of the Kirsten-ras proto-oncogene, indicates conservation of a syntenic group in these three mammalian species.

The presumed co-existence of PLP and PTH in both teleost and avian species suggests the divergence of PLP and PTH from a common ancestral gene was an early evolutionary event. The pituitary gland, along with the corpuscles of Stannius, has previously been identified as being involved in the metabolism of calcium in fish (80). Using specific radioimmunoassays for PTH, PLP and stanniocalcin (a hypocalcemic fish peptide) salmon pituitary extracts were shown to contain substantial quantities of all three peptides (81). The relative concentrations of PTH:PLP:stanniocalcin following HPLC fractionation were 13:100:400 ng/mg of protein. Although immunocytochemical staining of stanniocalcin was demonstrated in sections of pituitary and brain neither PLP or PTH immunoreactivity was demonstrated, despite a previous report of PTH immunostaining in goldfish pituitary glands (82). This represents the only study to date reporting the presence of PLP in teleosts and must therefore be viewed with caution while awaiting verification.

The avian PLP gene on the other hand has been cloned and partially characterized using a chicken genomic DNA library probed with a human coding region fragment (83,84). The open reading frame contained in

positive clones had 69% identity at the nucleotide level with Exon IV of the human gene along with precisely conserved 5' and 3' splice junctions (Fig 3). The single copy gene encodes mature peptides of 139 and 141 amino acids, compared with 141 amino acids in the rat and 139, 141 or 173 amino acids in the human. Although only a single promoter was identified, Northern analysis of RNA from embryonic chicken tissue revealed widespread distribution of a 1.5 kb transcript as well as a minor 1.2 kb message suggesting the potential for alternative splicing of the chicken gene.

9. IN VIVO bioactivity of synthetic PLP

Once PLP had been cloned and sequenced, the commercial availability of synthetic PLP fragments enabled investigators to examine the extent to which this novel peptide both mimicked and differed from PTH in its biological activity. The primary objectives of the following experiments were therefore not only to determine if PLP was capable of eliciting the hypercalcemia of malignancy syndrome when circulating IN VIVO but also to explain the apparent differences in bioactivity between PLP and PTH when they appeared to exert their effects through the same receptor Previous studies had identified amino acids 1-34 of the PTH molecule to be both necessary and sufficient for receptor binding and activation, triggering changes in mineral homeostasis associated with that hormone (2). Initial studies therefore examined the bioactivity of synthetic NH_2-terminal fragments of PLP and compared them with the effects of NH_2-terminal fragments of PTH.

9.1 Effect on the kidney. When infused into acutely TPTX'd rats at a

rate of 1nmol/hour for 4 hours both PLP 1-34 and PTH 1-34 increased the fractional excretion of phosphate and cAMP, decreased urinary calcium excretion and prevented the fall in plasma calcium seen in vehicle infused controls (85) In addition to truncated amino-terminal fragments, recombinent full-length PLP 1-141 was tested in the same IN VIVO system and found to be equipotent with PLP 1-34 and PTH 1-34 in decreasing calcium excretion while increasing that of phosphate and cAMP (86) Co-infusion of PLP 1-34 with the PTH antagonist PTH 7-34 in a 50 fold excess resulted in inhibition of these PLP stimulated changes (87) Infusion of the peptides for 16 hours resulted in more pronounced changes in plasma calcium and phosphate as well as a rise in circulating $1,25(OH)_2D_3$ (88). In short term experiments (4h) the changed biochemical indices were most likely due to the altered renal handling of divalent ions whereas over the longer term (16h) changes could have been a function of altered responses in kidney, gut (given the rise in $1,25(OH)_2D_3$) or bone. The 16h infusion was therefore repeated in TPTX, nephrectomized (NPX) animals fed a low calcium diet to determine the relative contribution being made by bone to the PLP/PTH induced hypercalcemia. The drastically reduced response suggested an important renal component, either directly through reabsorption or indirectly through increased absorption as a function of the raised $1,25(OH)_2D_3$ levels.

9.2 Effect in bone. A direct catabolic effect of PLP on bone was demonstrated by infusing TPTX rats for 48h before removing the tibial mataphysis for histomorphometric analysis (89). Equivalent, dose-dependent increases in the number of osteoclasts/mm^2 of bone were noted for PLP and

PTH treated animals compared with those receiving only a maintenance dose of PTH. These changes were accompanied by equivalent increases in plasma calcium and decreases in plasma phosphate for the two peptides. Repeated sub cutaneous injections of PLP or PTH over a 3d period in mice reportedly elicited the same degree of increased calvarial resorption (90) but the calcemic response was significantly greater to PLP than to PTH, perhaps reflecting a species difference or altered metabolism of the two peptides as a function of the route of administration. Alternatively the quantitation of osteoclasts may not be a sufficiently sensitive method of demonstrating altered bone metabolism.

Quantitative histomorphometry of rat long bones following a 12d course of low dose PLP 1-34 or PTH 1-34 treatment indicated an increase in bone mass and bone forming surfaces in PTH treated rats whereas the bones of PLP treated animals were indistinguishable from control bones.(91). This anabolic response to PTH 1-34 was later shown to be severely attenuated in hypophysectomised rats (92). Administration of growth hormone or PTH alone produced little change in bone mass or calcium content compared with vehicle treated control animals. However, co-administration of growth hormone and PTH restored the anabolic response. As growth hormone has no direct effect on appositional bone growth it was most likely acting as a competence factor for some intermediate. Perhaps the lack of anabolic response to PLP reflects an inability of that peptide to interact with the intermediate in question. The apparent lack of anabolic activity in PLP treated animals compared with PTH treated animals could help to explain the "uncoupled" bone turnover associated with the hypercalcemia of malignancy syndrome.

It could therefore be concluded from these IN VIVO studies that, with the notable exception of anabolic activity in the skeleton, PLP 1-34 elicited the same spectrum of biological activity as PTH 1-34 when infused into rats and that this activity appeared to be a function of the peptides binding to a common receptor in both kidney and bone.

10. Receptor binding and activation.

10.1 Binding characteristics. Using a variety of synthetic amino-terminal radioligands of PLP and PTH the characteristics of peptide binding were determined in both renal and osseous cells (93-95). Without exception the two peptides demonstrated the same reversible, time and temperature-dependent binding to a single class of high affinity (1.5 nmol) low capacity (10 fmol/ 10^5 cells) receptor. When affinity labeled and cross-linked with either radioligand the ligand-receptor complex migrated on SDS gels with an apparent molecular size of 80 kD. Each peptide specifically competed for binding with either radioligand in a dose dependent manner and with parallel inhibition curves. Given the limited (within the first 13 residues) sequence homology between PLP and PTH, and the fact that PTH 25-34 had been identified as critical to PTH receptor occupancy (96), these identical binding characteristics were somewhat puzzling. The lack of sequence homology in the 25-34 domains of PLP and PTH suggested that similarity in peptide conformation was sufficient for equivalent receptor interactions with either peptide. This hypothesis was validated when synthetic PLP 14-34 and PTH 14-34, which share no sequence homology, were shown to compete for binding to a common receptor on ROS cells (97) and in renal cortical membranes (98). PLP and PTH were therefore shown to bind

specifically to a membrane-bound component with the same apparent physicochemical and binding properties.

10.2 Adenylate cyclase activation. Unlike the strikingly similar binding characteristics displayed by PLP and PTH in both bone and kidney cells the data for adenylate cyclase activation and inhibition appears to be less well defined. In renal membrane assays PLP was either equipotent (85,90) or less potent (88,94) than PTH in adenylate cyclase activation whereas in intact bone cell systems PLP was either equipotent (85,88,90) or more potent (94,99) than PTH in stimulating cAMP production. Preincubation of opossum kidney and rat osteosarcoma cells (85) and of human osteosarcoma cells (100) with either peptide induced homologous desensitization, to a second challenge with the same peptide, as well as desensitization to the other peptide. On the other hand, preincubation with testosterone or estrogen reduced both PLP and PTH stimulated increases in adenylate cyclase activity in the human bone cells but not in those derived from the rat.(101,102). Both PLP 3-34 and PTH 3-34 demonstrated the same weak agonist activity when tested at high concentrations IN VITRO and especially IN VIVO (85). In addition, PLP 3-34 as well as PTH 3-34 served as competitive antagonists IN VITRO using either PLP 1-34 or PTH 1-34 as agonist (85,93). On the other hand, PLP 7-34 was a more effective antagonist to PLP 1-34 stimulated activity (103,104) as was PTH 7-34 to PTH 1-34 stimulated activity (104). These apparent discrepancies could reflect differences arising from the use of whole cell versus broken cell preparations, from interspecies differences or perhaps from the existence of multiple receptor sub-classes.

10.3 Cytosolic calcium mobilization. Apart from activating the adenylate cyclase pathway, binding of PTH to its membrane-bound receptor has been shown to induce transient increases in intracellular calcium (iCa^{++}) in both kidney (105) and bone (106) derived cells. Both inositol 1,4,5 triphosphate-stimulated release of calcium from intracellular stores (107,108) and calcium influx from the extracellular environment (109) have been identified as sources of calcium involved in these transient elevations in iCa^{++}. PLP 1-34 was shown to induce a rise in cytosolic calcium in suspensions of UMR-106 rat osteosarcoma cells which was partially inhibited by the competitive antagonist PTH 3-34 and duplicated by PLP 1-141 and PTH 1-84 (110). Neither chelation of extracellular Ca with EGTA nor treatment with a voltage-dependent Ca^{++} channel antagonist influenced this PLP/PTH induced calcium transient. Taken together with evidence documenting PLP/PTH stimulated increases in inositol triphosphates (111-112) these findings suggest that release of calcium from intracellular stores, rather than an influx from the extracellular pool, was the major source for the increase in cytosolic calcium in these experiments. These observations were corroborated and extended by others who demonstrated both increased intracellular calcium and cAMP accumulation were stimulated in a dose dependent and equivalent manner by PLP 1-34, PTH 1-34 and PLP 1-141 (104).

It is known that the first two amino acids of PTH are essential for full activation of the adenylate cyclase pathway while amino-terminal truncated fragments are either weakly active (PTH 3-34) or inactive (PTH 7-34). However, evidence exists for partial agonist activity for both PTH 3-34 and PTH 7-34 in the stimulation of iCa^{++} when measurements were made

in cell monolayers (113,114). This demonstration of alternate transduction mechanisms,perhaps requiring different molecular domains for activation, but apparently activated in an equivalent manner by PLP and PTH via the same receptor, opens the possibility of cell-specific coupling patterns which could modulate the biological response to the different peptides.

11. Role of PLP in fetal development

11.1 Placental calcium transfer. In late gestation the syncytiotrophoblast constitutes the major cell population separating maternal and fetal compartments and is the site of active nutrient and ion transport from mother to fetus. The existence of a "calcium pump" within this cell layer, which maintains the fetus in a state of hypercalcemia relative to the mother has been postulated since before Albright's time Initial studies attempted to identify PTH as the hypercalcemic factor responsible for maintaining the placental calcium gradient. However, circulating levels of both amino terminal and carboxy terminal PTH were decreased in the term fetus compared with the mother and yet PTH-like bioactivity was considerably elevated.(115). A strong positive correlation was demonstrated between the calcium gradient and the bioactivity present in the fetal circulation suggesting the calcium pump was being directed from the fetal side. Identification of PTH receptors on both maternal and fetal sides of the syncytiotrophoblast (116) reinforced the hypothesis that a factor binding to the PTH receptor could be influencing calcium transport through this tissue. An Australian group using IN SITU perfusion of sheep placenta as a working model had shown that fetal TPTX IN UTERO

resulted in a rapid decline in fetal plasma calcium and abolition of the placental calcium gradiant. (117). Infusion of PTH 1-34 over the short term failed to re-establish the gradient whereas long term infusion of large doses of PTH did. The late increase in fetal plasma calcium was later shown to be a function of calcium release from fetal bone rather than from increased placental transport (118). Infusion of partially purified extracts of either fetal parathyroid gland or the BEN cell lung tumor, from which PLP was purified, were both capable of reinstating the placental calcium gradient on a short term basis (119). When extracts of fetal parathyroid glands were bioassayed (119,120) after treatment with anti-PTH antibody they showed residual PTH-like activity which was later identified by radioimmunoassay as PLP (121). Therefore the physiological hypercalcemia in the fetal lamb, associated with increased PTH-like bioactivity but decreased immunoreactive PTH, appeared to be mediated by the same factor as that associated with the hypercalcemia of malignancy.

11.2 Autocrine modulator of growth More recent studies have identified PLP, using a variety of techniques, in both fetal and placental tissue of the rat (122), the sheep (123) and man (124). The widespread distribution pattern, which appears to shift during ontogeny, is consistent with an autocrine/paracrine role in the growth of these mammals as well as a possible role in fetal calcium homeostasis.

The succeeding chapters of this thesis will describe the work I have done examining the role played by PLP in the pathogenesis of hypercalcemia, the regulation of its expression in a model of tumor progression, and its mechamism of action in a non-classical target tissue.

REFERENCES

1. Case Records of the Massachusetts General Hospital (Case 27461). 1941 N Engl J Med. 225 789-791.

2. Habener J.F, Rosenblatt M and Potts J.T. 1984 Parathyroid hormone: biochemical aspects of biosynthesis, secretion, action, and metabolism. Physiol Rev. 64 985-1040.

3. Talmage R.V, Cooper G.W and Toverud S.U. 1982 The physiological significance of calcitonin. In Bone and Mineral Research 1 W.A.Peck Ed. Excerta Medica 74-143.

4. Stewart A.F, Horst R, Deftos L, Cadman E.C, Lang R and Broadus A.E 1980 Biochemical evaluation of patients with cancer-associated hypercalcemia. New Engl J Med 303 1377-1383.

5. Stewart A.F, Vignery A, Silverglate A, Ravin N.D, LiVolsi V, Broadus A.E and Baron R. 1982 Quantitative bone histomorphometry in humoral hypercalcemia of malignancy· uncoupling of bone cell activity. J Clin Endocrinol Metab 55 219-227.

6. Sherwood L.M, O'Riordan J.L.H, Aurbach G.D and Potts J.T. 1967 Production of parathyroid hormone by nonparathyroid tumors. J Clin Endocrinol Metab 27 140-144.

7. Benson R.C, Riggs B.L, Pickard B.M and Arnaud C.D. 1973 Radioimmunoassay of parathyroid hormone in hypercalcemic patients with malignant disease. Amer J Med 56 79-84.

8. Shaw J.W, Oldham S.B, Rosoff L, Bethune J.E and Fichman M.P. 1976 Urinary cyclic AMP analyzed as a function of the serum calcium and parathyroid hormone in the differential diagnosis of hypercalcemia. J Clin Invest 59 14-21.

9. Hirshorn J.E, Vrhovsek E and Posen S. 1979 Carcinoma of the breast associated with hypercalcemia and the presence of parathyroid hormone-like substances in the tumor. J Clin Endocrinol Metab 48 217-221.

10. Simpson E.L. Mundy G.R, D'Souza S.M, Ibbotson K.J, Bockman M.D and Jacobs J.W. 1983 Absence of parathroid hormone messenger RNA in nonparathyroid tumors associated with hypercalcemia. New Engl J Med 309 325-330.

11. Yoshimoto K, Yamasaki R, Sakai H, Tezuka U, Takahashi M, Iizuka M. Sekiya T and Saito S. 1989 Ectopic production of parathyroid hormone by a small cell lung cancer in a patient with hypercalcemia. J Clin Endocrinol Metab 68 976-981.

12. Nussbaum S.R, Gaz R.D and Arnold A. 1990 Hypercalcemia and ectopic secretion of parathyroid hormone by an ovarian carcinoma with rearrangement of the gene for parathyroid hormone. New Engl J Med 323 1324-1328.

13. Robertson R.P, Baylink D.J, Marini J.J and Adkison H.W. 1975 Elevated prostaglandins and suppressed parathyroid hormone associated with hypercalcemia and renal cell carcinoma. J Clin Endocrinol Metab 41 164-167.

14. Seyberth H.W, Segre G.V, Morgan J.L, Sweetman B.J, Potts J.T and Oates J.A. 1975 Prostaglandins as mediators of hypercalcemia associated with certain types of cancer. New Engl J Med 293 1278-1283

15. Mundy G.R, Wilkinson R and Heath D.A. 1983 Comparative study of available medical therapy for hypercalcemia of malignancy. Amer J Med 74 421-432.

16. Franklin R.B and Tashjian A.H. 1975 Intravenous infusions of prostaglandin E_2 raises plasma calcium in the rat. Endocrinology 97 240-243.

17. Voelkel E.F, Tashjian A.H, Franklin R, Wasserman E and Levine L. 1975 Hypercalcemia and tumor prostaglandins: the VX_2 carcinoma model in the rabbit. Metabolism 24 973-986.

18. Trechsel U, Taylor C.M, Bonjour J.P and Fleisch H. 1980 Influence of prostaglandins and of cyclic nucleotides on the metabolism of 25 hydroxyvitamin D_3 in primary chick kidney cell culture. Biochem Biophys Res Comm 93 1210-1216.

19. Doppelt S.H, Slovik D.M, Neer R.M, Nolan J, Zusman R.M and Potts J.T. 1982 Gut mediated hypercalcemia in rabbits bearing VX_2 carcinoma: new mechanism for tumor-induced hypercalcemia. Proc Nat Acad Sci (USA) 79 640-644.

20. Mundy G.R. 1° The hypercalcemia of malignancy revisited. Kidney Int 31 142-155.

21. Tashjian A.H, Voelkel E.F, Lazzaro M, Singer F.R, Roberts A.B, Derynck R, Winkler M.E and Levine L. 1985 α and ß human transforming growth factors stimulate prostaglandin production and bone resorption in cultured mouse calvaria. Proc Nat Acad Sci (USA) 82 4535-4538.

22. Ibbotson K.J, D'Souza S.M, Smith D.D, Carpenter G and Mundy G.R. 1984 EGF receptor antiserum inhibits bone resorbing activity produced by a rat Leydig cell tumor associated with the humoral hypercalcemia of malignancy. Endocrinology 116 469-471.

23. Insogna K.L, Weir E.C, Wu T.L, Stewart A.F, Broadus A.E, Burtis W.J and Centrella M. 1987 Co-purification of transforming growth factor ß-like activity with PTH-like and bone resorbing activities from a tumor associated with humoral hypercalcemia of malignancy. Endocrinology 120 2183-2185.

24. Centrella M, McCarthy T and Canalis E. 1988 Skeletal tissue and transforming growth factor ß. FASEB J 2 3066-3073.

25. Horton J.E, Raisz L.G, Simmons H.A, Oppenheim J.S and Mergenhagen S.E. 1972 Bone resorbing activity in supernatant fluid from cultured human peripheral blood leukocytes Science 177 793-795

26. Canalis E, McCarthy T and Centrella M. 1988 Growth factors and the regulation of bone remodeling. J Clin Invest 81 277-281.

27. Johnson R.A, Boyce B.F, Mundy G.R and Roodman G.D. 1989 Tumors producing human tumor necrosis factor induce hypercalcemia and osteoclastic bone resorption in nude mice. Endocrinology 124 1424-1427.

28. Jacobson J.O, Bringhurst F.R, Harris N.L, Weitzman S.A and Aisenberg A.C. 1988 Humoral hypercalcemia in Hodgkin's disease Clinical and laboratory evaluation. Cancer 163 917-923.

29. Breslau N.A, McGuire J.L, Zerwech J.E, Frenkel E.P and Pak C Y.C. 1984 Hypercalcemia associated with increased calcitriol levels in three patients with lymphoma. Ann Int Med 100 1-7.

30. Honda S, Yamaguchi K, Miyake Y, Hayashi N, Adachi N, Kinoshita K, Ikehara O, Kimura S, Kinoshita T, Shimotohngc K, Shimoyama M and Abe K. 1988 Production of parathyroid hormone-related protein in adult T-cell leukemia cells. Jpn J Cancer Res 79 1264-1268.

31. Fukumoto S, Matsumoto T, Watanabe T, Takahashi H, Miyoshi I and Ogata E. 1989 Secretion of parathyroid hormone-like activity from human T-cell lymphotropic virus type 1-infected lymphocytes. Cancer Res 49 3849-3852.

32. Motokura T, Fukumoto S, Matsumoto T, Takahashi S, Fujita A, Yamashita T, Igarashi T and Ogata E 1989 Parathyroid hormone-related protein in adult T-cell leukemia-lymphoma. Ann Int Med 111 484-488.

33. Gkonos P.J, Hayes T, Burtis W, Jacoby R, McGuire J Baron R and Stewart A. 1984 Squamous carcinoma model of humoral hypercalcemia of malignancy. Endocrinology 115 2384-2390.

34. D'Souza S.M, vIbbotson K.J, Smith D.D and Mundy G.R. 1984 Production of a macromolecular bone resorbing factor by the hypercalcemic variant of the Walker rat carcinosarcoma. Endocrinology 115 1746-1752.

35. Strewler G.J, Wronski T.J, Halloran B.P, Miller S.C, Leung S.C, Williams R.D and Nissenson R.A. 1986 Pathogenesis of hypercalcemia in nude mice bearing a human renal carcinoma. Endocrinology 119 303-310.

36. Weir E.C, Insogna K.L, Brownstein D.G, Bander N.H and Broadus A.E. 1988 In Vitro adenylate cyclase stimulating activity predicts the occurrence of humoral hypercalcemia of malignancy in nude mice. J Clin Invest. 81 818-821.

37. Abramson E.C, Kukla L.J, Shevrin D.H, Lad T.E, McGuire W.P and Kukreja S.C. 1984 A model for malignancy-associated humoral hypercalcemia. Calcif Tiss Int 36 563-567.

38. Kukreja S.C, Rosol T.J, Shevrin D.H and York P.A. 1988 Quantitative bone histomorphometry in nude mice bearing a human squamous cell lung cancer. J Bone Min Res 3 341-346.

39. Kukreja S.C, Shevrin D.H, Wimbiscus S.A, Ebeling P.R, Danks J.A, Rodda C.P, Wood W.I and Martin T.J. 1988 Antibodies to parathyroid hormone-related protein lower serum calcium in athymic mouse models of malignancy associated hypercalcemia due to human tumors. J Clin Invest 82 1798-1802.

40. Shipley P.J, Shevrin D.H and Kukreja S.C. 1988 Increased renal calcium reabsorption in an animal model of hypercalcemia of human malignancy. J Bone Min Res 3 555-560.

41. Kukreja S.C, Rosol T.J, Wimbiscus S.A, Shevrin D.H, Grill V, Barengolts E.I and Martin T.J. 1990 Tumor resection and antibodies to parathyroid hormone-related protein cause similar changes on bone histomorphometry in hypercalcemia of cancer. Endocrinology 127 305-310.

42. Mehdizadeh S, Alaghband-Zadeh J, Gusterson B, Arlot M, Bradbeer J.N and Loveridge N. 1989 Bone resorption and circulating PTH-like bioactivity in an animal model of hypercalcemia of malignancy. Biochem Biophys Res Comm 161 1166-1171.

43. Marks S.C. 1983 The origin of the osteoclast. J Oral Pathol 12 226-256.

44. Takahashi N, Kujita T, MacDonald B.R, Bird A, Mundy G.R, McManus L.M, Miller M, Boyde A, Jones S.J and Roodman G.D. 1989 Osteoclast-like cells form in long-term human bone marrow but not in peripheral blood cultures. J Clin Invest 83 543-550.

45. Rice B.F, Ponthier R.L and Miller M.C. 1971 Hypercalcemia and neoplasia: a model system. Endocrinology 88 1210-1216.

46. Rice B.F, Roth L.M, Cole F.E, MacPhee A.A, Davis K, Ponthier R.L and Sternberg W.H. 1975 Hypercalcemia and neoplasia: biologic, biochemical, and ultrastructural studies of a hypercalcemia producing Leydig cell tumor of the rat. Lab Invest 33 428-439.

47. Sica D.A, Martodam R.R, Aronow J and Mundy G.R. 1983 The hypercalcemic rat Leydig cell tumor: A model of the humoral hypercalcemia of malignancy. Calcif Tiss Int 35 287-293.

48. Spiegal A.M, Saxe A.W, Deftos L.J and Brennan M.F. 1983 Humoral hypercalcemia caused by a rat Leydig-cell tumor is associated with suppressed parathyroid hormone secretion and increased urinary cAMP excretion. Horm Metabol Res 15 299-304.

49. Tam C.S, Heersche J N.M, Santora A and Spiegal A.M. 1984 Skeletal response in rats following the implantation of hypercalcemia-producing Leydig cell tumors. Metabolism 33 50-53.

50. Insogna K.L, Stewart A.F, Vignery A.M.C, Weir E, Namnum P A, Baron R.E, Kirkwood J.M, Deftos L M and Broadus A.E. 1984 Biochemical and histomorphometric characterization of a rat model for humoral hypercalcemia of malignancy. Endocrinology 114 888-896.

51. Rizzoli R, Caverzasio J, Fleisch H and Bonjour J.P. 1986 Parathyroid hormone-like changes in renal calcium and phosphate reabsorption induced by Leydig cell tumor in thyroparathyroidectomized rats. Endocrinology 119 1004-1009.

52. Walker A.T, Stewart A.F, Korn E.A, Shiratori T, Mitnick M.A and Carpenter T.O. 1990 Effect of parathyroid hormone-like peptides on 25-hydroxyvitamin D-1a-hydroxylase activity in rodents. Am J Physiol E297-303.

53. Goltzman D, Henderson B and Loveridge N. 1980 Cytochemical bioassay of parathyroid hormone: characteristics of assay and analysis of circulating forms. J Clin Invest 65 1309-1317

54. Goltzman D, Stewart A.F and Broadus A E 1981 Malignancy-associated hypercalcemia: evaluation with a cytochemical bioassay for parathyroid hormone. J Clin Endocrinol Metab 53 899-904

55. Stewart A.F, Insogna K.L, Goltzman D and Broadus A.E 1983 Identification of adenylate cyclase-stimulating activity and cytochemical glucose-6-phosphate dehydrogenase-stimulating activity in extracts of tumors from patients with humoral hypercalcemia of malignancy. Proc Natl Acad Sci (USA) 80 1454-1458

56. Nissenson R.A, Abbott S.R, Teitelbaum A P, Clark O.H and Arnaud C.D. 1981 Endogenous biologically active human parathyroid hormone measurement by a guanyl nucleotide-amplified renal adenylate cyclase assay. J Clin Endocrinol Metab 52 840-844.

57. Rodan S.B, Insogna K.L, Vignery A.M.C, Stewart A.F, Broadus A.E, D'Souza S.M, Bertolini D.R, Mundy G.R and Rodan G.A. 1983 Factors associated with humoral hypercalcemia of malignancy stimulate adenylate cyclase in osteoblastic cells. J Clin Invest 72 1511-1515.

58. Raisz L.G. 1965 Bone resorption in tissue culture. Factors influencing the response to parathyroid hormone. J Clin Invest 103-116.

59. Rabbani S.A, Mitchell J, Roy D.R, Kremer R, Bennett H.P.J and Goltzman D. 1986 Purification of peptides with parathyroid hormone-like bioactivity from human and rat malignancies associated with hypercalcemia. Endocrinology 118 1200-1210.

60. Moseley J.M, Kubota M, Diefenbach-Jagger H, Wettenhall R.E.H, Kemp B.E, Suva L.J, Rodda C.P, Ebeling P.R, Hudson P.J, Zajac J.D and Martin T.J. 1987 Parathyroid hormone-related protein purified from a human lung cancer cell line. Proc Natl Acad Sci (USA) 84 5048-5052.

61. Suva L.J, Winslow G.A, Wettenhall R.E.H, Hammonds R.G, Moseley J.M, Diefenbach-Jagger H, Rodda C.P, Kemp B.E, Rodriguez H, Chen E.Y, Hudson P.J, Martin T.J and Wood W.I. 1987 A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. Science 237 893-896.

62. Burtis W.J, Wu T, Bunch C, Wysolmerski J.J, Insogna K.L, Weir E.C, Broadus A.E and Stewart A.F. 1987 Identification of a novel 17,000-dalton parathyroid hormone-like adenylate cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy. J Biol Chem 262 7151-7156.

63. Mangin M, Webb A.C, Dreyer B.E, Posillico J.T, Ikeda K, Weir E.C, Stewart A.F, Bander N.H, Milestone L, Barton D.E, Francke U and Broadus A.E. 1988 Identification of a cDNA encoding a parathyroid hormone-like peptide from a human tumor associated with humoral hypercalcemia of malignancy. Proc Natl Acad Sci (USA) 85 597-601.

64. Merendino J.J, Insogna K.L, Milstone L.M, Broadus A.E and Stewart A.F. 1986 A parathyroid hormone-like protein from cultured human keratinocytes. Science 231 388-390.

65. Mangin M, Ikeda K, Dreyer B.E, Milstone L and Broadus A.E. 1988 Two distinct tumor-derived parathyroid hormone-like peptides result from alternantive ribonucleic acid splicing. Mol Endocrinol 1988 2 1049-1055.

66. Strewler G.J, Stern P.H, Jacobs J.W, Eveloff J, Klein R.F, Leung S.C, Rosenblatt M and Nissenson R.A. 1987 Parathyroid hormonelike protein from human renal carcinoma cells: structural and functional homology with parathyroid hormone. J Clin Invest 80 1803-1807.

67. Thiede M.A, Strewler G.J, Nissenson R.A, Rosenblatt M and Rodan G.A. 1988 Human renal carcinoma expresses two messages encoding a parathyroid hormone-like peptide: evidence for the alternative splicing of a single-copy gene. Proc Natl Acad Sci (USA) 85 4605-4609.

68. Yasuda T, Banville D, Rabbani S.A, Hendy G.N and Goltzman D. 1989 Rat parathyroid hormone-like peptide:comparison with the human homologue and expression in malignant and normal tissue. Mol Endocrinology 3 518-524.

69. Thiede M.A and Rodan G.A. 1988 Expression of a calcium-mobilizing parathyroid hormone-like peptide in lactating mammary tissue. Science 242 278-280.

70. Yasuda T, Banville D, Hendy G.N and Goltzman D. 1989 Characterization of the human parathyroid hormone-like peptide gene. J Biol Chem 264 7720-7725.

71. Mangin M, Ikeda K, Dreyer B.E and Broadus A.E. 1989 Isolation and characterization of the human parathyroid hormone-like peptide gene. Proc Natl Acad Sci (USA) 86 2408-2412.

72. Suva L.J, Mather K.A, Gillespie M.T, Webb G.C, Ng K.W, Winslow G.A, Wood W.I, Martin T.J and Hudson P.J. 1989 Structure of the 5' flanking region of the gene encoding human parathyroid-hormone-related protein (PTHrP) Gene 77 95-105.

73. Mangin M, Ikeda K, Dreyer B.E and Broadus A E. 1990 Identification of an up-stream promoter of the human parathyroid hormone-related peptide gene. Mol Endocrinol 4 851-858.

74. Karaplis A.C, Yasuda T, Hendy G.N, Goltzman D and Banville D. 1990 Gene-encoding parathyroid hormone-like peptide: nucleotide sequence of the rat gene and comparison with the human homologue Mol Endocrinol 4 441-445.

75. Shaw G and Kamen R. 1986 A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46 659-667.

76. Ikeda K, Lu C, Weir E.C, Mangin M and Broadus A.E. 1990 Regulation of parathyroid hormone-related peptide gene expression by cycloheximide. J Biol Chem 265 5398-5402.

77. Comings D.E. 1972 Evidence for ancient tetraploidy and conservation of linkage groups in mammalian chromosomes. Nature 238 455-457.

78. Szpirer C, Riviere M, Hanson C, Levan G and Hendy G.N. 1991 Assignment of the rat parathyroid hormone-like peptide gene to chromosome 4: evidence for conserved synteny between human chromosome 12, mouse chromosome 6 and rat chromosome 4. Cytogenet Cell Genet 56 193-195.

79. Hendy G.N, Sakaguchi A.Y, Lalley P.A, Martinez L, Yasuda T, Banville D and Goltzman D. 1990 Gene for parathyroid hormone-like peptide is on mouse chromosome 6 Cytogenet Cell Genet 53 80-82.

80. Parsons J.A, Gray D, Rafferty B and Zanelli J.M. 1978 Evidence for a hypercalcemic factor in the fish pituitary immunologically related to mammalian parathyroid hormone. IN: Endocrinology of calcium metabolism, edited by Copp D.H and Talmage R.V. 111-114.

81. Fraser R.A, Kaneko T, Pang P.K.T and Harvey S. 1991 Hypo- and hypercalcemic peptides in fish pituitary glands. Am J Physiol R622-626.

82. Kaneko T and Pang P.K.T. 1987 Immunocytochemical detection of parathyroid hormone-like substance in the goldfish brain and pituitary gland. Gen Comp Endocrinol 68 147-152.

83. Thiede M.A and Rutledge S.J. 1990 Nucleotide sequence of a parathyroid hormone-related peptide expressed by the 10 day chicken embryo. Nucl Acids Res 18 3062.

84. Schermer D T, Chan S.D.H, Bruce R, Nissenson R.A, Wood W.I and Strewler G.J. 1991 Chicken parathyroid hormone-related protein and its expression during embryologic development. J Bone Min Res 6 149-155.

85. Rabbani S.A, Mitchell J, Roy D.R, Hendy G.N and Goltzman D 1988 Influence of the amino-terminus on IN VITRO and IN VIVO biological activity of synthetic parathyroid hormone-like peptides of malignancy. Endocrinology 123 2709-16.

86. Zhou H, Leaver D.D, Moseley J.M, Kemp B, Ebeling P.R and Martin T.J. 1989 Actions of parathyroid hormone-related protein on the rat kidney IN VIVO. J. Endocrinol 122 229-235.

87. Horiuchi N, Hongo T and Clemens T.L. 1990 The synthetic parathyroid hormone-related protein is inhibited by a parathyroid hormone antagonist in rats IN VIVO. J Bone Min Research 5 541-545.

88. Horiuchi N, Caulfield M.P, Fisher J.E, Goldman M.E, McKee R.L, Reagan J E, Levy J.J, Nutt R.F, Rodan S.B, Schofield T.I, Clemens T.I and Rosenblatt M. 1987 Similarity of synthetic peptide from human tumor to parathyroid hormone IN VIVO and IN VITRO. Science 238 1566-1568.

89. Thompson D.D, Seedor J.G, Fisher J.E, Rosenblatt M and Rodan G.A. 1988 Direct action of the parathyroid hormone-like human hypercalcemic factor on bone. Proc Natl Acad Sci (USA) 85 5673-5677.

90. Yates A.J.P, Gutierrez G.E, Smolens P, Travis P S, Katz M.S, Aufdemorte T.B, Boyce B.F, Hymer T.K, Poser J.W and Mundy G.R. 1988 Effects of a synthetic peptide of a parathyroid-hormone related protein on calcium homeostasis, renal calcium reabsorption and bone metabolism IN VIVO and IN VITRO in rodents. J. Clin. Invest. 81 932-938.

91. Hock J.M, Fonesca J, Gunness-Hey M, Kemp B E and Martin T.J. 1989 Comparison of the anabolic effects of synthetic parathyroid hormone-related protein (PTHrP) 1-34 and PTH 1-34 on bone in rats. Endocrinology 125 2022-2027.

92. Hock J.M and Fonesca J. 1990 Anabolic effect of human synthetic parathyroid hormone-(1-34) depends on growth hormone. Endocrinology 127 1804-1810.

93. Shigeno C, Yamamoto I, Kitamura N, Noda T, Lee K, Sone T, Shiomi K, Ohtaka A, Fujii N, Yajima H and Konishi J. 1988 Interaction of human parathyroid hormone-related peptide with parathyroid hormone receptors in clonal rat osteosarcoma cells J Biol Chem 263 18369-18377.

94. Nissenson R.A, Diep D, and Strewler G.J. 1988 Synthetic peptides comprising the amino-terminal sequence of a parathyroid hormone-like protein from human malignancies: Binding to parathyroid hormone receptors and activation of adenylate cyclase in bone cells and kidney. J Biol Chem 263 12866-12877.

95. Jueppner H, Abou-Samra A, Uneno S, Gu W, Potts J.T and Segre G V. 1988 The parathyroid hormone-like peptide associated with humoral hypercalcemia of malignancy and parathyroid hormone bind to the same receptor on the plasma membrane of ROS 17/2.8 cells. J Biol Chem 263 8557-8560.

96. Nussbaum S.R, Rosenblatt M, and Potts J.T. 1980 Parathyroid hormone-renal receptor interactions Demonstration of two receptor binding domains. J Biol Chem 255 10183-10186.

97. Abou-Samra A, Uneno S, Jueppner H, Keutmann H, Potts J.T, Segre G.V and Nussbaum S.R. 1989 Non-homologous sequences of parathyroid hormone-related peptide bind to a common receptor on ROS 17/2.8 cells. Endocrinology 125 2215-2217.

98. Caulfield M.P, McKee R.L, Goldman M.E, Duong L.T, Fisher J.E, Gay C.T, DeHaven P.A, Levy J.J, Roubini E, Nutt R.F, Chorev M and Rosenblatt M. 1990 The bovine renal parathyroid hormone (PTH) receptor has equal affinity for two different amino acid sequences: The receptor binding domains of PTH and PTH-related protein are located within the 14-34 region Endocrinology 127 83-87.

99. Kemp B.E, Moseley J.M, Rodda C.P, Ebeling P.R, Wettenhall R.E.H, Stapleton D, Diefenbach-Jagger H, Ure F, Michelangeli V.P, Simmons H.A, Raisz L.G and Martin T.J. 1987 Parathyroid hormone-related protein of malignancy: active synthetic fragments. Science 238 1568-1570.

100. Fukayama S, Bosma T.J, Goad D.L, Voelkel E.F and Tashjian A.H. 1988 Human parathyroid hormone (PTH)- related protein and human PTH: comparative biological activities on human bone cells and bone resorption. Endocrinology 123 2841-2848.

101. Fukayama S and Tashjian A.H. 1989 Direct modulation by estradiol of the response of human bone cells (SAOS-2) to human parathyroid hormone (PTH) and PTH-related protein Endocrinology 124 397-401.

102. Fukayama S and Tashjian A.H. 1989 Direct modulation by androgens of the response of human bone cells (SAOS-2) to human parathyroid hormone (PTH) and PTH-related protein. Endocrinology 125 1789-1794.

103. Nagasaki K, Yamaguchi K, Miyake Y, Hayashi C, Honda S, Urakami K, Miki K, Kimura S, Watanabe T and Abe K. 1989 IN VITRO and IN VIVO antagonists against parathyroid hormone-related protein. Biochem Biophys Res Comm. 158 1036-1042.

104. Donahue H.J, Fryer M.J and Heath H. 1990 Structure-function relationships for full-length recombinant parathyroid hormone-related peptide and its amino-terminal fragments: effects on cytosolic calcium ion mobilization and adenylate cyclase activation in rat osteoblast-like cells. Endocrinology 126 1471-1477.

105. Hruska K, Moskowitz D, Esbrit P, Civitelli R, Westbrook S and Huskey M. 1987 Stimulation of inositol triphosphate and diacylglycerol production in renal tubular cells by parathyroid hormone. J Clin Invest 79 230-239.

106. Reid I.R, Civitelli R, Halstead L.R, Avioli L.V and Hruska K.A. 1987 Parathyroid hormone acutely elevates intracellular calcium in osteoblast-like cells. Am J Physiol 253 E45-50.

107. Civitelli R, Reid I.R, Westbrook S, Avioli L.V and Hruska K.A. 1988 PTH elevates inositol polyphosphates and diacylglycerol in a rat osteoblast-like cell line. Amer J Physiol 255 E660-667.

108. Farndale R.W, Sandy J.R, Atkinson S.J, Pennington S.R, Meghji S and Meikle M. 1988 Parathyroid hormone and prostaglandin E_2 stmulate both inositol phosphates and cAMP accumulation in mouse osteoblast cultures. Biochem J 252 263-268.

109. Yamaguchi D.T, Hahn T.J, Iida-Klein A, Kleeman C.R and Muallem S. 1987 Parathyroid hormone-activated calcium channels in an osteoblast-like clonal osteosarcoma cell line. J Biol Chem 262 7711-7718.

110. Civitelli R, Martin T.J, Fausto A, Gunsten S.L, Hruska K A and Avioli L.V. 1989 Parathyroid hormone-related peptide transiently increases cytosolic calcium in osteoblast-like cells: comparison with parathyroid hormone. Endocrinology 125 1204 1210.

111. Civitelli R, Hruska K.A, Shen V and Avioli L V. 1990 Cyclic AMP-dependent and calcium-dependent signals in parathyroid hormone function. Exper Gerontol 25 223-231.

112. Short A.D, Brown B.L and Dobson P.R.M. 1991 The effect of retinoic acid on parathyroid hormone and parathyroid hormone-related peptide-induced intracellular calcium in a rat osteosarcoma cell line, UMR 106. J Endocrinol 129 75-81.

113. Donahue H.J, Fryer M.J, Eriksen E.F and Heath H 1988 Differential effects of parathyroid hormone and its analogues on cytosolic calcium ion and cAMP levels in cultured rat osteoblast-like cells. J Biol Chem 263 13522-13527.

114. Fujimori A, Cheng S, Avioli L.V and Civitelli R. 1991 Dissociation of second messenger activation by parathyroid hormone fragments in osteosarcoma cells. Endocrinology 128 3032-3039

115. Allgrove J, Adami S, Manning R.M and O'Riordan J.L.H. 1985 Cytochemical bioassay of parathyroid hormone in maternal and cord blood. Arch Dis Childhood 60 110-115.

116. Lafond J, Auger D, Fortier J and Brunette M.G. 1988 Parathyroid hormone receptor in human placental syncytiotrophoblast brush border ans basal plasma membranes. Endocrinology 123 2834-2840.

117. Care A.D, Caple I.W and Pickard D.W. 1985 The roles of the parathyroid and thyroid glands on calcium homeostasis in the ovine fetus. In: The physiological development of the fetus and newborn. Ed. Jones C.T and Nathanielsz P.W. London: Academic Press 135-140.

118. Caple I.W, Heath J.A, Pham T.T, Farrugia W, Wark J.D, Care A.D and Martin T.J. 1989 The role of the parathyroid glands, PTH, PTHrP and elevated plasma calcium in bone formation in fetal lambs. J Bone Min Res 4S1 S262.

119. Rodda C.P, Kubota M, Heath J.A, Ebeling P.R, Moseley J.M, Care A.D, Caple I.W and Martin T.J. 1988 Evidence for a novel parathyroid hormone-related protein in fetal lamb parathyroid glands and sheep placenta: comparisons with a similar protein implicated in humoral hypercalcaemia of malignancy. J Endocrinol 117 261-271.

120. Loveridge N, Caple I.W, Rodda C, Martin T.J and Care A.D. 1988 Further evidence for parathyroid hormone-related protein in fetal parathyroid glands of sheep. Quart J Exptl Physiol 73 781-784.

121. Abbas S.K, Pickard D.W, Illingworth D, Storer J, Purdie D.W, Moniz C, Dixit M, Caple I.W, Ebeling P.R, Rodda C.P, Martin T.J and Care A.D. 1990 Measurement of parathyroid hormone-related protein in extracts of fetal parathyroid glands and placental membranes. J Endocrinol 124 319-325.

122. Senior P.V, Heath D.A and Beck F. 1991 Expression of a parathyroid hormone-related protein mRNA in the rat before birth: demonstration by hybridization histochemistry. J Mol Endocrinol 6 281-290.

123. MacIsaac R.J, Caple I.W, Danks J.A, Diefenbach-Jagger H, Grill V, Moseley J.M, Southby J and Martin T.J. 1991 Ontogeny of parathyroid hormone-related protein in the ovine parathyroid gland. Endocrinology 129 757-764.

124. Moniz C, Burton P.B.J, Malik A.N, Dixit M, Banga J.P, Nicolaides K, Quirke P, Knight D.E and McGregor A.M. 1991 Parathyroid hormone-related peptide in normal fetal development. J Mol Endocrinol 5 259-266.

CHAPTER II CIRCULATING CONCENTRATIONS OF PARATHROID HORMONE-LIKE PEPTIDE IN MALIGNANCY AND IN HYPERPARATHYROIDISM

Preface

Identification in tumors associated with hypercalcemia of a peptide sharing substantial amino-terminal sequence homology with parathyroid hormone appeared to provide some insight into a common paraneoplastic syndrome. However, the frequency with which it was produced by tumors IN VIVO and its exact role in the pathogenesis of malignancy associated hypercalcemia remained to be defined. This chapter describes the development of an amino-terminal radioimmunoassay for parathyroid hormone-like peptide (PLP) and its application to the measurement of circulating concentrations of the peptide in cancer patients and in patients with hyperparathyroidism.

Abstract

We have examined circulating concentrations of a parathyroid hormone-like peptide (PLP) in patients with malignancies and in patients with hyperparathyroidism The radioimmunoassay employed reacts with synthetic amino-terminal fragments of PLP but not with parathyroid hormone. Elevated plasma PLP concentrations were observed in 50% of patients with malignancy and hypercalcemia and in 15% of normocalcemic cancer patients, mean values being higher in the former group. Detectable plasma PLP concentrations were found in 2 of 39 control subjects. In two patients with breast cancer plasma PLP declined concomitant with a reduction in tumor burden Adenocarcinoma of the breast and squamous cell carcinomas were most frequently associated with high plasma PLP levels although a variety of histologic types were represented. The presence of metastases on bone scans did not correlate with either the severity of hypercalcemia or with the extent of PLP elevation. Increased concentrations of plasma PLP were also observed in 4 out of 20 patients with primary hyperparathyroidism and in 5 out of 16 patients with chronic renal failure and secondary hyperparathyroidism Gel filtration analysis of immunoreactive PLP in plasma from two hypercalcemic breast cancer patients revealed heterogeneity, with in each case, both large (greater than 15 Kilodaltons) and small (6-7 Kilodaltons) molecular weight amino-terminal moieties. The results document the presence of PLP in the circulation of patients with cancer and are consistent with a pathogenetic role for PLP in the hypercalcemia of malignancy irrespective of whether skeletal metastases have occurred. PLP may also contribute to the skeletal and/or renal manifestations of hyperparathyroid states.

Introduction

Hypercalcemia is a relatively common cause of morbidity due to malignancies and may complicate virtually all histological types. The concept of a humoral pathogenesis for this complication of malignancy has received considerable study (1-8) and a number of potential factors which might act in either endocrine or paracrine modes have been implicated over the years (9). The necessity to search for a systemic factor arose in part from the association of hypercalcemia with malignancy in the absence of skeletal metastases, although an endocrine mediator was suggested by Albright even in the presence of osteolytic lesions (1). Parathyroid hormone (PTH) per se, in most cases, seemed not to be the pathogenetic agent of the hypercalcemia of malignancy syndrome (10,11). Nevertheless, a biologically related, but immunologically distinct substance appeared to be a reasonable candidate as a mediator (12). Recent studies examining this issue led to the identification in both human neoplasms (13-15) and in a rat model of the human syndrome (16), of a PTH-like peptide (PLP). This material has been cloned and sequenced (16-20) and synthetic fragments prepared. These have been shown to mimic closely the effect of PTH by acting at common renal and osseous receptors (21-27) Nevertheless, the role of this peptide as a pathogenetic factor in the hypercalcemia of malignancy remains unclear. In the present study, we have applied a recently developed radioimmunoassay (RIA) for PLP to the examination of circulating concentrations and for evidence of its association with the hypercalcemia of malignancy syndrome. Furthermore, we have compared circulating concentrations in malignancy with those occuring in hyperparathyroid states.

Subjects and Methods

Subjects

Patients with malignancy seen between March and June 1988 in the inpatient wards of the Royal Victoria Hospital or Montreal General Hospital or in the outpatient services of the Royal Victoria or Jewish General Hospitals were prospectively examined. Those with hypercalcemia (plasma calcium greater than 2.62 mM) included 18 men and 21 women aged 31 to 78 (mean age of 60 years). Normocalcemic cancer patients included 31 men and 43 women aged 31-87 (mean age 58 years). Evidence for metastatic involvement of bone was sought by means of 99^{m}TC-diphosphonate bone scans in 41 of the patients studied. Control subjects, who were all normocalcemic and without evidence of cancer, parathyroid disease or renal disease, included 17 men and 22 women, aged 23 to 75 (mean age 52 years). Patients with primary hyperparathyroidism included 3 men and 17 women, aged 18 to 76 (mean age 58 years) without evidence of renal impairment. All were documented to have adenomas at the time of surgery. 12 men and 4 women (aged 30 to 78, mean 49 years) undergoing chronic hemodialysis at the Royal Victoria Hospital were also studied. The mean duration of hemodialysis in these patients was 4.5 years.

Blood Samples

Blood samples for assay were collected in heparinized (143 units per 10 ml tube) vacutainer tubes (Becton Dickinson) and kept at 4°C for up to 2 hours prior to centrifugation. Plasma was then immediately frozen and stored at -20°C for up to 2 weeks before assay. Samples from patients

2 hours prior to centrifugation. Plasma was then immediately frozen and stored at -20°C for up to 2 weeks before assay. Samples from patients undergoing chronic hemodialysis were taken pre-dialysis.

Analytical Methods

The PLP radioimmunoassay employed an antiserum, 5087/3, at a final dilution of 1:25,000. This antiserum was raised in female New Zealand white rabbits to human (h)PLP-(1-34) which was synthesized using solid phase techniques (Armand Frappier Institute, Montreal, Quebec, Canada) Animals were innoculated at multiple (8-10) sites subcutaneously with a solution containing 1250 μgm of PLP-(1-34), 250 μgm of metBSA, 650 μl of Freunds complete adjuvant and 650 μl of 0.9% NaCl/rabbit for the primary immunization. Animals were given their first boost 6 weeks later with 1300 μl of solution containing one third the peptide and metBSA as the primary immunization and bled via an ear vein ten days later Subsequent boosts were at 4 week intervals, the collection of blood being made 10-12 days later. The antiserum employed for these studies was obtained after the third booster. Synthetic hPLP-(1-34) was also employed as a standard and 0.3 ml aliquots of heparinized patient plasma were assayed in triplicate in a final volume of 0.5 ml containing sodium barbital buffer, pH 8.0. Outdated blood bank plasma was employed as an assay "blank" and to dilute standards. Non-equilibrium incubations were carried out for 5 days at 4°C· antibody added to standards and unknowns on day 1, ^{125}I-labeled [Tyr$^{\bullet}$]hPLP-(1-34) prepared by the lactoperoxidase technique (28) added on day 3 followed by phase separation on day 5 using 1 ml of a mixture of 0 3% dextran (Sigma) and 3% alkaline charcoal (Norit A). The stability of ^{125}I-

labeled PLP during the assay was established as being greater than 93% by incubating 0.3 ml of patient plasma in parallel in the absence of antibody. The interassay coefficient of variation was 19% (n=5) and the intraassay coefficient of variation was 6% (n=10). Synthetic [Tyr°]hPLP-(1-34) and [Tyr34]hPLP-(1-34) (Peninsula Laboratories Belmont, CA), and synthetic hPTH-(1-84) and hPTH-(1-34) (Bachem Fine Chemicals Incorporated, Torrance, CA) were used for determination of radioimmunoassay characteristics.

The PTH assay was a two-site, immunoradiometric assay measuring "intact" PTH-(1-84) (29) (Nichols Institute, San Juan, Capistrano, CA). One antiserum binds the 1-34 sequence of hPTH and the other is specific for the 39-84 portion of the hPTH molecule.

Plasma calcium levels were determined by atomic absorption spectrophotometry (Perkin Elmer Model 703). Serum creatinine, blood urea nitrogen, albumin, total protein and gamma glutamyl transpeptidase (GGT) were determined by autoanalyzer (Technicon Laboratories, New York, NY). Serum carcinoembryonic antigen (CEA) was determined by ELISA assay (Boehringer Mannheim).

Gel Filtration Chromatography

Gel filtration was performed as previously described (30) with Bio Gel P-100 (100-200 mesh) on 1.2 x 40 cm columns at 4°C eluting with a buffer of 0 1M ammonium acetate, pH5.0. Fractions (1 ml) were collected, lyophilized, reconstituted with 300 µl of OBBP and radioimmunoassayed for PLP The column void volume and salt volume were determined with blue dextran and $Na^{125}I$ respectively. Iodinated synthetic hPTH(1-84), hPTH(7-

84), hPTH(1-34) and human calcitonin (CT) with molecular weights of 9500, 8800, 4100 and 3400 respectively (Bachem Fine Chemicals Incoporated) were employed for calibration.

Statistical Analysis

Data are expressed as mean ± SEM and statistical comparisons were based on the Student's t-test.

Results

Characteristics of the Radioimmunoassay

Increasing concentrations of synthetic hPLP-(1-34) and of synthetic [Tyr°]hPLP-(1-34) produced a dose-dependent and equivalent inhibition of binding of ^{125}I-labeled [Tyr°]hPLP-(1-34) to the antiserum (Fig. 1) Serial dilutions of plasma from a patient with malignancy (breast) and hypercalcemia also produced parallel inhibition of binding. However, the synthetic analogue [Tyr^{34}]hPLP(1-34) was only 5% as potent as hPLP-(1-34) in inhibiting binding, suggesting that an important epitope was present in the carboxyl end of the molecule Neither hPTH-(1-34) nor hPTH-(1-84) cross-reacted when added up to 2.5 μgm/tube demonstrating the specificity of the assay for immunoreactive PLP.

Plasma PLP Concentrations in Malignancy and in Parathyroid Disorders

Plasma concentrations of PLP, PTH and calcium were measured concomitantly in samples from 39 hypercalcaemic cancer patients, 74

normocalcaemic cancer patients, 20 patients with primary hyperparathyroidism and 16 patients with chronic renal failure and secondary hyperparathyroidism (Fig. 2). Mean levels of PLP were highest in patients with malignancy and hypercalcemia but were also elevated in 4 out of 20 patients with primary hyperparathyroidism and in 5 out of 16 patients with secondary hyperparathyroidism. Only 2 out of 39 normocalcemic controls had detectable levels (Fig. 2). Mean PTH levels were below the mean normal level in patients with malignancy and hypercalcemia, were indistinguishable from normal in patients with malignancy but without hypercalcemia, and were elevated in primary and secondary hyperparathyroidism.

Patients harbouring a variety of different malignancies were examined (Table 1). Elevated plasma PLP concentrations were found in association with hypercalcemia in all pathologic types studied but were most frequent with adenocarcinoma of the breast and squamous cell carcinoma. In the absence of hypercalcemia, elevated plasma PLP concentrations were less often observed in patients with each tumour type but were still commonly seen in association with breast cancer (Table 2)

Bone scans were obtained in 41 patients with malignancies of various types (Table 3). In this subset, PLP concentrations in hypercalcemic patients were again significantly higher than were PLP concentrations in normocalcemic patients (Table 4). Neither serum calcium values nor PLP concentrations were significantly different between patients with and without evidence of skeletal metastases in either hypercalcemic or normocalcemic sub-groups.

In one patient with squamous carcinoma of the ethmoid sinus

reduction of serum calcium with saline hydration, furosemide and mithramycin was unaccompanied by a significant alteration in PLP concentration (Fig. 3A). In two patients with breast adenocarcinoma PLP concentrations fell concomitant with tumour cytoreducti n and serum calcium concentrations declined in parallel (Fig. 3B and 3C).

Heterogeneity of Circulating Immunoreactive PLP

The pattern of circulating immunoreactive PLP in the plasma of two patients with breast adenocarcinoma and hypercalcemia was examined using gel-filtration analysis (Fig. 4). The predominant immunoreactive form in both cases eluted close to the void volume. The molecular weight of this moiety (A), based on its Kd, was estimated as greater than 15,000 daltons. Additional small molecular weight immunoreactivity (B) of approximately 6-7000 daltons was observed in each case.

Discussion

Previous studies have documented PLP production by malignancies and have demonstrated PLP in conditioned medium from tumors maintained in cell culture. Our studies provide direct evidence for the presence of PLP in the circulation of patients with malignancy, the highest concentrations being measured in those patients with hypercalcemia. Studies in which marked decreases in circulating PLP occurred as tumor mass was reduced by chemotherapy provided evidence in support of the tumor-derived origin of this material. Reductions in serum calcium by means other than anti-neoplastic therapy produced no significant change in plasma PLP Inasmuch as only two control subjects had detectable PLP with this immunoassay, the

majority of normal individuals must have circulating levels, if any, clearly below 0.15 ng/ml. The inability to detect circulating concentrations in the vast majority of control subjects does not diminish the value of the PLP immunoassay as a tumor marker but, in fact, may facilitate the interpretation of easily detectable concentrations as abnormal Indeed, immunoreactive calcitonin gained widespread utility as a clinical tumor marker for medullary thyroid carcinoma (31) even before suitable radioimmunoassays capable of definitively measuring normal concentrations were developed. As with calcitonin, the presence of an elevated PLP level must be interpreted in conjunction with the clinical setting, since both peptides may be elevated in malignant or non-malignant disorders A more sensitive radioimmunoassay might demonstrate elevated PLP concentrations in a larger percentage of patients with cancer and also more readily detect PLP levels in normal subjects. If PLP functions physiologically as a growth and/or differentiation factor with an autocrine or paracrine mode of action (14,16,32) mean normal circulating levels would remain undetectable even with a highly sensitive assay.

In previous studies employing a cytochemical bioassay for PTH (12) we and others (33) documented the presence of elevated PTH-like bioactivity in the circulation of patients with malignancy and hypercalcemia. The present study confirms and extends those results using a specific radioimmunoassay based on the known amino acid sequence of PLP. Our gel chromatographic data indicate that heterogeneity of circulating PLP occurs Analyses of cloned cDNAs (17-20) and of the gene encoding PLP (34,35) have predicted the existence of molecular PLP forms of 139, 141 and 173 amino acids, each being biosynthesized as a precursor extended at

the amino-terminus. The major immunoreactive material in our studies was found to be of high molecular weight, consistent with the presence of one or more of these species, or precursor forms, and/or of protein-bound moieties in the circulation. The aim of the present gel filtration studies was not to precisely identify this high molecular weight material but to determine whether heterogeneity of amino-terminal immunoreactive PLP occurs in the circulation. In the present studies smaller molecular weight amino-terminal immunoreactivity was also detected in the plasma of patients with malignancy. Small molecular weight entities with PTH-like bioactivity have previously been identified in tumor tissue extracts (8) and in conditioned medium of cultured tumor cells (15) Furthermore, multiple circulating forms, including some of small molecular weight, were observed by us in our earlier analyses with the cytochemical bioassay (12). The heterogeneity observed in those studies was somewhat greater perhaps due to the higher sensitivity of the bioassay In view of the fact that structure-function studies have demonstrated that PTH-like bioactivity resides in amino terminal fragments (21-26), both large and small molecular weight immunoreactive material may contribute to the sum of circulating bioactivity but with differing potencies. The precise chemical nature of the circulating amino-terminal PLP forms and their biopotencies remains to be elucidated in future studies.

Despite the fact that the concept of a PTH-like endocrine mediator of malignancy-associated hypercalcemia has classically been associated with squamous cell and renal cell carcinomas our studies demonstrate the association of PLP with a variety of cancer types, most frequently with breast carcinoma. Indeed, one of the first specimens of purified PLP

suitable for sequencing was extracted from a breast cancer (14). Our results are consistent with reports of increased urinary cyclic AMP excretion (2,3) and abnormalities in renal calcium and phosphate handling (7) in patients with carcinoma of the breast and hypercalcemia, suggesting the presence of a humoral factor with activity similar to that of PTH. Furthermore, our results extend the recent finding of PLP production by hyperplastic (lactating) mammary tissue (36) to PLP over-production by neoplastic mammary tissue. Hypercalcemia was shown to correlate with PLP concentration whereas the presence or absence of skeletal metastases did not. These findings are in agreement with previous reports stating that hypercalcemia was more likely due to tumor production of a humoral factor with PTH-like activity with respect to bone resorption, renal phosphate threshold and renal calcium handling than to focal skeletal metastases (4,6). Consequently, PLP as a pathogenetic factor in maligancy-induced hypercalcemia may be more widespread than previously anticipated, and endocrine mechanisms associated with increased PLP may contribute to the development of hypercalcemia even when localized skeletal resorption occurs.

Reductions in circulating PLP commensurate with chemotherapy correlated with a decline in serum calcium in two patients with breast cancer and hypercalcemia. These observations, taken together with previous evidence of the capacity of synthetic fragments and analogues of PLP to increase serum calcium in animals via skeletal and renal mechanisms (21-25) and the reported capacity of injected antiserum raised against PLP to partially reduce tumor induced hypercalcemia in nude mice (37), all point to a major role for PLP in the pathogenesis of malignancy-induced

hypercalcemia. Nevertheless, in our current study, a number of cases of malignancy were associated with elevated PLP concentrations even in the absence of hypercalcemia. These results are consistent with previous findings of elevated nephrogenous cyclic AMP excretion in patients with malignancies in the absence of hypercalcemia (3,38) Consequently counter-regulatory mechanisms may maintain normocalcemia in these patients but be absent or insufficient in those patients developing hypercalcemia. Alternatively, increased circulating immunoreactive forms of lesser bio-potency may occur in plasma of cancer patients who remain normocalcemic Gel filtration analysis of molecular forms of PLP in the plasma of cancer patients who are normocalcemic will be required for comparison with profiles seen in the presence of hypercalcemia to clarify this issue. Finally, increased circulating PLP may contribute to, but not be sufficient for, induction of hypercalcemia. Thus, a variety of cytokines and growth factors have been implicated in the development of hypercalcemia associated with malignancy (9) and our studies do not exclude a role for these agents as part of a multifactorial pathogenetic pathway. PLP concentrations were found to be augmented in several patients with primary hyperparathyroidism due to adenomas, and in some cases of chronic renal failure with secondary hyperparathyroidism. Recent studies have documented the presence of mRNA encoding PLP in parathyroid adenomas (39), and our current findings suggest that such adenomas may co-secrete both PLP and PTH. The occasional presence of a second circulating hypercalcemic agent in primary hyperparathyroidism may explain the relatively low concentrations of immunoreactive plasma PTH sometimes measured in this disorder. This emphasizes the need to consider

concentrations of both peptides in hyperparathyroid states especially inasmuch as our previous studies in animals indicated that IN VIVO, interaction of these two peptides at receptor sites may occur (23). Finally, the biological consequences of elevated PLP in uremia remain to be determined. The observation that only a sub-set of functionally anephric patients manifested increased circulating PLP concentrations indicates that increased production rather than simply diminished renal clearance was the predominant determinant of those elevated levels. Having now established that amino-termin.i PLP circulates in some patients with renal failure, the possibility of this factor contributing to renal osteodystrophy appears to be a fruitful area for further exploration.

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References

1. Case Records of the Massachusetts General Hospital (Case 27461) 1941 N Engl J Med 225 789-791.

2. Shaw JW, Oldham SB, Rosoff L, Bethune JE, Fichman MP 1977 Urinary cyclic AMP analyzed as a function of the serum calcium and parathyroid hormone in the differential diagnosis of hypercalcemia J Clin Invest 59 14-21.

3. Rude RK, Sharp CF Jr , Fredericks RS, Oldham SB, Elbaum N, Link J, Irwin L, Singer FR 1981 Urinary and nephrogenous adenosine 3',5'-monophosphate in the hypercalcemia of malignancy J Clin Endo Metab 52 765-771.

4. Ralston SH, Fogelman I, Gardner MD, Boyle IT 1982 Hypercalcemia and metastatic bone disease· is there a causal link? Lancet 5 903-905.

5. Stewart AF, Horst R, Deftos LJ, Cadman EC, Lang R, Broadus AE 1980 Biochemical evaluation of patients with cancer-associated hypercalcemia. Evidence for humoral and non-humoral groups N Engl J Med 303 1377-1383.

6. Ralston SH, Fogelman I, Gardner MD, Boyle IT 1984 Relative contribution of humoral and metastatic factors to the pathogenesis of hypercalcemia in malignancy Br Med J 288 1405-1408.

7. Percival RC, Yates AJP, Gray RES, Galloway J, Rogers K, Neal FE, Kanis JA 1985 Mechanisms of malignant hypercalcemia in carcinoma of the breast. Br Med J 289 812-813.

8. Rabbani SA, Mitchell J, Roy DR, Kremer R, Bennett HPJ, Goltzman D 1986 Purification of peptides with parathyroid hormone-like bioactivity from human and rat malignancies associated with hypercalcemia. Endocrinol 118 1200-1210

9. Mundy GR 1988 Hypercalcemia of malignancy revisited. J Clin Invest 82 1-6.

10. Powell D, Singer FR, Murray TM, Minkin C, Potts JT Jr 1973 Nonparathyroid hypercalcemia in patients with neoplastic diseases N Engl J Med 289 176-181.

11. Simpson EL, Mundy GR, D'Souza, SM, Ibbotson KJ, Bockman MD, and Jacobs JW 1983 Absence of parathyroid hormone messenger RNA in nonparathyroid tumors associated with hypercalcemia. N Engl J Med 309 325-330.

12. Goltzman D, Stewart AF, Broadus AE 1981 Malignancy-associated hypercalcemia: evaluation with a cytochemical bioassay for parathyroid hormone. J Clin Endocrinol Metab 53 899-904.

13. Moseley JM, Kubota M, Diefenbach-Jagger H, Wettenhall REH, Kemp BE, Suva LJ, et al 1987 Parathyroid hormone-related protein purified from a human lung cancer cell line. Proc Natl Acad Sci (USA) 84 5048-5052.

14. Stewart AF, Wu T, Goumas D, Burtis WJ, Broadus AE 1987 N-terminal amino acid sequence of two novel tumor-derived adenylate cyclase-stimulating proteins: identification of parathyroid hormone-like and parathyroid hormone-unlike domains. Biochem Biophys Res Commun 146 672-678

15. Strewler GJ, Stern PH, Jacobs JW, Eveloff J, Klein RF, Leung SC, et al 1987 Parathyroid hormone-like protein from human renal carcinoma cells. Structural and functional homology with parathyroid hormone. J Clin Invest 80 1803-1807.

16 Yasuda T, Banville D, Rabbani SA, Hendy GN, Goltzman D 1989 Rat parathyroid hormone-like peptide: Comparison with the human homologue and expression in malignant and normal tissue. Molec Endocrinol. In Press.

17. Suva LJ, Winslow GAA, Wettenhall REH, Hammonds RG, Moseley JM, Diefenbach-Jagger H, et al 1987 A parathyroid hormone-related protein implicated in malignant hypercalcemia: Cloning and expression. Science 237 893-896.

18. Mangin M, Webb AC, Dreyer BE, Posillico JT, Ikeda K, Weir EC, et al 1988 Identification of a cDNA encoding a parathyroid hormone-like peptide from a human tumor associated with humoral hypercalcemia of malignancy. Proc Natl Acad Sci USA 85 597-601.

19. Thiede MA, Strewler GJ, Nissenson RA, Rosenblatt M, Rodan GA 1988 Human renal carcinoma expresses two messages encoding a parathyroid hormone-like peptide: Evidence for the alternative splicing of a single-copy gene. Proc Natl Acad Sci USA 85 4605-4609.

20. Mangin M, Ikeda K, Dreyer BE, Milstone L and Broadus AE. 1988 Two distinct tumor-derived parathyroid hormone-like peptides result from alternantive ribonucleic acid splicing. Mol Endocrinol 1988 2 1049-1055.

21. Horiuchi N, Caulfield MP, Fisher JE, Goldman ME, Mc Kee RL, Reagan JE, et al 1987 Similarity of synthetic peptide from human tumor to parathyroid hormone *in vivo* and *in vitro*. Science 238 1566-1568.

22. Kemp BE, Moseley JM, Rodda CP, Ebeling PR, Wettenhall REH, Stapleton D, et al 1987 Parathyroid hormone-related protein of malignancy: Active synthetic fragments. Science 238 1568-1569.

23. Rabbani SA, Mitchell J, Roy DR, Hendy GN, Goltzman D 1988 Influence of the amino-terminus on in vitro and in vivo biological activity of synthetic parathyroid hormone-like peptides of malignancy. Endocrinology 123 2709-2716.

24. Stewart AF, Mangin M, Wu T, Goumas D, Insogna KL, Burtis WJ, et al 1988 Synthetic human parathyroid hormone-like protein stimulates bone resorption and causes hypercalcemia in rats. J Clin Invest 81 596-600.

25. Yates AJP, Gutierrez GE, Smolens P, Travis PS, Katz MS, Aufdemorte TB, et al 1988 Effects of a synthetic peptide of a parathyroid hormone-related protein on calcium homeostasis, renal tubular calcium reabsorption, and bone metabolism in vivo and in vitro in rodents. J Clin Invest 81 932-938.

26. Rodan SB, Noda M, Wesolowski G, Rosenblatt M, Rodan GA 1988 Comparison of postreceptor effects of 1-34 human hypercalcemia factor and 1-34 human parathyroid hormone in rat osteosarcoma cells. J Clin Invest 81 924-927

27. Jueppner H, Abou-Samra AB, Uneno S, Gu WX, Potts JT Jr , Segre GV 1988 The parathyroid hormone-like peptide associated with humoral hypercalcemia of malignancy and parathyroid hormone bind to the same receptor on the plasma membrane of ROS 17/2.8 cells. J of Biol Chem 26 8557-8560

28. DeMay M, Mitchell J, Goltzman D 1985 Comparison of the binding of parathyroid hormone and its amino-terminal fragments to renal and osseous receptors. Am J Physiol E437-443

29. Nussbaum SR, Zahnadnik RJ, Lavigne JR, Brennan GL, Nozawa-Ung K, Kim LY, et al 1987 A highly sensitive two-site immunoradiometric assay of parathyrin (PTH) and its clinical utility in evaluating patients with hypercalcemia. Clin Chem 33 1364-1368

30. Goltzman D, Bennett H.P.J, Koutsilieris M, Mitchell J, Rabbani S.A, Rouleau M. 1986 Studies of the multiple molecular forms of bioactive parathyroid hormone and parathyroid hormone-like substances. Recent Prog Horm Res 42 665-703

31. Goltzman D, Potts JT Jr, Ridgway C, Maloof F 1974 Calcitonin as a tumor marker: use of the radioimmunoassay for calcitonin in the post-operative evaluation of patients with medullary thyrold carcinoma. N Engl J Med 290 1035-1039.

32. Insogna KL, Stewart AF, Morris CA, Hough LM, Milstone LM, Centrella M 1989 Native and synthetic analogue of the malignancy-associated parathyroid hormone-like protein have in vitro transforming growth factor-like activity J Clin Invest 83 1057-1060

33. Loveridge N, Kent GN, Heath DA, Jones EL 1985 Parathyroid hormone bioactivity in a patient with severe osteitis fibrosa cystica due to malignancy· renotropic actions of a tumour extract as assessèd by cytochemical bioassay. Clin Endocrinol 22 135-146.

34. Mangin M, Ikeda K, Dreyer BE, Broadus AE 1989 Isolation and characterization of the human parathyroid hormone-like peptide gene. Proc Natl Acad Sci (USA) 86 2408-2412.

35. Yasuda T, Banville D, Hendy GN, Goltzman D 1989 Characterization of the human parathyroid hormone-like peptide gene: functional and evolutionary aspects J Biol Chem In Press.

36. Thiede MA, Rodan GA 1988 Expression of a calcium-mobilizing parathyroid hormone-like peptide in lactating mammary tissue. Science 242 278-280.

37. Kukreja SC, Shevrin DH, Wimbiscus SA, Ebeling PR, Danks JA, Rodd CP, et al 1988 Antibodies to parathyroid hormone-related protein lower serum calcium in athymic mouse models of malignancy-associated hypercalcemia due to human tumors. J Clin Invest 82 1798-1802.

38 Krukeja SH, Shemerdiak WP, Lad TE, Johnson PA 1980 Elevated nephrogenous cyclic AMP with normal serum parathyroid hormone levels in patients with lung cancer. J Clin Endo Metab 51 167-169

39 Ikeda K, Weir EC, Mangin M, Dannies PS, Kinder B, Deftos LJ, et al 1988 Expression of messenger ribo· ·cleic acids encoding a parathyroid hormone-like peptide in ɔrmal human and animal tissues with abnormal expression in hum , arathyroid adenomas. Mol Endocrinol 2 1230- 1236.

Table 1. Distribution of PLP according to cancer type.

Type	Number	Number with ↑ PLP	%
Breast	31	12	39
Squamous[a]	20	6	30
Renal cell	1	1	100
Colo-rectal	10	3	30
Lung (non-squamous)[b]	9	1	11
Hematologic[c]	30	4	13
Miscellaneous[d]	11	3	27
Total	112	30	27

a Includes squamous cell carcinoma of head and neck (11), lung (3), esophagus (1) and cervix (5).

b Includes small (6) and non-small cell (3) types.

c Includes B-cell (16), T-cell (2) and unclassified (3) lymphomas, chronic leukemias (2) and myelomas (7).

d Includes pancreatic (1), ovarian (1), medullary thyroid (1), melanoma (2), neural (2) and primary site unknown (4).

Table 2. Distribution of elevated PLP in cancer types with elevated or normal calcium

Type	Hypercalcemic			Normocalcemic		
	Occurrence		PLP ng eq/ml mean±SEM	Occurrence		PLP ng eq/ml mean±SEM
	cases	%		cases	%	
Breast	6/10	60	0.61±0.17	6/21	29	0.39±0.08
Squamous	5/10	50	1.05±0.43	1/10	10	0.25±0.05
Renal cell	1/1	100	0.83	-	-	-
Colo-rectal	2/5	40	0.61±0.25	1/5	20	0.33±0.11
Lung(non-squamous)	1/3	33	0.83±0.52	0/6	0	-
Hematologic	2/6	33	0.42±0.16	2/24	8	0.33±0.09
Miscellaneous	2/3	67	1.59±1.05	1/8	13	0.43±0.21

Table 3. Tumor types in patients with bone scans

Tumor Type	Metastases	No Metastases
Breast	13	7
Squamous[a]	3	5
Renal cell	0	1
Lung (non-squamous)[b]	0	3
Hematologic[c]	6	3
Total	22	19

a Includes squamous cell carcinomas of the head and neck (6), lung (1) and esophagus (1)

b Includes small (2) and non-small cell (1) types

c Includes multiple myeloma (6) and lymphoma (3)

Table 4. PLP values and calcium values in patients with bone scans

	Hypercalcemic		Normocalcemic	
	Serum calcium mM/L mean±SEM	Plasma PLP ng eq/ml mean±SEM	Serum calcium mM/L mean±SEM	Plasma PLP ng eq/ml mean±SEM
All scans	3.14±0.16	0.91±0.29	2.24±0.03	0.33±0.07[a]
Positive scans	3.06±0.17	1.04±0.56	2.24±0.05	0.25±0.04
Negative scans	3.21±0.26	0.79±0.29	2.24±0.05	0.41±0.13
p[b]	NS	NS	NS	NS

[a] Significantly different from plasma PLP in hypercalcemic patients $p < 0.05$

[b] p values for differences between groups with positive compared with negative scans

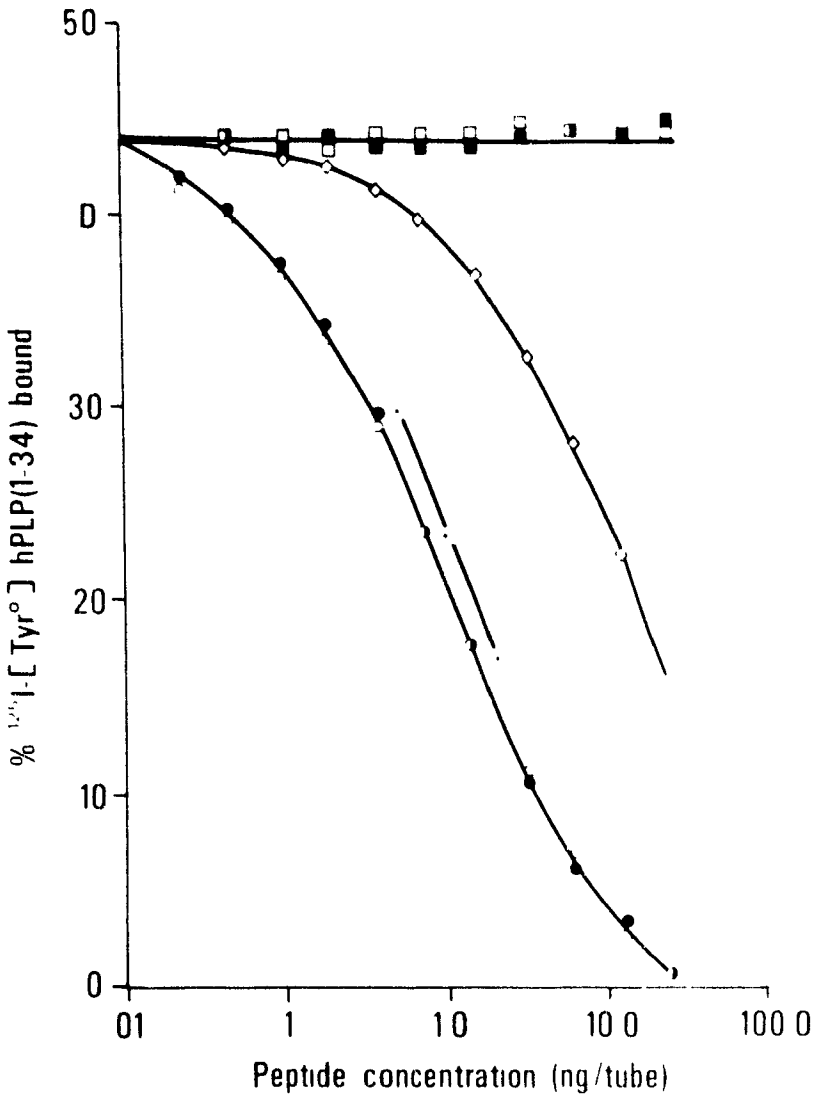

Figure 1. Inhibition of binding of ^{125}I-[Tyr°]hPLP1-34) to a rabbit antiserum raised against hPLP(1-34) by hPLP(1-34)(●), [Tyr°]hPLP(1-34) (○), [Tyr34] hPLP(1-34)(◇), hPTH(1-34)(■), and hPTH(1-84) (□) Also depicted is inhibition of binding by 300 ul, 150 ul and 75 ul of plasma from a patient with adenocarcinoma of the breast and hypercalcemia ( ) Each point is the mean of three individual determinations which varied by less than 7 percent from the mean. D represents the detection limit of the assay which was 0.15 ng eq PLP-(1-34)/ml. Details of the radioimmunoassay are provided in Methods.

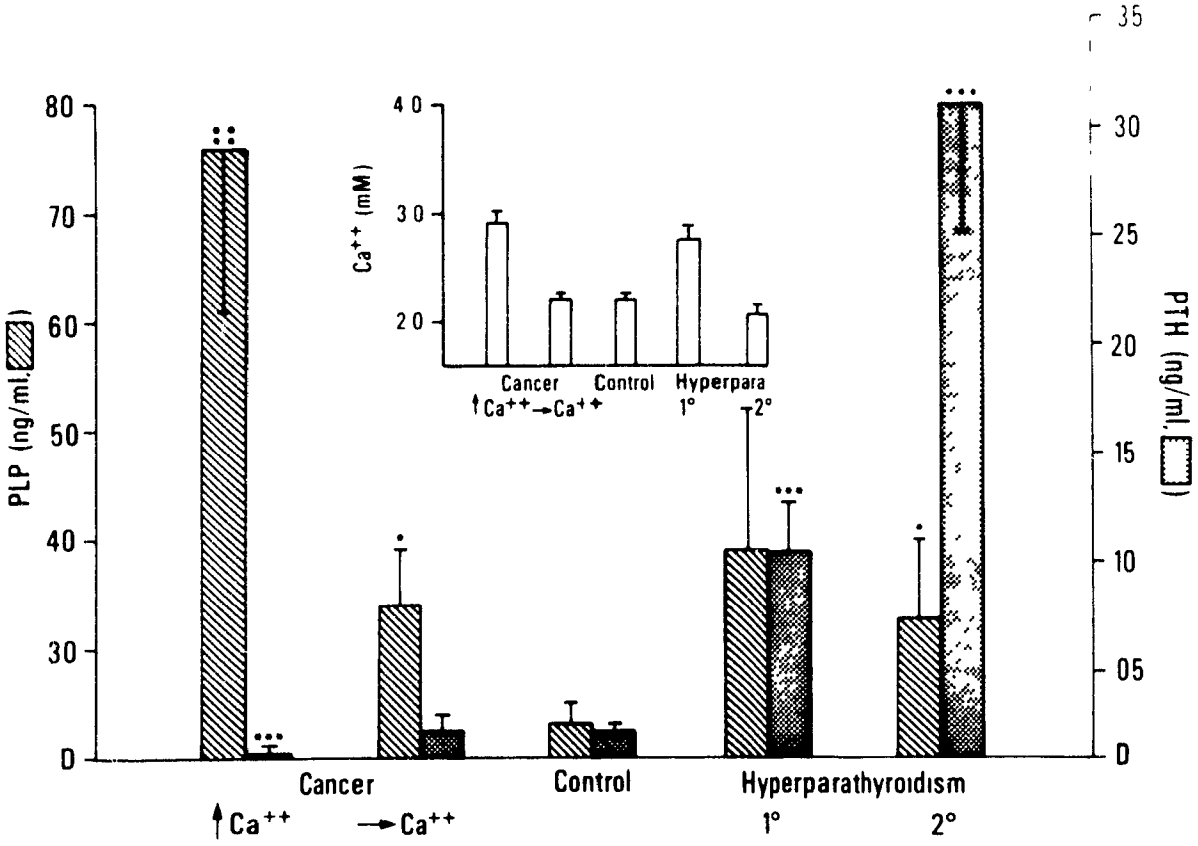

Figure 2. Concentrations of immunoreactive (i)PLP(▨) and of iPTH(▩) in the plasma of control subjects and in patients with cancer and hypercalcemia (↑Ca++), cancer and normocalcemia (→Ca++), primary (1°) hyperparathyroidism (HPT) and secondary (2°) HPT. Samples with an undetectable level were assigned a value equal to the detection limit of the assay (D) Each bar represents the mean ± SEM of concentrations in each group. Total plasma calcium (Ca++) in the same groups is shown in the inset. PLP, PTH and calcium values were determined as described in Methods Serum albumin and total protein values were within normal limits in each subject in whom plasma calcium was determined. Significant difference from control * $p<0.05$ ** $p<0.01$ and *** $p<0.001$. Significant difference from $\rightarrow Ca^{++}$ •• $p<0.02$. Calcium levels were significantly higher than control in $\uparrow Ca^{++}$ and 1° HPT, were not different from control in $\rightarrow Ca^{++}$, and were significantly lower than control in 2° HPT.

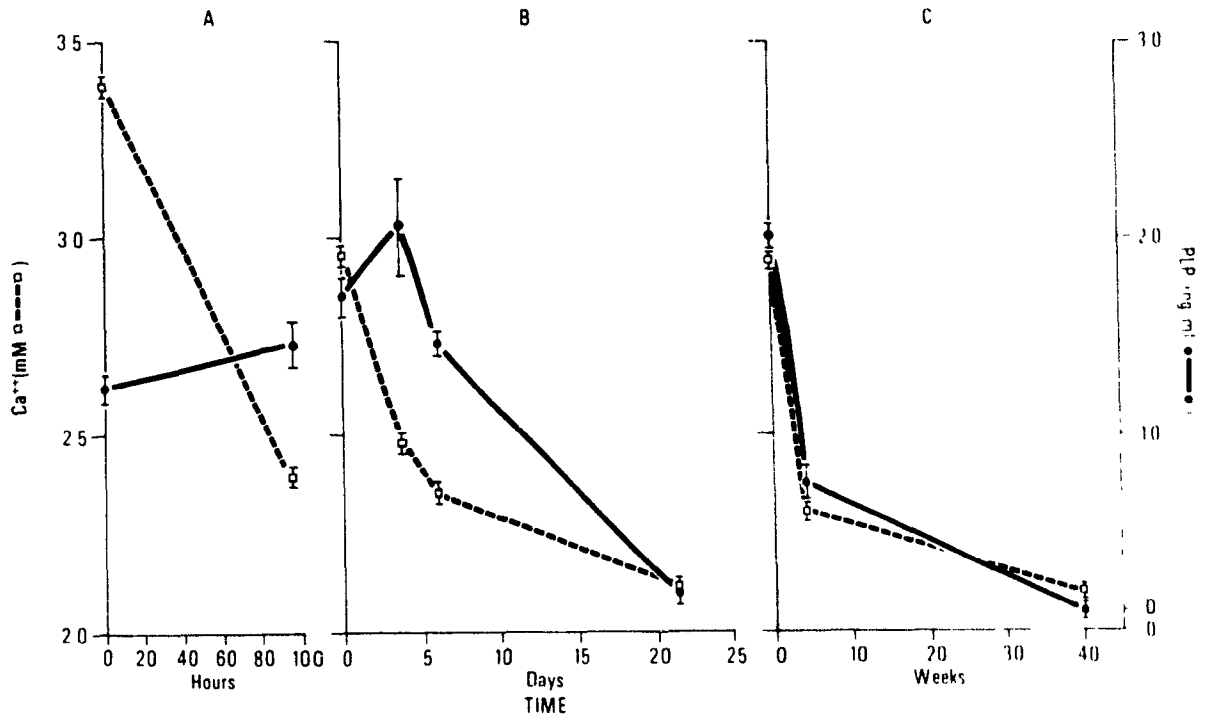

Figure 3. Plasma iPLP(●) and calcium(□) concentrations in a patient with squamous cell carcinoma of the ethmoid sinus treated only with saline, furosemide and mithramycin for control of hypercalcemia **(panel A)** and in two patients with breast adenocarcinoma metastatic to the liver **(panels B and C)** treated with chemotherapy Each value is the mean ± SEM of triplicate determinations In patient B, serum CEA fell from 901 ng/ml to 33 ng/ml and serum GGT from 1566 SI units/L to 253 SI units/L during three weeks of intermittent mitomycin C and mitoxantrone chemotherapy In patient C, serum CEA fell from 541 ng/ml to 9.5 ng/ml and serum GGT from 327 SI units/L to 22 SI units/L during forty weeks of intermittent courses of cyclophosphamide, adriamycin and 5-fluorouracil therapy Both patients receiving chemotherapy, therefore, displayed substantial biochemical evidence of a reduction in tumour mass concomitant with decreases in plasma calcium and plasma PLP

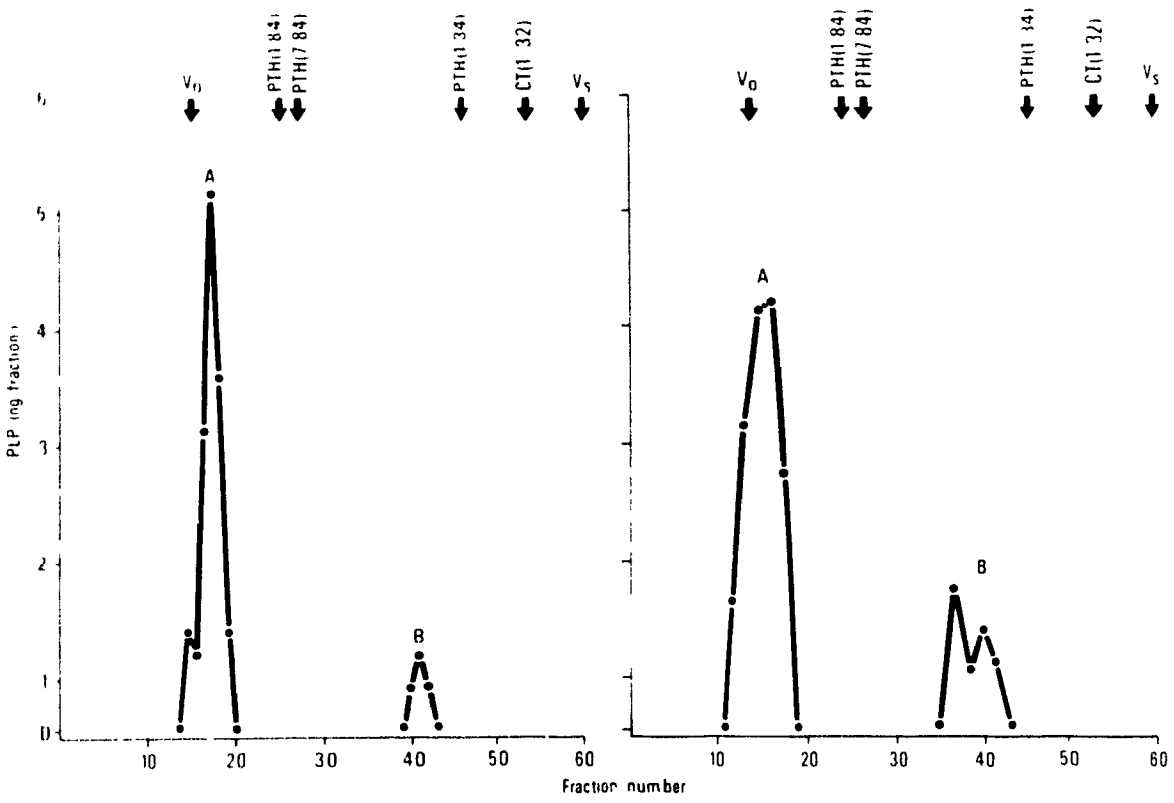

Figure 4. Gel filtration profiles of immunoreactive PLP after chromatography on Bio-Gel P-100 of 3.0 ml of plasma from each of two patients with breast carcinoma and hypercalcemia Vertical arrows from left to right denote respectively the elution position of the void volume (Vo), hPTH (1-84), hPTH(7-84), hPTH (1-34), hCT(1-32) and the salt volume (Vs).

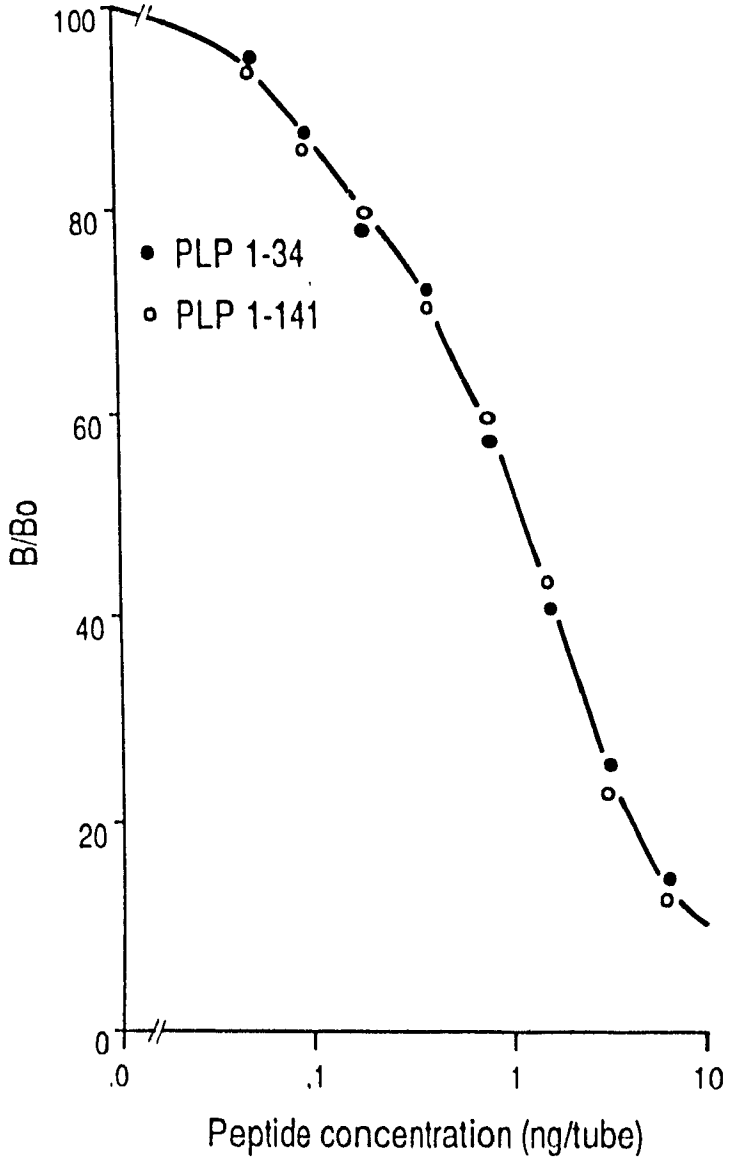

Figure 1. Inhibition of binding of ^{125}I-[Tyr°]hPLP1-34) to a rabbit antiserum raised against hPLP(1-34) by hPLP(1-34)(●) and recombinant hPLP(1-141) (○). Each point is the mean of three individual determinations which varied by less than 7 percent from the mean. Binding is expressed as a percent of total specific binding (Bo).

CHAPTER III EFFECTS OF PASSIVE IMMUNIZATION AGAINST PARATHYROID HORMONE-LIKE PEPTIDE AND PARATHYROID HORMONE IN HYPERCALCEMIC TUMOR-BEARING RATS AND IN NORMOCALCEMIC CONTROLS

Preface

The preceeding work examining circulating concentrations of PLP in patients with neoplasia provided strong evidence for a pathogenetic role for this peptide in malignancy associated hypercalcemia. In addition, identification of elevated levels of PLP in some patients with hyperparathyroidism suggested co-secretion of PLP and PTH from the parathyroid gland. In Chapter III, I present data from passive immunization experiments which were designed to assess and compare the relative roles played by PLP and PTH in the mineral metabolism of both normal rats and rats with malignancy associated hypercalcemia.

Abstract

Passive immunization using antisera raised against rat parathyroid hormone-like peptide (rPLP)(1-34) and rat parathyroid hormone (rPTH)(1-84) was used to assess and compare the roles played by PLP and PTH in modulating mineral metabolism in hypercalcemic rats bearing the Rice-500 Leydig cell tumor and in normocalcemic control animals. Treatment of tumor-bearing rats with antiserum to PLP resulted in a rapid and sustained reversal of their biochemical abnormalities which was associated with an increase in longevity. Reduction of plasma calcium was shown to be a function of early (5 h) neutralization of PLP bioactivity in the kidney whereas neutralizing effects in bone appeared later (24-48 h) and were more prolonged. Immunization of normal animals with antiserum to PTH resulted in a hypocalcemic episode of smaller magnitude and shorter duration compared with that achieved in hypercalcemic animals immunized against PLP and appeared to be unassociated with neutralization of distal tubular effects. Neutralization of proximal tubular and skeletal actions of PTH appeared to occur in a manner analagous to that seen for PLP in tumor-bearing animals but were of shorter duration. These studies suggest that in normal animals the action of PTH in the skeleton and/or on vitamin D metabolism contributes considerably more than renal transport activity to the maintenance of normocalcemia and that compensatory mechanisms rapidly restore homeostasis. PTH appears to be the major modulator of calcium homeostasis in normal rats with PLP playing a minor role, if any. In tumor-bearing rats, mechanisms to restore calcium levels to baseline are delayed after PLP immunoneutralization and PLP appears to be both necessary and sufficient for the hypercalcemic state

Introduction

Although a number of animal models are available for investigating the pathogenesis of malignancy-associated hypercalcemia, the male Fischer rat implanted with the Rice-500 Leydig cell tumor is probably the best characterized (1,2) and most closely resembles the human disorder (3,4). Hypercalcemia and hypophosphatemia are associated with changes in the renal handling of calcium, phosphorus and cAMP (5) and in bone there is increased resorption and decreased formation (2,6). A parathyroid hormone-like peptide (PLP) recently isolated, cloned and sequenced from human neoplasms associated with hypercalcemia (7-9) was subsequently cloned and sequenced in the rat using the Rice-500 tumor (10). The human and rat peptides are virtually identical from residue 1-113 and share 62% sequence homology with human parathyroid hormone (hPTH) in the first 13 amino acids. These peptides appear to be products of a gene family (11,12). Synthetic amino-terminal fragments of PLP, although immunologically distinct from PTH (13-15), have been shown to mimic PTH-like bioactivity both IN VIVO and IN VITRO (16-19), suggesting a role for the peptide in the pathogenesis of the hypercalcemic syndrome in this rat model. Peptides, such as transforming growth factors, have also been identified as possible contributory agents in the pathogenesis of the hypercalcemia associated with the Leydig tumor model (20,21). Previous studies using nude mice implanted with human tumors associated with hypercalcemia (13,22-24) have demonstrated the presence of PTH-like bioactivity. Treatment of those mice with antiserum against PLP transiently reduced the serum calcium levels in some animals (13). Consequently, although those studies demonstrated that PLP was of major importance in the pathogenesis

of malignancy-associated hypercalcemia, they may not have excluded the presence of other contributory factors in the hypercalcemia associated with that model.

In the present study we have used the hypercalcemic, Leydig tumor-bearing rat as well as normocalcemic, non-tumor-bearing rats to evaluate the effect of passive immunization on biochemical indices of mineral metabolism. Antisera raised against synthetic rPLP(1-34) and synthetic rPTH(1-84) were employed to examine and compare the roles played by endogenous concentrations of PLP and PTH as regulatory factors.

Materials and Methods

Antisera

Polyclonal antiserum (5084/4) to PLP (anti-PLP) was raised in New Zealand white rabbits to synthetic rPLP(1-34) (Armand Frappier Institute, Montreal, QC, Canada). Animals were immunized at multiple (8-10) sites, subcutaneously at four week intervals and bled ten days after each immunization. The antiserum employed was obtained after the fourth boost.

Polyclonal antiserum (R51) to PTH (anti-PTH) was raised in New Zealand white rabbits to synthetic rPTH(1-84) (Bachem Inc., Torrance, CA) using the same protocol as for PLP. Antiserum obtained on the second boost was employed for this study.

Normal rabbit serum (NRS) was obtained prior to commencement of the PLP immunization protocol. Characterization of binding of the two antisera were determined by radioimmunoassay as previously described (15). In brief, increasing concentrations of rPLP(1-34) and rPTH(1-34) were

incubated with either antiserum 5084/4 (final dilution 1 0,000) and ^{125}I-labeled [Tyr^{0}] rPLP(1-34) as tracer or with antiserum R51 (final dilution 1:18,000) and ^{125}I-labeled rPTH(1-84) as tracer. Phase separation was performed with dextran-charcoal (15).

Intact Cell Adenylate Cyclase

Adenylate cyclase assays were performed using UMR 106 cells as described previously (17). Confluent cells in 24 well cluster plates were incubated for 2 h with 0.5 uCi/well [^{3}H] adenine, washed twice with Hank's Balanced Salt Solution and incubated with peptides pre-treated for 45 min at room temperature with normal rabbit serum or antiserum diluted 1:1000 with MEM/HANKS (Gibco Labs. Grand Isl. N.Y.). 10 min incubations were carried out at room temperature in 0.5 ml MEM/HANKS containing 0.1% BSA and 1.0 mM 3-isobutyl-1-methylxanthine (Sigma Chem. St.Louis Mo). The reaction was stopped by aspiration of the medium and immediate addition of 0.5 ml of 10% trichloroacetic acid. Approximately 3000 cpm [^{14}C]cAMP were added in a carrier solution and [^{3}H]cAMP formed was isolated from other cyclic nucleotides by the method of Salomon et al. (25)

Animal Protocols

Male Fischer 344 rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 200-220 gms (10 weeks of age) were used in all studies. Animals were housed individually and maintained on tap water ad libitum and standard rodent chow (Rat chow #5012, Ralston Purina Canada Inc., Lasalle, QC) containing 1% calcium and 0.74% phosphorus. Urine collected over 4 h periods from animals having access only to 2% dextrose

was stored with 50 ul 6N HCL/5 ml urine to ensure stability of cAMP. Blood samples (from the caudal artery) were collected into heparinized tubes at the end of the urine collection at the time of implantation of the Rice-500 Leydig cell tumor (CONTROL), between 14 and 17 days post implantation (TIME 0) and at 5, 24, 48, 72, 96 and in some instances 144 h following treatment. Untreated, hypercalcemic animals were sacrificed at the 24 h time point while those treated with PTH antiserum or normal rabbit serum were exsanguinated at 48 h. Animals treated with PLP antiserum were sacrificed at either 96 or 144 h post treatment. Untreated hypercalcemic animals, if not sacrificed, expired 24 to 72 h after Time 0. All plasma and urine samples were stored at -20C until the time of analysis.

The Rice-500 Leydig cell tumor was obtained from the National Cancer Institute Breast Cancer Task Force Bank (EEG Mason Research Institute, Worcester, MA). Tumor was minced in normal saline to give a suspension of 1 part tumor:1 part saline. Tumor suspension (0.2 ml) was then injected into the left flank of each test animal. Control animals were weight matched and maintained under identical conditions.

Using a 23 g needle, 0.6 ml of undiluted antiserum to PLP, antiserum to PTH, or normal rabbit serum was injected into the internal jugular vein of lightly anesthetized rats.

Analytical Methods

Plasma and urinary calcium and magnesium levels were determined by atomic absorption spectrophotometry (Perkin Elmer, Model 703). Plasma phosphate, plasma alkaline phosphatase, urinary phosphate and urinary creatinine were determined by autoanalyser (Technicon Laboratories, New

York, NY). Urinary cAMP was assayed by RIA using the Amersham cAMP [125] assay system (Amersham International Pk, Amersham, U.K.). Insufficient plasma sample was available for determination of magnesium or 1,25 dihydroxyvitamin D ($1,25(OH)_2D_3$) concentrations.

Statistical Analysis

All results are expressed as the mean±standard error of mean (SEM) and statistical comparisons are based on a one-way analysis of variance (ANOVA) using a Bonferroni adjustment where appropriate (26).

Results

Characterization of Antisera

Direct binding studies demonstrated that antiserum 5084/4 raised against synthetic rPLP(1-34) bound the NH_2 terminal region of PLP but not PTH and that antiserum R51 raised against synthetic rPTH(1-84) bound the NH_2 terminal region of PTH but not PLP (Fig. 1).

At a dilution of 1:1000, antiserum raised against synthetic rPLP(1-34) was capable of a 20 to 25-fold inhibition of rPLP(1-34) stimulated adenylate cyclase activity but had no effect on rPTH(1-34) stimulated activity in UMR 106 cells (Fig. 2, panel A). Similarly, antiserum raised against rPTH(1-84), at a dilution of 1:1000, had a 20 to 25-fold inhibitory effect on rPTH(1-34) stimulated activity but not on rPLP(1-34) stimulated adenylate cyclase activity (Fig. 2, panel B).

Effects of Immunization Against PLP in Hypercalcemic Rats

Following transplantation with the Rice-500 tumor, animals continued to gain weight and showed little change in biochemical indices for 10-14 days. After this time they became hypercalcemic and hypophosphatemic (Fig. 3, panel A) and, if left untreated, rapidly became moribund and died (Table 1). Hypercalcemic animals treated with a bolus dose of antiserum to PLP showed a significant decrease in plasma calcium, accompanied by a rise in plasma phosphate, by 5 h following immunization. The decline in body weight was retarded and plasma calcium decreased into the normal range and remained normal for 3 days during which time alkaline phosphatase levels rose (Fig. 3, panel B). These animals continued to live beyond the mean survival time of untreated hypercalcemic rats, their mean plasma calcium levels at the time of sacrifice remaining lower than the final values obtained in untreated rats (Table 1).

The early changes in plasma calcium and phosphate were accompanied by reciprocal changes in urinary calcium and phosphate, i.e. increased calcium excretion and decreased phosphate excretion, in association with a reduction in urinary cAMP (Fig. 4, panel B). Urinary calcium was maintained at a low level from 24-144 h post-immunization while phosphate and cAMP excretion returned to pre-immunization levels and remained at those levels up to 96 h. Urine phosphate rose thereafter. In contrast, in untreated animals no such reductions of calcium, phosphate and cAMP in the urine occurred (Fig. 4, panel A).

Comparison of Treatment with anti-PLP to treatment with anti-PTH or NRS in Hypercalcemic and Normocalcemic Rats

Biochemical parameters in normocalcemic, control rats and in

hypercalcemic, tumor-bearing rats were examined following injections of anti-PLP, anti-PTH or NRS.

In control rats, a moderate and transient decrease in plasma calcium was observed after treatment with anti-PTH. No change was observed in plasma calcium after anti-PLP or NRS treatment (Fig. 5A). In contrast, tumor-bearing rats sustained a much greater and more prolonged reduction in plasma calcium following treatment with anti-PLP whereas no such reduction in plasma calcium was observed in anti-PTH and NRS treated animals (Fig. 5B). Reductions in urinary calcium excretion were seen in all control animals following treatment, however, greater reductions were observed in the anti-PLP and anti-PTH treated animals compared with NRS treated rats (Fig. 5C). Tumor-bearing rats treated with anti-PLP demonstrated a significant increase in renal calcium excretion by 5 h followed by a sustained decrease in urinary calcium. In contrast, treatment with either anti-PTH or NRS resulted in an immediate decrease followed by a rise to pre-immunization levels of calcium excretion (Fig. 5D). There was an increase in urine magnesium (Fig. 6) similar to that seen in urinary calcium (Fig. 5D) at 5 h post treatment with anti-PLP in tumor-bearing rats.

Plasma phosphate was transiently increased above the normal range by treatment with anti-PTH in control rats (Fig. 7A) and increased to within the normal range by treatment with both anti-PTH and anti-PLP in hypercalcemic rats (Fig. 7B). However, while plasma phosphate remained above pre-immunization levels in anti-PLP treated tumor-bearing animals it rapidly returned to sub-normal levels in anti-PTH treated tumor-bearing rats. Urinary phosphate (Fig. 7C and 7D) and cAMP (Fig. 8A and 8B)

excretion decreased in control animals in response to anti-PTH and in tumor-bearing animals in response to anti-PLP.

Discussion

We have examined the effects on biochemical indices of mineral homeostasis of passive immunization with antiserum to PLP and with antiserum to PTH in rats bearing the Rice-500 Leydig cell tumor and in normocalcemic control rats. Despite substantial amino acid sequence homology within the first thirteen residues of PLP(1-34) and PTH(1-34) and the interaction of these peptides at common renal and skeletal receptors (17,27,28), specific blocking antibodies were developed. These were found to selectively bind PLP and PTH and immunoneutralize PLP and PTH stimulation of adenylate cyclase activity IN VITRO with approximately equivalent potencies. When PLP antiserum was administered to rats bearing the Rice 500 Leydig cell tumor, many of the biochemical abnormalities associated with this malignancy were reversed and the longevity of the animals was increased. Most dramatic, perhaps, was the normalization of plasma calcium which was sustained for several days (Figs 3B, 5B). Previous studies, using nude mice implanted with human tumors associated with hypercalcemia, demonstrated that passive immunization with PLP antiserum reduced the tumor-induced elevation in serum calcium in some of those mice (13). Our studies using a different model of malignancy-associated hypercalcemia therefore confirm and extend those studies. The normalization of plasma calcium concentrations observed in our studies following treatment with PLP antiserum appears to indicate that PLP per se is both necessary and sufficient as the mediator of abnormal calcium

homeostasis in this model of tumor-induced hypercalcemia. Thus, any role for other factors, such as transforming growth factors, in the maintenance of hypercalcemia in this disorder would seem to be secondary to the effects of circulating PLP, perhaps as local mediators or modulators of the actions of PLP in its target tissues of bone and kidney These issues await further investigation.

In our studies the earliest effect of PLP immunoneutralization was an augmentation of urinary calcium and magnesium excretion which coincided with a rapid decrease in plasma calcium. This undoubtedly reflects the well-documented influence of PLP on enhancing renal tubular calcium reabsorption in a manner analogous to that of PTH (17-19) Magnesium in the kidney may share common distal tubular transport mechanisms with calcium (29). Consequently it was of interest to observe a small early increase in magnesium excretion in tumor-bearing animals passively immunized with PLP antiserum, which coincided with augmented calcium excretion. The role of the kidney in the development and maintenance of hypercalcemia in the rat Leydig cell tumor model is attested to by the observation that hypercalcemia precedes hype calciuria (1,30) and by the finding that Leydig tumor-bearing rats treated with WR-2721, an inhibitor of tubular calcium reabsorption, sustain a rapid reduction in plasma calcium associated with an early increase in urinary calcium excretion (31). Considering the qualitative and quantitative similarity in the renal handling of calcium in rats and humans (32) and clinical observations documenting early involvement of the kidney in the development of malignancy-associated hypercalcemia (33,34), renal mechanisms appear to play an important role in tumor-induced hypercalcemia in both the rat

Leydig tumor model and in the human disorder. Our studies demonstrate that the mediator of these renal effects, at least in the rat model, is PLP

Following the initial augmentation of renal calcium excretion, urinary calcium decreased in tumor-bearing rats passively immunized with PLP antiserum. In view of the fact that plasma calcium levels did not increase, the reduced urinary calcium excretion was almost certainly the result of a reduced filtered load of calcium. This was most likely consequent to diminished efflux of calcium from the skeleton but may have also been due to reduced $1,25(OH)_2D_3$ production. Thus infusion of PLP into thyroparathyroidectomized rats is known to increase $1,25(OH)_2D_3$ (16), and Leydig tumor bearing rats are known to have high circulating levels of $1,25(OH)_2D_3$ (2), in contrast to the low levels found in humans with malignancy-associated hypercalcemia (3,35).

The apparent inhibition of PLP-induced skeletal action was sustained for some time. The elevation in plasma alkaline phosphatase which occurred during this interval may have represented an attempt at bone repair and reflected cessation of the marked inhibition of bone formation which has been noted in malignancy-associated hypercalcemia (2,3,6) The important role of the skeleton in the maintenance of hypercalcemia in both rat (30) and human malignancies (36) has been well-documented. Our findings implicating PLP in the maintenance of hypercalcemia via skeletal action are, therefore, consistent with studies attributing a central role to PLP in stimulating osteoclastic bone resorption (18) and producing hypercalcemia in thyroparathyroidectomized rats through skeletal as well as renal mechanisms (37).

The influence of endogenous PLP on renal function in the Leydig

tumor-bearing rats was also manifested by reductions in urinary phosphate and cAMP excretion following injection with PLP antiserum The cAMP effect was also previously noted in passive immunization experiments in nude mice implanted with human tumors associated with hypercalcemia (13). The decrease in renal phosphate excretion observed in our studies was associated with an early increase in plasma phosphate (followed by a decline suggesting neutralization of PLP-induced skeletal mobilization of phosphate). By 144 h after immunization the reappearance of PLP-induced phosphaturia undoubtedly contributed to the further decline in plasma phosphate.

In contrast to the effects of passive immunization with PLP antiserum, immunoneutralization of PTH produced little or no effect on the biochemical parameters measured in Leydig tumor-bearing rats including plasma calcium concentrations and urinary excretion of phosphate, cAMP and magnesium. Transient effects on urine calcium and plasma phosphate appeared to occur, which could possibly reflect the presence of low circulating concentrations of non calcium-suppressible PTH. However, PTH is unlikely to play a significant role in the biochemical abnormalities observed in these animals.

In normal animals, passive immunization with PTH antiserum resulted in reductions in plasma and urinary calcium, an increase in plasma phosphate, and concomitant decrease in urinary phosphate and cyclic AMP excretion. These effects were the same as those observed after passive immunization of Leydig tumor-bearing rats with PLP antiserum, reflecting the similar spectra of bioactivities of PTH and PLP. However, the magnitude and duration of the effects of immunoneutralization of PTH were

generally less than were those following anti-PLP treatment. In the normal rat this could in part have reflected rapid homeostatic adjustments involving a calcium-mediated increase in PTH secretion which overwhelmed the binding capacity of the antiserum. In contrast, no rapid restoration of the baseline calcium concentration occurred in the tumor-bearing rats, suggesting that plasma calcium is not a modulator of PLP secretion in Leydig cell tumors, but rather that these tumors secrete PLP constitutively or in response to other mediators. Consequently, the effects of PLP immunoneutralization would appear to be more slowly reversible in the tumor-bearing model where compensatory mechanisms would be deficient In addition to these quantitative differences, a qualitative difference in the renal handling of calcium was demonstrated. The early augmentation of urinary calcium and magnesium excretion following PLP neutralization in Leydig tumor-bearing rats was absent in normal rats treated with anti-PTH suggesting that PTH-mediated reabsorption in the ascending limb and distal tubule is of lesser importance in normal calcium homeostasis. The delayed reduction in plasma calcium in these animals was presumably due to an effect on the skeleton and/or on $1,25(OH)_2D_3$ production, indicating that the major component of extracellular fluid calcium control may reside in skeletal and/or vitamin D-mediated events rather than in renal transport effects of PTH

PLP immunoneutralization in normal animals resulted only in significantly decreased urinary calcium concentrations. Although effects on other parameters may have been obscured by rapid homeostatic changes following injection of PLP antiserum, it seems more likely that PLP plays little role in modulating mineral metabolism in the normal adult rat.

These studies, therefore, point to the central role of PLP in the maintenance of malignancy-associated hypercalcemia through renal and skeletal mechanisms and show that of the PLP/PTH family of peptides, the major modulator of normal calcium homeostasis in adult rats is PTH In addition, these studies indicate differential mechanisms of calcium regulation may be utilized by these peptides in the modulation of normal calcium metabolism versus malignancy-associated hypercalcemia

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REFERENCES

1 Sica DA, Martodam RR, Aronow J, Mundy GR 1983 The hypercalcemic rat Leydig cell tumor - A model of the humoral hypercalcemia of malignancy. Calcif Tissue Int 35:287

2 Insogna KL, Stewart AF, Vignery AM, Weir EC, Namnum PA, Baron RE, Kirkwood JM, Deftos LM. Broadus AE 1984 Biochemical and histomorphometric characterization of a rat model for humoral hypercalcemia of malignancy. Endocrinology 114:888

3. Stewart AF, Horst R, Deftos LJ, Cadman EC, Lang R, Broadus AE 1980 Biochemical evaluation of patients with cancer-associated hypercalcemia N Engl J Med. 303:1377

4. Goltzman D, Stewart AF, Broadus AE 1981 Malignancy-associated hypercalcemia: Evaluation with a cytochemical bioassay for parathyroid hormone J Clin Endocrinol Metab. 53:899

5. Rizzoli R, Caverzasio J, Fleisch H, Bonjour JP 1986 Parathyroid hormone-like changes in renal calcium and phosphate reabsorption induced by Leydig cell tumor in thyroparathyroidectomized rats. Endocrinology 119.1004

6. Tam CS, Heersche JNM, Santora A, Spiegel AM 1984 Skeletal response in rats following the implantation of hypercalcemia-producing Leydig cell tumors. Metabolism 33:50

7. Suva LJ, Winslow GA, Wettenhall REH, Hammonds RG, Moseley JM, Diefenbach-Jagger H, Rodda CP, Kemp BE, Rodriguez H, Chen EY, Hudson PJ, Martin TJ, Wood WI 1987 A parathyroid hormone-related protein implicated in malignant hypercalcemia: Cloning and expression. Science 237:893

8. Mangin M, Webb AC, Dreyer BE, Posillico JT, Ikeda K, Weir EC, Stewart AF, Bander NH, Milstone L, Barton DE, Francke U, Broadus AE 1988 Identification of a cDNA encoding a parathyroid hormone-like peptide from a human tumor associated with humoral hypercalcemia of malignancy Proc Natl Acad Sci (USA) 85:597

9. Thiede MA, Strewler GJ, Nissenson RA, Rosenblatt M, Rodan GA 1988 Human renal carcinoma expresses two messages encoding a parathyroid hormone-like peptide: Evidence for the alternative splicing of a single-copy gene. Proc Natl Acad Sci (USA) 85:4605

10. Yasuda T, Banville D, Rabbani SA, Hendy GN, Goltzman D 1989 Rat parathyroid hormone-like peptide: Comparison with the human homologue and expression in malignant and normal tissue. Mol Endocrinol. 3:518

11. Yasuda T, Banville D, Hendy GN, Goltzman D 1989 Characterization of the human parathyroid hormone-like peptide gene. functional and evolutionary aspects. J Biol Chem 264:7720

12. Mangin M, Ikeda K, Dreyer BE, Broadus AE 1989 Isolation and charaterization of the human parathyroid hormone-like peptide gene. Proc Natl Acad Sci (USA) 86:2408

13. Kukreja SC, Shevrin DH, Wimbiscus SA, Ebeling PR, Danks JA, Rodda CP, Wood WI, Martin TJ 1988 Antibodies to parathyroid hormone-related protein lower serum calcium in athymic mouse models of malignancy-associated hypercalcemia due to human tumors. J Clin Invest. 82:1798

14. Budayr AA, Nissenson RA, Klein RF, Pun KK, Clark OH, Diep D, Arnaud CD, Strewler GJ 1989 Increased serum levels of a parathyroid hormone-like protein in malignancy-associated hypercalcemia. Ann Int Med 111:807

15. Henderson J, Shustik C, Kremer R, Rabbani SA, Hendy GN, Goltzman D 1990 Circulating concentrations of parathyroid hormone-like peptide in malignancy and in hyperparathyroidism. J Bone Min Res 5:105

16. Horiuchi N, Caulfield MP, Fisher JE, Goldman ME, McKee RL, Reagan JE, Levy JJ, Nutt RF, Rodan SB, Schofield TL, Clemens TL, Rosenblatt M 1987 Similarity of synthetic peptide from human tumor to parathyroid hormone in vivo and in vitro. Science 238:1566

17. Rabbani SA, Mitchell J, Roy DR, Hendy GN, Goltzman D 1988 Influence of the amino-terminus on in vitro and in vivo biological activity of synthetic parathyroid hormone-like peptides of malignancy. Endocrinology 123:2709

18. Yates AJP, Gutierrez GE, Smolens P, Travis PS, Katz MS, Aufdemorte TB, Boyce BF, Hymer TK, Poser JW, Mundy GR 1988 Effects of a synthetic peptide of a parathyroid hormone-like protein on calcium homeostasis, renal tubular calcium reabsorption, and bone metabolism in vivo and in vitro in rodents. J Clin Invest 81:932

19. Zhou H, Leaver DD, Moseley JM, Kemp B, Ebeling PR, Martin TJ 1989 Actions of parathyroid hormone-related protein on the rat kidney in vivo. J Endocrinol. 122·229

20. Ibbotson KJ, D'Souza SM, Smith DD, Carpenter G, Mundy GR 1984 EGF receptor antiserum inhibits bone resorbing activity produced by a rat Leydig cell tumor associated with the humoral hypercalcemia of malignancy. Endocrinology 116:469

21. Insogna KL, Weir EC, Wu TL, Stewart AF, Broadus AE, Burtis WJ, Centrella M 1987 Co-purification of transforming growth factor B-like activity with PTH-like and bone resorbing activities from a tumor associated with humoral hypercalcemia of malignancy. Endocrinology 120:2183

22. Strewler GJ, Wronski TJ, Halloran BP, Miller SC, Leung SC, Williams RD, Nissenson RA 1986 Pathogenesis of hypercalcemia in nude mice bearing a human renal carcinoma. Endocrinology 119:303

23. Weir EC, Insogna KL, Brownstein DG, Bander NH, Broadus AE 1988 In vitro adenylate cyclase-stimulating activity predicts the occurrence of humoral hypercalcemia of malignancy in nude mice. J Clin Invest 81:818

24. Mehdizadeh S, Alaghband-Zadeh J, Gusterson B, Arlot M, Bradbeer JN, Loveridge N 1989 Bone resorption and circulating PTH-like bioactivity in an animal model of hypercalcemia of malignancy. Biochem Biophys Res Comm 161:1166

25. Salomon Y, Londos C, Rodbell M 1974 A highly sensitive adenylate cyclase assay. Analyt Biochem 58:541

26. Glantz SA 1981 Primer of Biostatistics. McGraw-Hill Inc.

27. Juppner H, Abou-Samra AB, Ureno S, Gu WX, Potts JT, Segre GV 1988 The parathyroid hormone-like peptdie associated with humoral hypercalcemia of malignancy and parathyroid hormone bind to the same receptor on the plasma membrane of ROS 17/2.8 cells. J Biol Chem 263:8557

28. Nissenson RA, Diep D, Strewler GJ 1988 Synthetic peptides comprising the amino-terminal sequence of a parathyroid hormone-like protein from human malignancies: binding to parathyroid hormone receptors and activation of adenylate cyclase in bone cells and kidney. J Biol Chem 263:12866

29. Harris CA, Burnatowska MA, Seely JF, Sutton RAL, Quamme GA, Dirks JH 1979 Effects of parathyroid hormone on electrolyte transport in the hamster nephron. Am J Physiol 236:F342

30. Martodam RR, Thornton KS, Sica DA, D'Souza SM, Flora L. Mundy GR 1983 The effects of dichloromethylene diphosphonate on hypercalcemia and other parameters of the humoral hypercalcemia of malignancy in the rat Leydig cell tumor. Calcif Tissue Int 35:512

31. Hirschel-Schotz S, Caverzasio J, Rizzoli R, Bonjour JP 1986 Normalization of hypercalcemia associated with a decrease in renal calcium reabsorption in Leydig cell tumor-bearing rats treated with WR-2721 J Clin Invest 78:319

32. Hugi K, Bonjour JP, Fleisch H 1979 Renal handling of calcium: influence of parathyroid hormone and 1,25-dihydroxyvitamin D3. Am J Physiol 236:F349

33. Percival RC, Yates AJP, Grey RES, Galloway J, Rogers K, Neal FE, Kanis JA 1985 Mechanisms of malignant hypercalcemia in carcinoma of the breast. Br Med J 291:776

34. Ralston SH, Boyce BF, Cowan RA, Gardner MD, Fraser WD, Boyle IT 1989 Contrasting mechanisms of hypercalcemia in patients with early and advanced hypercalcemia of malignancy. J Bone Min Res 4:103

35. Kukreja SC, York PA, Nalbantian-Brandt C, Shevrin DH, Favus MJ 1989 Effect of hypercalcemia-producing tumor on $1,25(OH)_2D_3$ biosynthesis in athymic mice. Am J Physiol 256:E309

36. Ralston SH, Alzaid AA, Gallacher SJ, Gardner MD, Cowan RA, Boyle IT 1988 Clinical experience with aminohydroxy-propylidene bisphosphonate (APD) in the management of cancer-associated hypercalcemia. Quart J Med 258:825

37. Rizzoli R, Caverzasio J, Chapuy MC, Martin TJ, Bonjour JP 1989 Role of bone and kidney in parathyroid hormone-related peptide-induced hypercalcemia in rats. J Bone Min Res 4:759

Table 1. Survival of Leydig tumor-bearing rats following development of hypercalcemia

	-PLP Antiserum		+PLP Antiserum	
Rat	Days[a]	Ca (mM)[b]	Days[a]	Ca (mM)[b]
1	3	3.94	6	2.56
2	1	3.83	4	3.00
3	3	3.51	6	3.00
4	1	3.69	6	3.63
5	2	3.30	6	3.12
6	2	3.27	6	2.97
7	1	4.66	6	2.97
8	1	3.69	6	3.14
9	2	3.44	6	3.11
Mean±SEM	1.8±0.3	3.70±0.1	5.8±0.2[c]	3.05±0.09[c]

[a] Survival in days of treated (+PLP antiserum) versus non-treated (-PLP antiserum) rats following development of hypercalcemia.

[b] Plasma calcium values are those obtained within 24 h of expiration of the non-treated animals and at the time of sacrifice of the treated animals.

[c] $p<0.01$ compared with non-treated animals by Student's t-test.

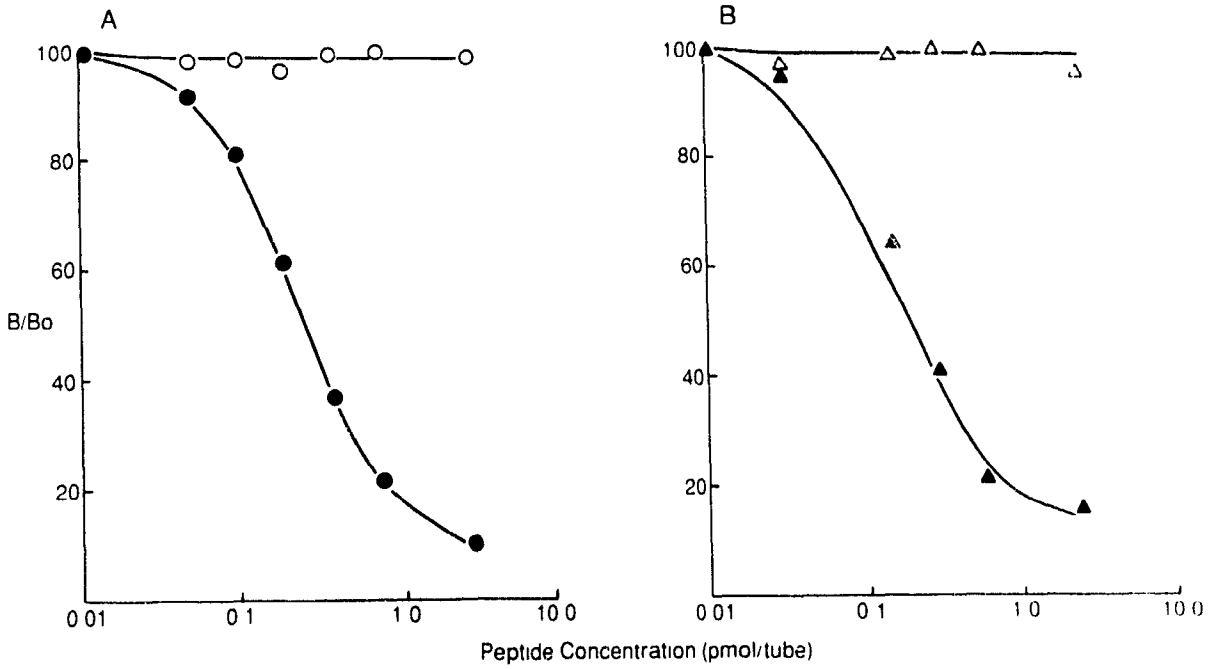

Figure 1. Panel A: Inhibition of binding (B) of ^{125}I-labeled [Tyr^{0}]rPLP(1-34) to rabbit antiserum 5084/4 [raised against rPLP-(1-34)] by rPLP(1-34) (●) and rPTH(1-34) (○). **Panel B:** Inhibition of binding of ^{125}I-labeled rPTH-(1-84) to rabbit antiserum R51 [raised against rPTH(1-84)] by rPTH(1-84) (▲) and rPLP(1-34) (△). Initial binding (Bo) to antiserum 5084/4 was 30% and to antiserum R51 was 29%. Each point is the mean of duplicate determinations which varied by less than 8% from the mean. Details of the radioimmunoassay are provided in Materials and Methods.

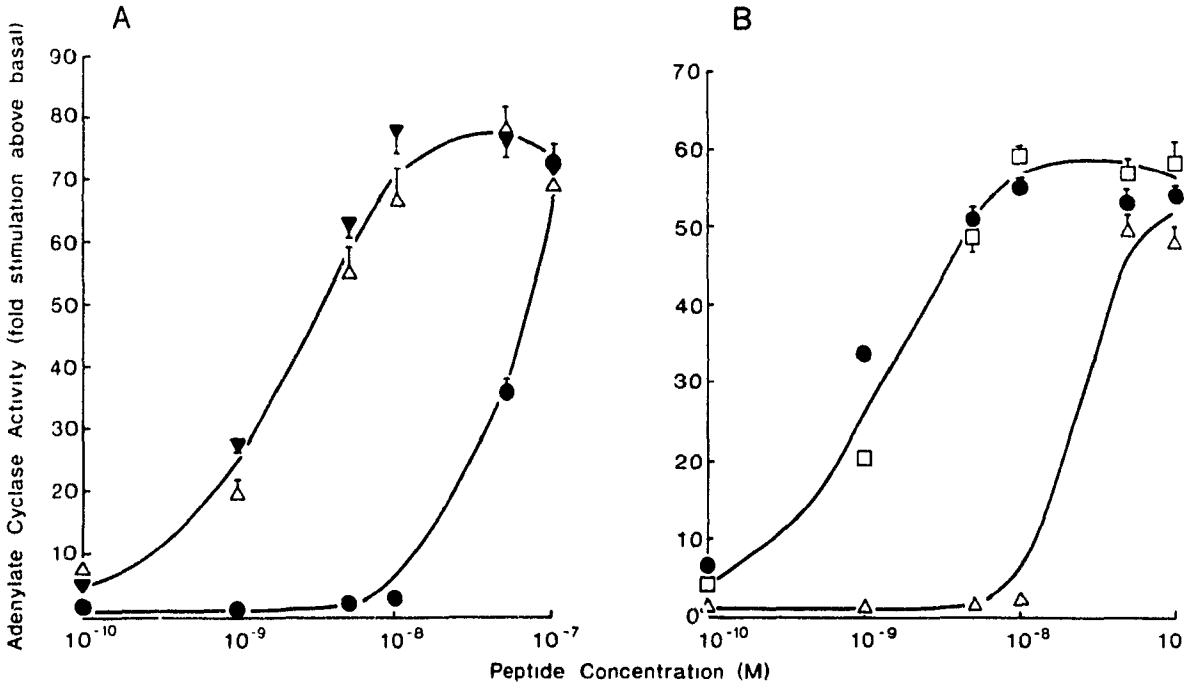

Figure 2. Effect on adenylate cyclase activity of increasing concentrations of rPLP(1-34) **(panel A)** pre-incubated with NRS (▼), anti-PLP (●) or anti-PTH (△) and of increasing concentrations of rPTH(1-34) **(panel B)** pre-incubated with NRS (□), anti-PLP (●) or anti-PTH (△). Adenylate cyclase activity was determined as described in Materials and Methods and is expressed as enzyme activity stimulated by each concentration of peptide divided by basal activity (fold stimulation above basal). Basal activity was 150±22cpm of [^{3}H]cAMP/10min/culture in panel A and 101±26cpm of [^{3}H]cAMP/10min/culture in panel B. Each point represents the mean±SEM of triplicate determinations.

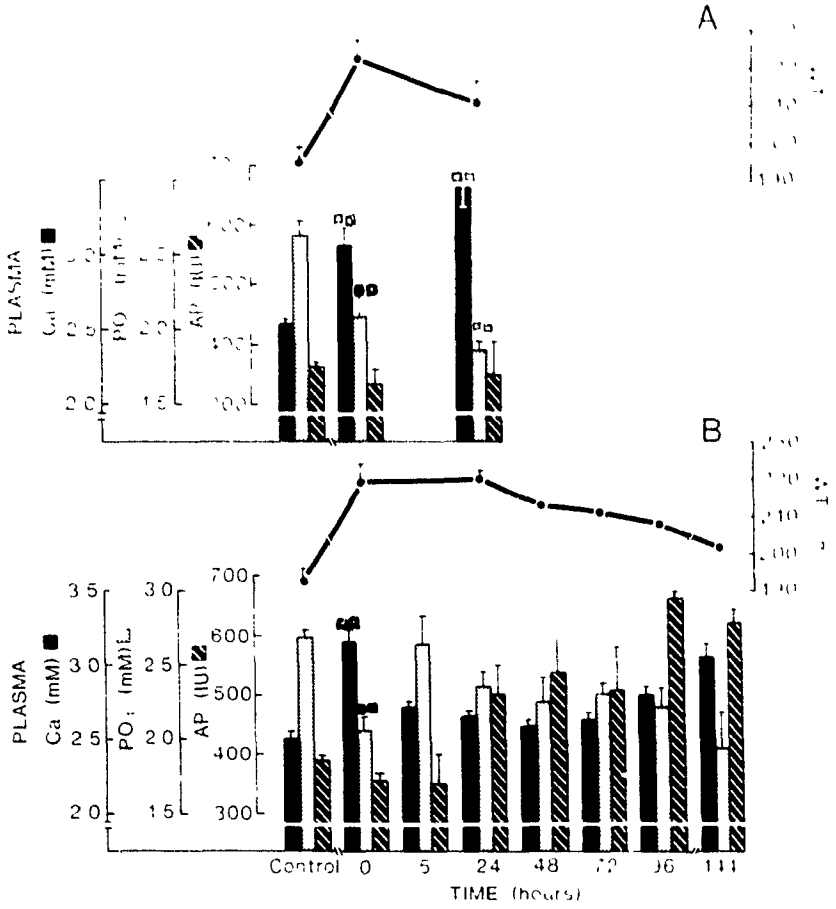

Figure 3. Effect of treatment with anti-PLP **(panel B)** compared with no treatment **(panel A)** on plasma calcium (Ca, ■), phosphate (PO_4, ▩), aklaline phosphatase (AP, ▨) and body weight (●—●) in hypercalcemic, tumor-bearing rats. Animals were bled via the caudal artery as described in Materials and Methods at the time of tumor implantation **(Control)**, immediately prior to treatment **(Time 0)** and at 5, 24, 48, 72, 96 and 144 h post-treatment. Untreated animals were sacrificed at 24 h. Each plasma calcium value represents the mean±SEM of determinations in 8-16 animals Phosphate and alkaline phosphatase results are based on the mean±SEM of 4-10 plasma sample pools Significant differences from Control are represented by ■■ ($p<0.01$). Significant differences from Time 0 are represented by * ($p<0.05$) and ** ($p<0.01$). Normal ranges for plasma calcium, phosphate and alkaline phosphatase are 2.41-2 65 mM, 2.55-3.49 mM and 314-400 international units per L respectively.

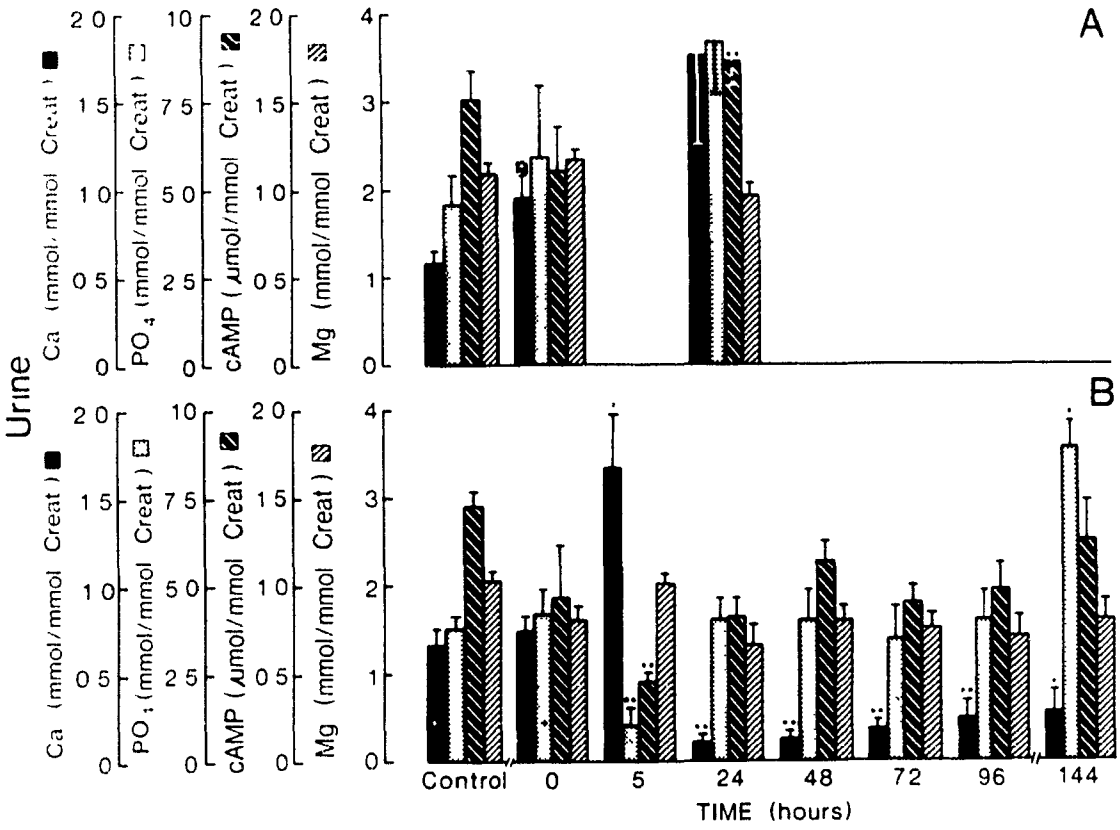

Figure 4. Effect of treatment with anti-PLP **(panel B)** compared with no treatment **(panel A)** on urinary calcium (Ca, mmol/mmol creatinine, ■), phosphate (PO_4 mmol/mmol creatinine, ▩), cAMP umol/mmol creatinine, ▧) and magnesium (Mg mmol/mmol creatinine, ▨) in hypercalcemic, tumor-bearing rats. Urine collections were made over 4 h periods as described in Materials and Methods at the time of tumor implantation **(Control)**, prior to treatment **(Time 0)** and 5, 24, 48, 72, 96, and 144 h post-treatment. Results represent the mean±SEM of determinations in 8-16 animals. Significant differences from Control are represented by ◘ ($p<0.05$). Significant differences from time 0 are represented by * ($p<0.05$) and ** ($p<0.01$). Normal ranges for urinary calcium, phosphate, cAMP and magnesium are 0.47-1.43 mmol/mmol creatinine, 2.3-7.0 mmol/mmol creatinine, 0.66-1.48 µmol/mmol creatinine and 2.1-2.7 mmol/mmol creatinine, respectively.

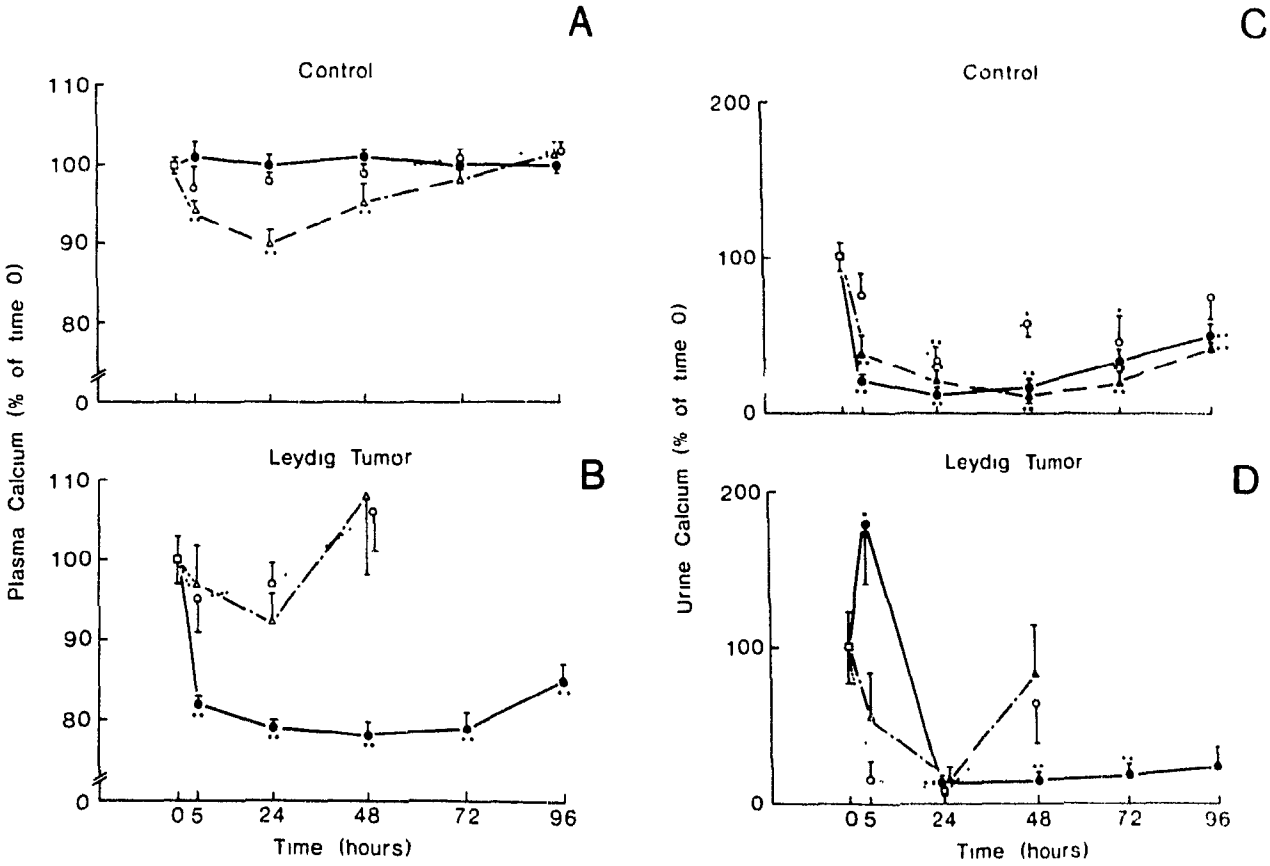

Figure 5. Effect of treatment with NRS (- - - -), anti-PLP (——), and anti-PTH (—•—) on plasma calcium in normocalcemic control rats **(panel A)** and in hypercalcemic tumor-bearing rats **(panel B)**, and on urine calcium in control rats **(panel C)**, and in rats with Leydig cell tumors **(panel D)**. Animals were bled and 4 h urine collections made as described in Materials and Methods. Results represent the mean±SEM of determinations in 5-10 animals and are expressed as percent of Time 0. Concentrations of plasma calcium at Time 0 in control and in tumor-bearing animals were 2.57±0.01 and 3.31±0.09 mM respectively. Concentrations of urine calcium at Time 0 in control and in tumor-bearing animals were 0.85±0.0 07 and 0.93±0.21 mmol/mmol creatinine respectively. Significant differences from time 0 at each time point are represented by * ($p<0.05$) and ** ($p<0.01$).

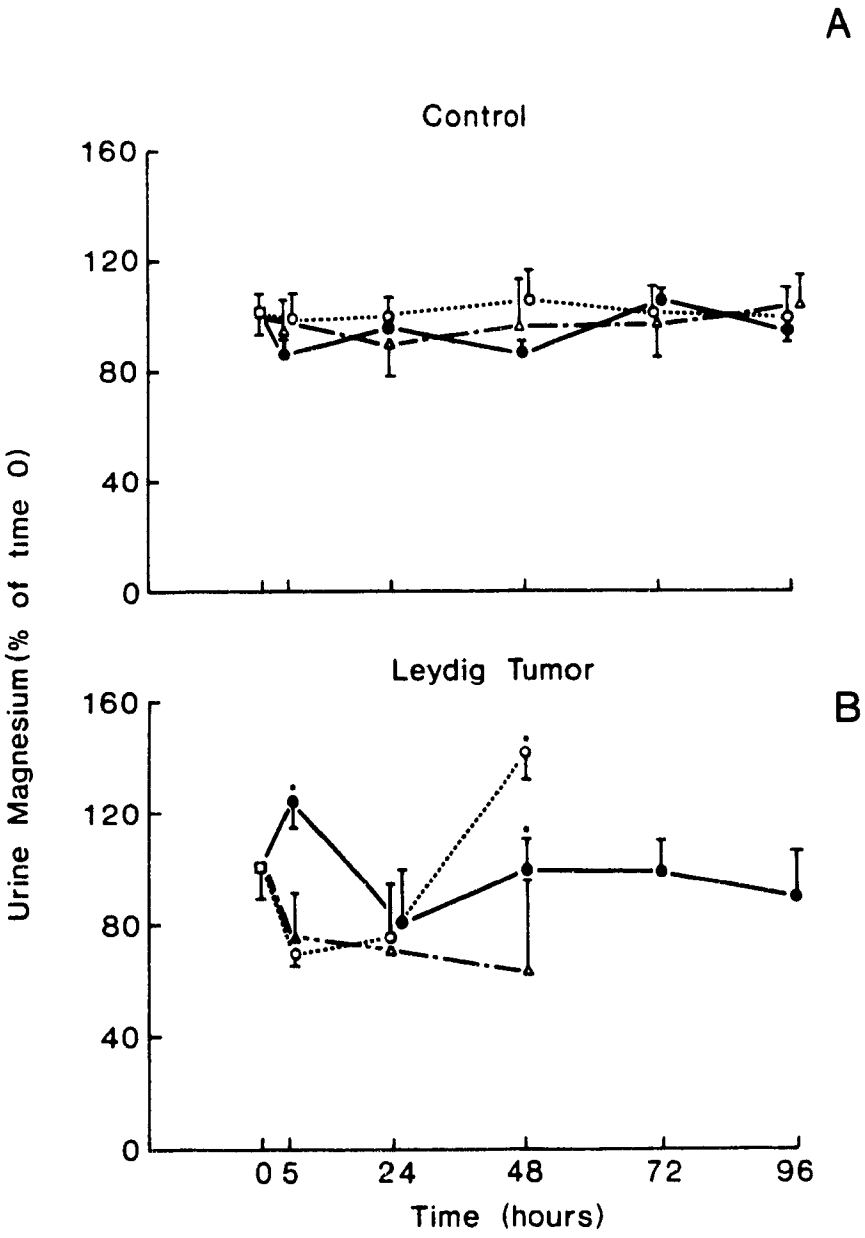

Figure 6. Effect of treatment with NRS (- - - -), anti-PLP (——), and anti-PTH (—•—) on urine magnesium in normocalcemic control rats **(panel A)** and in hypercalcemic tumor-bearing rats **(panel B)**. Urine collections were made as described in Materials and Methods. Results represent the mean±SEM of determinations in 5-10 animals and are expressed as percent of Time 0. Concentrations of urine magnesium at Time 0 in control and in tumor-bearing animals were 2.39±0.08 and 1.57±0.11 mmol/mmol creatinine respectively. Significant differences from Time 0 at each time point are represented by * ($p<0.05$) and ** ($p<0.01$).

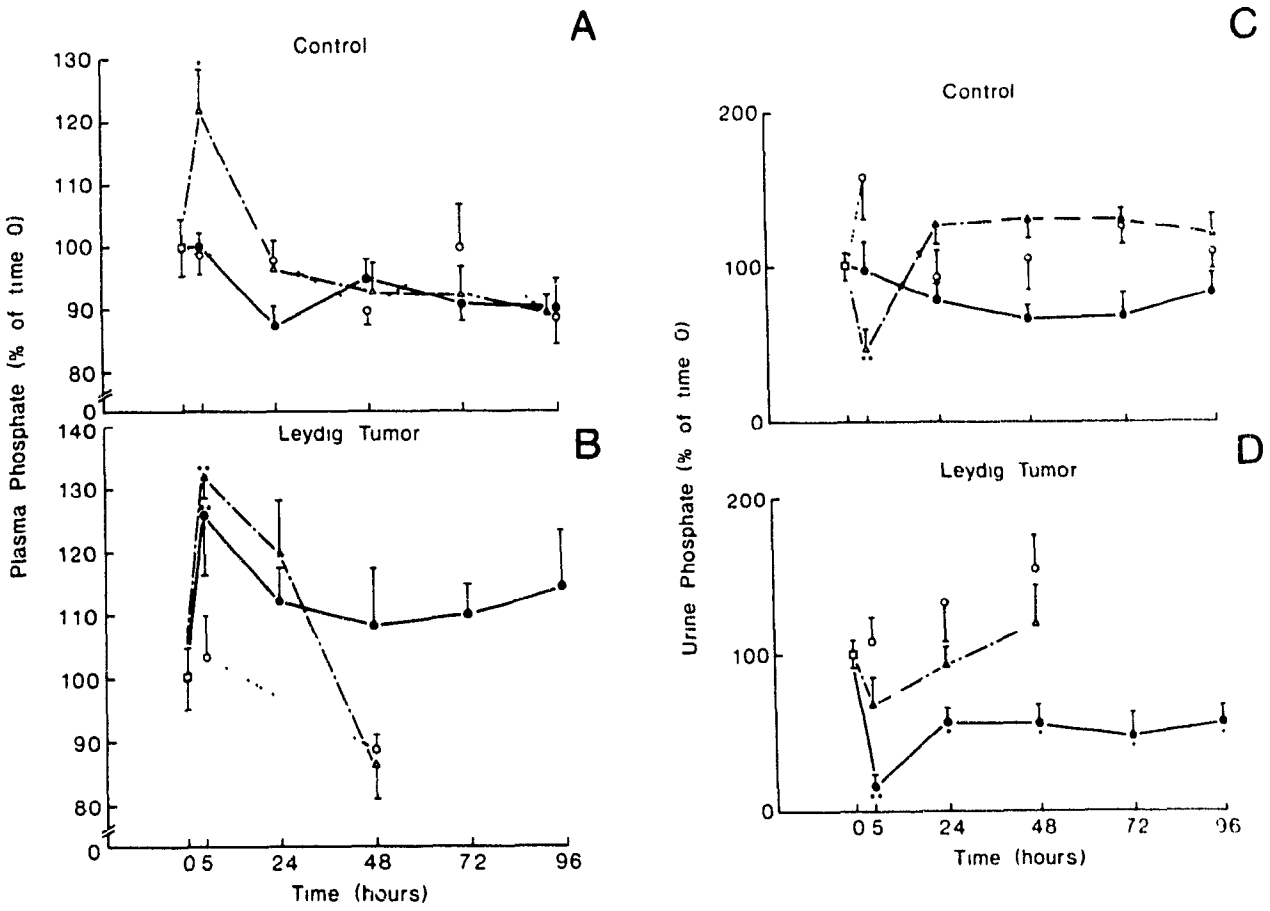

Figure 7. Effect of treatment with NRS (- - - -), anti-PLP (——), and anti-PTH (—•—) on plasma phosphate in normocalcemic control rats **(panel A)** and in hypercalcemic tumor-bearing rats **(panel B)**, and on urine phosphate in control rats **(panel C)** and in rats with Leydig cell tumors **(panel D)**. Animals were bled and urine collections made as described in Materials and Methods. Results represent the mean±SEM of determinations in 5-10 animals and are expressed as percent of Time 0 Concentrations of plasma phosphate at Time 0 in control and in tumor-bearing animals were 3.19±0.10 and 2.05±0.10 mM respectively. Concentrations of urine PO_4 at Time 0 in control and in tumor-bearing animals were 5.09±0.44 and 7.04±0.84 mmol/mmol creatinine respectively. Significant differences from time 0 at each time point are represented by * ($p<0.05$) and ** ($p<0.01$)

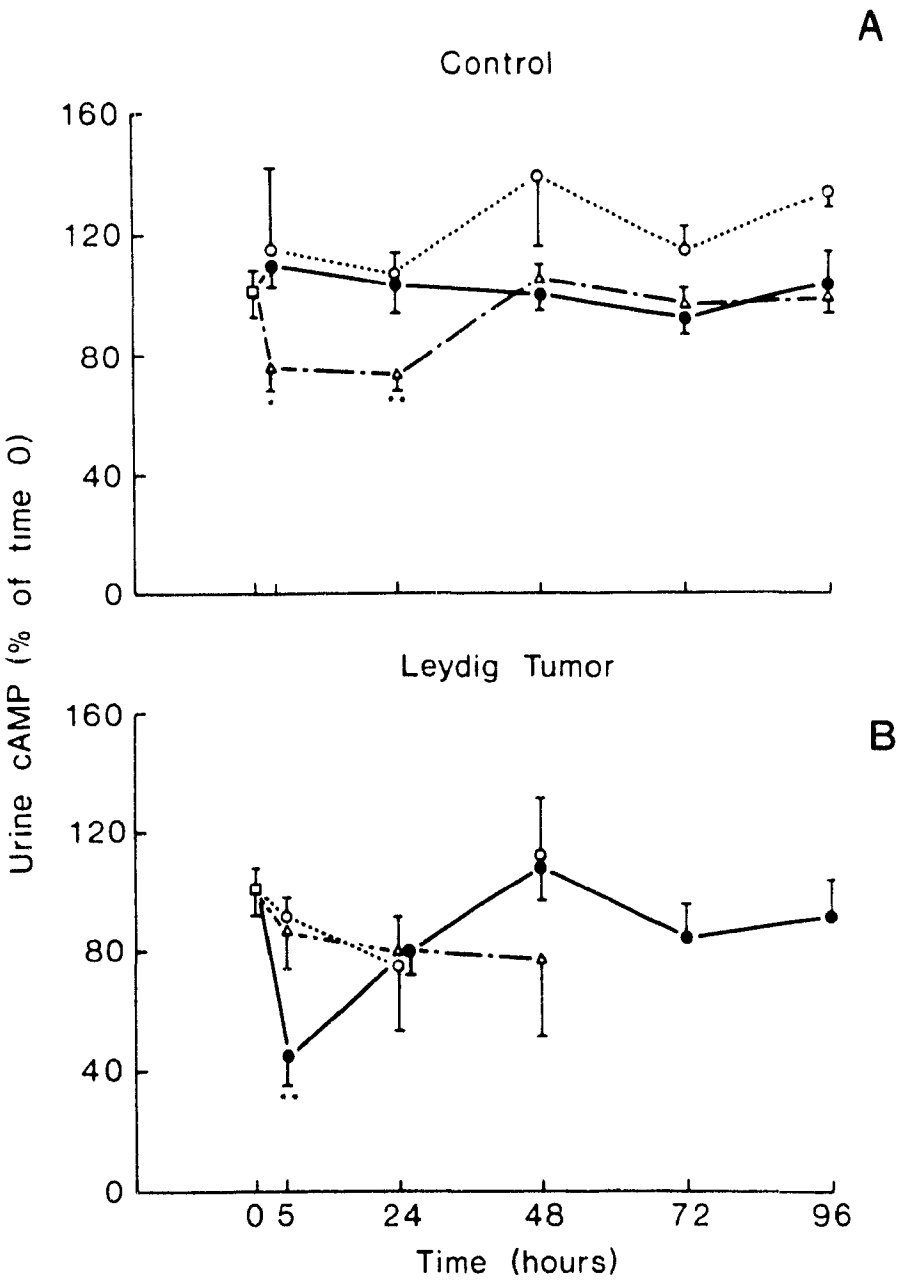

Figure 8. Effect of treatment with NRS (----), anti-PLP (——), and anti-PTH (—•—) on urine cAMP in normocalcemic control rats **(panel A)** and in hypercalcemic tumor-bearing rats **(panel B)**. Urine collections were made as described in Materials and Methods. Results represent the mean±SEM of determinations in 5-10 animals and are expressed as percent of Time 0. Concentrations of urine cAMP at Time 0 in control and in tumor-bearing animals were 1.07±0.08 and 1.04±0.08 umol/mmol creatinine respectively. Significant differences from Time 0 at each time point are represented by * ($p<0.05$) and ** ($p<0.01$).

CHAPTER IV DYSREGULATION OF PARATHYROID HORMONE-LIKE PEPTIDE EXPRESSION AND SECRETION IN A KERATINOCYTE MODEL OF TUMOR PROGRESSION

Preface

The observations made in Chapters II and III were consistent with a pathogenetic role for tumor derived PLP in the hypercalcemia of malignancy. The frequent association of squamous cell cancer with hypercalcemia and high circulating levels of PLP suggested overproduction of PLP by those tumors IN VIVO. As normal keratinocytes in culture had been shown to express and secrete PLP in a well regulated manner, I examined a human keratinocyte model of tumor progression IN VITRO for evidence of dysregulation of PLP production.

Abstract

Using a human keratinocyte model of tumor progression, we have examined the regulation of gene expression and secretion of a parathyroid hormone-like peptide (PLP) which has been implicated in the pathogenesis of hypercalcemia in malignancy. A rapid and transient induction of PLP mRNA in response to serum stimulation was demonstrated in both established (HPK1A) and malignant (HPK1A-ras) cells, however the dose dependent increases were greater in HPK1A than in HPK1A-ras. Significant inhibition of this induction was noted with the addition of 1, 25-dihydroxyvitamin D [$1,25(OH)_2D_3$] at a lower concentration in HPK1A than in HPK1A-ras. Amino-terminal PLP immunoreactivity and bioactivity correlated well ($R=0.98$) when measured in conditioned medium. In the absence of mitogenic stimuli, malignant keratinocytes (HPK1A-ras) secreted significantly more PLP than established (HPK1A) keratinocytes. However, in response to increasing concentrations of epidermal growth factor (EGF) and fetal bovine serum (FBS), PLP release was far greater from HPK1A (maximum 13x basal) than from HPK1A-ras (maximum 3x basal) cells In addition, $1,25(OH)_2D_3$ was more effective in inhibiting both basal and stimulated PLP secretion in HPK1A than in HPK1A-ras cultures. Reduction of extracellular Ca^{++} from 2.0 mM to 0.5 mM appeared to be more effective at an early time point in reducing PLP secretion from the established cells compared with the malignant cells. These studies therefore demonstrate a progressive dysregulation of PLP expression and secretion in human keratinocytes in the transformation from established to malignant phenotype and may have important implications for understanding the pathogenetic mechanisms involved IN VIVO in the development of hypercalcemia in malignancy.

Introduction

Hypercalcemia is a common cause of morbidity and mortality in patients harboring malignancy and tumors of squamous epithelial origin represent one of the largest sub-groups associated with hypercalcemia (1, 2). Parathyroid hormone-like peptide (PLP), sharing 62% amino acid sequence homology with parathyroid hormone (PTH) in the first 13 residues, has been purified, (3-5) cloned and sequenced (6-9) from malignancies associated with hypercalcemia and PLP specific radioimmunoassays (10-12) have identified elevated levels of this peptide in the plasma of hypercalcemic patients harboring a wide variety of tumors. Synthetic NH_2 terminal fragments of PLP were shown to bind to the PTH receptor in both kidney and bone (13) and when infused IN VIVO mimicked the actions of PTH (14). In addition, passive immunization of hypercalcemic, tumor-bearing rodents with antisera directed against the NH_2 terminus of PLP was effective in normalizing plasma calcium (15, 16). Taken together, these findings strongly implicate PLP as a pathogenetic agent in the hypercalcemia associated with malignancy. Identification of PTH-like bioactivity in squamous cell malignancies (17, 18) led to the examination of non-malignant human keratinocytes for the presence of this activity (19). Normal keratinocytes were shown to secrete a factor with parathyroid hormone-like bioactivity which was distinct in size and immunoreactivity from native PTH and subsequent studies confirmed PLP production by these cells. PLP has been detected in conditioned medium (CM) from normal keratinocytes (20) as well as in CM from a number of other normal and neoplastic cells in culture (21-23). Production of PLP

IN VITRO has been reported to be regulated by a variety of agents including growth factors and $1,25(OH)_2D_3$ (20), cycloheximide (24), calcitonin and chromogranin A (25) phorbol esters (26), glucocorticoids (27) and extracellular calcium (28, 22). Although normal keratinocytes in culture secrete PLP in a well regulated manner (20), little is known regarding the development of PLP overproduction during the process of transformation. In the present study, we have therefore used a recently developed IN VITRO multistep model for human epithelial cell carcinogenesis (29), to study PLP expression and secretion in established and malignant human keratinocytes in culture in response to epidermal growth factor (EGF), fetal bovine serum (FBS), 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and calcium, factors which have previously been shown to influence PLP expression (20) in cultured normal keratinocytes.

Materials and Methods

Normal Human Keratinocyte (NHK) and Culture Conditions. Normal human keratinocytes were prepared from skin tissue removed during breast reduction according to a previously published method (30). Briefly, cells were grown in KGM (Clonetics Corp., San Diego, CA) supplemented with 0.15 mM calcium (the complete medium required for clonal growth of primary keratinocytes) and were used on the second or third passage. Cells were seeded at a density of 10^6 cells/well in 6 well cluster plates (Becton Dickinson Labware; Lincoln Park, NJ). At 50% confluence, medium was replaced with fresh KBM (Clonetics) supplemented with 0.5 μg/ml hydrocortisone (Sigma Chem. Co., St. Louis, MO) and 5 μg/ml insulin

(Sigma) and cultures incubated for 24 h (basal conditions). Medium was again replaced with fresh KBM supplemented with insulin, hydrocortisone and with 10 ng/ml EGF (Sigma) in the absence or presence of 10^{-8} M $1,25(OH)_2D_3$. (A kind gift of Dr. M. Uskokovic, Hoffman-LaRoche, Nutley, NJ). Conditioned medium (CM) removed 24 h. later was processed for radioimmunoassay.

Cell Lines and Culture Conditions: Following transfection with HPV16 DNA, using the calcium phosphate/glycerol shock technique, human keratinocytes in culture acquired an indefinite lifespan (> 60 passages) compared with control cells transfected with calf thymus DNA which senesced after 6 passages (31). Southern blot analysis revealed that the immortalized HPK1A population had HPV16 genomes stably (> 2 yr) integrated at a single site within the host DNA. These immortalized cells were neoplastically transformed into the malignant HPK1A-ras line following polybrene-induced transfer of pSV_2ras DNA containing the neo selectable marker (29). Geneticin-resistant transformants demonstrated integrated copies of the exogenous H-ras gene and expressed high levels of p21 compared with the parental HPK1A cells. Table 1 summarizes the growth and differentiation properties of normal human keratinocytes compared with those of the immorcalized HPK1A and malignant HPK1A-ras cell lines. Apart from attaining an indefinite lifespan in culture, immortalized cells retained many features characteristic of normal keratinocytes, such as contact inhibition, production of normal amounts of extracellular matrix proteins and development of cell-specific differentiation markers when triggered to differentiate by loss of contact with substrata. Transformation of these

cells with an activated H-ras oncogene induced changes associated with the acquisition of a malignant phenotype, including loss of contact inhibition, colony formation in soft agar and production of invasive squamous cell carcinomas in nude mice. In addition, the malignant HPK1A-ras cells produced increased quantities of extracellular matrix glycoproteins (32), a feature which has been associated with inhibition of terminal differentiation in human keratinocytes (33).

Cell lines were maintained as stocks in Dulbecco's modified eagle medium (DMEM) (Gibco Labs; Grand Is., NY) supplemented with FBS (10%) (Gibco), 1x antibiotic-antimycotic (Gibco) and passaged once or twice weekly. For all experiments cells were seeded in DMEM containing FBS (10%) at a density of 2×10^5 cells/well for HPK1A and 10^5 cells/well for HPK1A-ras in 6 well cluster plates. At 50% confluence, medium was replaced with fresh, unsupplemented DMEM (basal conditions) and incubation continued for 24 h. Following this 24 h serum-free period, medium was replaced with fresh unsupplemented DMEM or medium containing various combinations of FBS, EGF and $1,25(OH)_2D_3$. Incubations were continued at 37°C with 5% CO_2 for the appropriate times. For experiments in which ambient calcium was varied, minimal essential medium (MEM) (Gibco) supplemented with $CaCl_2$ was used in place of DMEM.

Assay of Immunoreactive PLP (iPLP) in Conditioned Medium: Conditioned medium (CM), 1.5 ml/well, was removed at the appropriate times and centrifuged to remove debris. Duplicate aliquots of 200-500 µl were evaporated to dryness in a Speed-Vac (Savant Inst. Inc., Hicksville, NY) and stored at -20 °C until assayed. Dried medium was reconstituted with

300 μl of outdated blood bank plasma (OBBP) and radioimmunoassayed as described previously (11). Briefly, antiserum raised in rabbits against synthetic hPLP(1-34) (Institut Armand Frappier, Montreal, Canada) was used at a final dilution of 1:35,000 and synthetic hPLP(1-34), diluted in OBBP, was used as standard. The detection limit of the assay was 0.1 ng equivalents (eq) of hPLP(1-34)/ml of CM. PLP immunoreactivity was undetectable in equivalent amounts of unconditioned medium containing FBS (10%) or EGF (10 ng/ml) and treated in a similar manner. Results are expressed as ng equivalents of hPLP(1-34)/10^5 cells.

To determine the stability of PLP-(1-34), ~ 100,000 cpm/well of tracer used for the radioimmunoassay [^{125}I-Tyr^{0}PLP-(1-34)] was added to 50% confluent cultures of HPK1A and HPK1A-ras cells and to empty 6 well plates for control. CM was removed at timed intervals up to 72 hours and aliquots were tested for protein precipitable tracer using 10% trichloroacetic acid (TCA).

Assay of Bioactive PLP (bioPLP) in Conditioned Medium: Samples of CM kept frozen at -20°C were defrosted at the time of assay and mixed 1:1 with MEM/HANKS (Gibco) containing bovine serum albumin (BSA) (Gibco) and isobutylmethylxanthine (IBMX) (Sigma) to give a final concentration of 0.2% BSA and 1 mM IBMX. Following adjustment to pH 7.5 with dilute hydrochloric acid, samples were tested in the UMR 106 adenylate cyclase bioassay as described previously (34). Briefly, confluent layers of cells incubated for 2 h with 0.5 μCi/well [^{3}H]adenine (16 Ci/mM, New England Nuclear (NEN, Boston, MA) were washed twice with Hanks balanced salt solution (HBSS) (Gibco) and incubated for 10 minutes at 22°C with

keratinocyte CM prepared as described above. The reaction was stopped by aspiration of the medium and addition of 0.5 ml of TCA (10%). Approximately 2500 cpm [^{14}C]cAMP (53 mCi/ml:NEN) were added to measure recovery when [^{3}H]cAMP was isolated from other adenylated nucleotides by the method of Salomon et al (35). hPLP(1-34), assayed in multiple dilutions, was used as a standard. Bioactive PLP in 1.5 ml CM was corrected for cell numbers and expressed as ng equivalents hPLP(1-34)/10^6 cells.

Cell Counts: Following removal of CM, cells were trypsinized, dispersed and an aliquot taken for counting in a coulter counter (Coulter Electronics, Beds. UK). Remaining cells were centrifuged at low speed, rinsed with phosphate buffered saline (PBS), lysed with 4M guanidine thiocyanate, 25mM trisodium citrate 1mM EDTA and 1mM ß-mercaptoethanol (GTC mix), and stored at -70°C for subsequent analysis by Northern or dot blot hybridization.

RNA Analysis: For Northern blot hybridization, GTC extracts were purified by cesium chloride gradient centrifugation (36) and 10 μg of total RNA was electrophoresed on a 1.1% agarose-formaldehyde gel (20). For dot blot hybridization, samples were processed as described previously (37). Air dried filters were baked for 2 h at 80°C then hybridized (38) with a 537-bp SacI, HindIII restriction fragment encoding exon III (coding region) of the human PLP gene. This restriction fragment was labeled with [^{32}P]dCTP (ICN Biomedicals Canada Ltd., Mississauga, ON) by the random primer method (Amersham Canada Ltd., Oakville, ON). Following a 24 h incubation at

42°C, filters were washed twice for 30 minutes with 2 x SSC, 0.1% SDS at 50°C (1 x SSC is 0.15 M sodium chloride, 0.015 M trisodium citrate). Autoradiography of filters was carried out at -70°C using XAR film (Eastman Kodak Co., Rochester, NY) with two intensifying screens Dot blot intensity was analysed by laser densitometry (Ultroscan XL, LKB Instruments Inc., Gaithersburg, MD). Gels were stained with ethidium bromide to ensure equivalent quantities of RNA were loaded into all lanes. In addition, filters were probed with an 800-bp BamH1 restriction fragment of rat cyclophilin as a control for PLP mRNA changes.

Statistical Analysis: All results are expressed as the mean ± SEM of replicate determinations and statistical comparisons based on the Student's t-test. A probability value of <0.01 was considered to be significant.

Results

Effect of FBS and $1,25(OH)_2D_3$ on PLP Gene Expression

Northern blot analysis of total RNA from FBS stimulated HPK1A-ras cells revealed the presence of a predominant 1.6 kb PLP transcript and a minor 2.1 kb transcript which reached maximum expression at 6 h, returning to basal levels thereafter (Fig. 1). Addition of $1,25(OH)_2D_3$ (10^{-7} M) to the culture medium produced a slight inhibition of this early increase in PLP mRNA and an apparent enhancement at 24h. Similar results were observed for HPK1A cells, except that a sustained inhibition was noted at 24h. Dot blot analysis of total RNA from HPK1A and HPK1A-ras cells

treated with FBS (10%) revealed a time-dependent increase in PLP mRNA which was greater in HPK1A-ras than in HPK1A (Fig 2A). However, when these increases were expressed as a fold stimulation above basal, values were far greater for HPK1A than for HPK1A-ras (Fig. 2B). In addition, varying concentrations of FBS elicited dose-dependent increases in the induction of PLP mRNA which, in absolute units, were greater in HPK1A-ras than in HPK1A (Fig. 3A), but when expressed as a fold stimulation above basal, these increases were far greater for HPK1A than for HPK1A-ras (Fig. 3B). Analysis of the inhibition of FBS-stimulated PLP mRNA expression by varying concentrations of $1,25(OH)_2D_3$ revealed significant inhibition at lower concentrations in HPK1A cells than in HPK1A-ras cells (Fig. 3C).

Stability of ^{125}I-Tyr^{0}PLP-(1-34) in Culture

After 72 h in culture, ^{125}I-Tyr^0 PLP-(1-34) was shown to be ~70% protein precipitable (Table 2). No differences were observed between samples incubated in the presence or absence of cells or between samples taken from HPK1A compared with HPK1A-ras cultures.

Time Course of Unstimulated PLP Secretion

To determine the characteristics of PLP secretion in established (HPK1A) and malignant (HPK1A-ras) human keratinocytes, we first measured iPLP in CM removed at timed intervals from cells maintained under basal conditions (Fig 4). PLP secretion increased linearly in CM of both cell lines, however, HPK1A-ras secreted significantly more iPLP than HPK1A at all times tested.

Time Course of FBS and EGF Stimulated PLP Secretion: Correlation of Immunoreactive with Bioactive PLP

The PLP secretory response to exogenous growth factors was next examined. Addition of FBS (10%) to cultures of HPK1A-ras elicited a time dependent augmentation in iPLP (Fig. 5) which exceeded that seen in CM from HPK1A-ras cultured under basal conditions (Fig. 4). Using adenylate cyclase stimulation in UMR 106 osteosarcoma cells as an index of bioactivity, bioactive PLP in CM was shown to correlate highly significantly ($r=0.98$, $p<0.01$) with iPLP (Fig. 5 insert) although bio PLP at each time point was approximately 50% of the immunoreactive value (Fig. 5). A similar time-dependent enhancement of iPLP release by FBS was seen in HPK1A cultures and a similar correlation between bioPLP and iPLP was seen in CM harvested from FBS stimulated cultures of HPK1A over the same time course. In all subsequent experiments, to facilitate multiple measurements, only iPLP was determined. Addition of EGF (10 ng/ml) produced a time-dependent increase in iPLP secretion in CM from HPK1A-ras which was significantly greater than the increase in iPLP in CM from HPK1A-ras cultured under basal conditions (Fig. 6) and similar results were also obtained for HPK1A.

Dose Dependency of EGF and FBS Stimulated PLP Secretion

The concentration dependent effect of EGF and FBS on PLP secretion was next examined. Increasing concentrations of either EGF (Fig. 7 panel A and B) or FBS (Fig. 7 panel C and D) produced progressive increases in iPLP in CM from cultures of both HPK1A and HPK1A-ras. Although the level of iPLP after 48h in basal conditions in HPK1A CM was 8-fold less than in

HPK1A-ras CM (panels A and C), EGF and FBS-stimulated increases, when expressed as a fold stimulation above basal (panels B and D), were far greater for HPK1A (maximum 13 x basal) than for HPK1A-ras (maximum 3 x basal).

Effect of $1,25(OH)_2D_3$ on EGF and FBS Stimulated PLP Secretion

Addition of $1,25(OH)_2D_3$ (10^{-8} M) to EGF (10 ng/ml) stimulated cultures of NHK and HPK1A produced a significant reduction in PLP secretion, which was greater in NHK than in HPK1A cells (Table 3). No reduction in PLP secretion was seen in EGF stimulated cultures of HPK1A-ras cells treated with 10^{-8} M $1,25(OH)_2D_3$ (Table 3). iPLP was next measured in CM from unstimulated (basal conditions) cultures of HPK1A and HPK1A-ras in the presence or absence of $1,25(OH)_2D_3$ (10^{-8} M) (Table 4). Basal secretion was reduced to a greater extent in HPK1A than in HPK1A-ras. Increasing concentrations of $1,25(OH)_2D_3$, were next shown to inhibit PLP secretion into CM in both EGF stimulated (Fig. 8A) and FBS stimulated (Fig. 8B) cultures of HPK1A and HPK1A-ras, however a greater degree of inhibition was observed in HPK1A than in HPK1A-ras cultures. In addition, $1,25(OH)_2D_3$ (10^{-8} M) was capable of sustained inhibition of PLP secretion at 72h in HPK1A (14% in EGF stimulated cultures and 35% in FBS stimulated cultures) but not in HPK1A-ras, where there was a small augmentation in PLP secretion in $1,25(OH)_2D_3$ (10^{-8} M) treated cultures at 72h (+9% in EGF stimulated cultures and +4% in FBS stimulated cultures).

Effect of Extracellular Calcium Concentration on EGF Stimulated PLP Secretion

A significant reduction in iPLP at 24 h was noted in CM from both HPK1A and HPK1A-ras cells when the calcium concentration of the culture medium was reduced from 2.0 mM Ca^{++} to 0.5 mM Ca^{++}, the reduction in HPK1A cells being greater than that in HPK1A-ras cells (Table 5). By 72 h, both cell lines maintained in 0.5 mM Ca medium produced approximately 50% of the PLP produced by the same cell type maintained in 2.0 mM Ca.

Discussion

We have examined the regulation of PLP production at the level of gene expression and of secretion in a model of human keratinocyte tumor progression. PLP mRNA levels were clearly increased by serum stimulation within 6 hours in both established and malignant cells and then quickly returned to reduced levels. The rapid and transient induction of PLP mRNA in response to mitogenic stimuli has previously been observed by us in normal human keratinocytes IN VITRO (20) and by others in mammary tissue IN VIVO in response to elevations in serum prolactin (39). This time course is analogous to the temporal response to growth factors of some "immediate-early genes", such as c-myc, which have been implicated in cell cycle regulation (40). The transience of PLP gene expression which we observed has also been noted previously (20, 27, 41) and may be a function of decreased mRNA stability conferred by multiple AU motifs present in the 3' untranslated region of the PLP mRNA (8, 42). Superinduction of PLP message expression following inhibition of protein synthesis by cycloheximide (24), another characteristic of rapidly induced genes, lends support to this hypothesis. These characteristics shared with early

response genes may therefore strengthen the postulated role assigned to PLP in modulating cell growth and/or differentiation.

We have previously demonstrated the presence of cis acting regions responsive to growth factors and serum within the PLP promoter region in transient transfection studies with normal human keratinocytes in culture (20). In the present study, both established and malignant cell lines increased PLP gene expression in response to serum however the stimulatory response was greater in the established than in the malignant cells. Whether the site of altered PLP gene induction in the malignant cells resides in altered activation of nuclear transcription or at other regulatory loci remains to be determined.

Normal human keratinocytes in culture have been shown to metabolize $25(OH)_2D_3$ to its active metabolite $1,25(OH)_2D_3$ IN VITRO (43). In addition, $1,25(OH)_2D_3$ receptors have been identified in skin and in cultured keratinocytes (44, 45) and $1,25(OH)_2D_3$ has also been shown to inhibit growth and stimulate differentiation (46,47) in normal human keratinocytes, perhaps in an autocrine manner. In previous studies we demonstrated that $1,25(OH)_2D_3$ inhibited PLP gene expression within hours in normal human keratinocytes, at least in part by inhibiting gene transcription (20). A comparable reduction in c-myc mRNA levels, within the same time frame in response to $1,25(OH)_2D_3$ has been noted by others in these cells (47). In the present study, an inhibitory effect of $1,25(OH)_2D_3$ on inducible PLP gene expression was present in the established cell line, HPK1A, but was markedly diminished in the malignant variant. Consequently, inhibitory as well as stimulatory control loci of PLP gene expression appeared dysregulated in the progression to the malignant

state.

We next assessed the characteristics of PLP secretion in this model PLP concentrations were determined in unextracted conditioned medium using an NH_2-terminal radioimmunoassay which correlated well with bioactive concentrations. Differences in absolute levels found between the two methods may be due to the molecular species of PLP released into conditioned medium which might differ in reactivity, relative to the synthetic PLP-(1-34) standard, in the radioimmunoassay relative to the bioassay. Consequently, all PLP values were expressed as ng-equivalents of the synthetic PLP-(1-34) standard rather than as absolute units. The secretory rate of PLP from unstimulated cultures of malignant HPK1A-ras cells was approximately three times that of the established HPK1A cells, however maximal concentrations of PLP secretion in response to EGF and FBS were similar in both cell lines. This occurred because the increment of stimulation above basal was far less for HPK1A-ras than for HPK1A cells, suggesting a decreased dependence on exogenous growth factors by the malignant keratinocytes. This reduced augmentation of the secretory response to exogenous growth factors therefore paralleled the reduced augmentation of PLP mRNA which was observed in the malignant cells. Increased production of endogenous growth stimulatory factors, for example TGFα (48), could be one explanation for this phenomenon. Alternatively, altered and/or constitutively active EGF receptors might explain the relative independence from exogenous ligand by the malignant cells.

We also demonstrated an increasing resistance to $1,25(OH)_2D_3$ inhibition of both unstimulated and growth factor (GF) stimulated PLP secretion in the progression from established to malignant phenotype. 10^{-8}

M $1,25(OH)_2D_3$ was most effective in reducing PLP secretion in EGF stimulated normal human keratinocytes (NHK). Although $1,25(OH)_2D_3$ (10^{-8} M) was capable of a sustained, significant reduction of both basal and GF enhanced PLP secretion in HPK1A cells, a higher concentration of $1,25(OH)_2D_3$ (10^{-7} M) was required to significantly inhibit PLP secretion in HPK1A-ras cultures. Furthermore, in these cells, a small increase was observed in PLP secretion at 72h preceded by an increase in PLP mRNA at 24h. Reduced responsiveness to $1,25(OH)_2D_3$ at the secretory level therefore paralleled the diminished responsiveness seen at the level of gene expression. This resistance to the action of $1,25(OH)_2D_3$ may be due to functional defects in either the steroid or DNA binding domains of the receptor protein, to defects in other trans activating factors concomitantly required for vitamin D action at the level of gene expression, or to alterations in the vitamin D response elements in the PLP promoter or in the regulatory regions of other genes involved in modulating effects of $1,25(OH)_2D_3$ on PLP production. Further studies will be required to define the precise mechanisms.

The concentration of extracellular calcium has been shown to regulate both PTH and PLP secretion IN VITRO. However, whereas low ambient calcium stimulates PTH and PLP secretion in parathyroid cells (22, 37, 49), rat Leydig tumor cells, which release PLP in culture, respond to decreased levels of calcium with diminished secretion of PTH-like bioactivity (28). In the present studies we have shown that a reduction in extracellular calcium results in a greater initial decrease in iPLP secretion into the culture medium by established than malignant keratinocytes. This reduction of PLP secretion may be due to a change in

differentiation/proliferation of the cells induced by the low calcium or to a direct effect on PLP production. As previous studies in normal keratinocytes have failed to demonstrate effects of extracellular calcium on PLP gene promoter activity, further studies will be required to determine if the calcium induced modulation of PLP secretion seen in these transformed cells is accomplanied by modulation at the transcriptional level.

Our studies therefore show, with respect to PLP production, that several regulatory mechanisms present in normal, human keratinocytes are altered in the progression to the malignant phenotype Since squamous cell cancers represent an important component of malignancies which overproduce PLP IN VIVO and are associated with hypercalcemia, these findings may have important implications for understanding the pathogenetic mechanisms involved IN VIVO in the development of hypercalcemia in malignancy.

Acknowledgements

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References

1. Muggia, F.M. Overview of cancer-related hypercalcemia: epidemiology and etiology. Seminars in Oncology, 17: 3-9, 1990.

2. Broadus, A E., Mangin, M., Ikeda, K., Insogna, K.I., Weir, E.C., Burtis, W.J., and Stewart, A.F. Humoral hypercalcemia of cancer: Identification of a novel parathyroid hormone-like peptide. New Eng. J. Med , 319· 556-563, 1988.

3. Moseley, J.M., Kubota, M., Diefenbach-Jagger, H., Wettenhall, R.E.H., Kemp, B.E., Suva, L.J., Rodda, C.P., Ebeling, P.R., Hudson, P.J., Zajac, J.D. and Martin, T.J. Parathyroid hormone-related protein purified from a human lung cancer cell line. Proc. Natl. Acad. Sci.(USA), 84: 5048-5052, 1987.

4. Stewart, A.F., Wu, T., Goumas, D., Burtis, W.J. and Broadus, A.E. N-terminal amino-acid sequence of two novel tumor-derived adenylate cyclase-stimulating proteins: Identification of parathyroid hormone-like and parathyroid hormone-unlike domains. Biochem. Biophys. Res. Commun. 146: 672-678, 1987.

5. Strewler, G.J., Stern, P.H., Jacobs, J.W., Eveloff, J., Klein, R.F., Leung, S.C., Roseblatt, M. and Nissenson, R.A. Parathyroid hormone-like protein from human renal carcinoma cells. Structural and functional homology with parathyroid hormone. J. Clin. Invest. 80: 1803-1807, 1987.

6. Suva, L.J., Winslow, G.A.A., Wettenhall, R.E.H., Hammonds, R.G., Moseley, J.M., Diefenbach-Jagger, H., Rodda, c.P. Kemp, B.E., Rodriguez, H., Chen, E.Y., Hudson, P.J., Martin, T. J. and Wood, W.K. A parathyroid hormone-related protein implicated in malignant hypercalcemia· Cloning and expression. Science, 237: 893-896, 1987.

7. Mangin, M., Webb, A.C., Dreyer, B.E., Posillico, J.T., Ikeda, K., Weir, E.C., Stewart, A.F., Bander, N.H., Milstone, L., Barton, D.E., Francke, U. and Broadus, A.E. Identification of a cDNA encoding a parathyroid hormone-like peptide from a human tumor associated with humoral hypercalcemia of malignancy. Proc. Natl. Acad. Sci. (USA), 85: 597-601, 1988.

8. Thiede, M.A., Strewler, G.J., Nissenson, R.A., Rosenblatt, M. and Rodan, G.A. Human renal carcinoma expresses two messages encoding a parathyroid hormone-like peptide: Evidence for the alternative splicing of a single copy gene. Proc. Natl. Acad. Sci. (USA), 85: 4605-4609, 1988.

9. Yasuda, T., Banville, D., Rabbani, S.A., Hendy, G.N. and Goltzman, D. Rat parathyroid hormone-like peptide: comparison with the human homologue and expression in malignant and normal tissue. Mol. Endocrinol., 3: 518-525, 1989.

10. Budayr, A.A., Nissenson, R.A., Klein, R.F., Pun, K.K., Clark, O.H., Diep, D., Arnaud, C.D. and Strewler, G.J. Increased serum levels of a parathyroid hormone-like protein in malignancy-associated hypercalcemia. Ann. Int. Med. 111: 807-812, 1989.

11. Henderson, J.E., Shustik, C., Kremer, R., Rabbani, S.A , Hendy, G.N. and Goltzman, D. Circulating concentrations of parathyroid hormone-like peptide in malignancy and in hyperparathyroidism. J. Bone Min. Res. 5: 105-113, 1990.

12. Burtis, W.J., Brady, T.G., Orloff, J.J., Ersbak, J.B., Warrell, R.P., Olson, B.R., Wu, T.L., Mitnick, M.A.E., Broadus, A.E. and Stewart, A.F. Immunochemical characterization of circulating parathyroid hormone-related protein in patients with humoral hypercalcemia of cancer. N. Eng. J. Med. 322: 1106-1112, 1990.

13. Orloff, J.J., Wu, T.L. and Stewart, A.F. Parathyroid hormone-like proteins: Biochemical responses and receptor interactions. Endocrine Rev. 10: 476-495, 1989.

14. Goltzman, D., Hendy, G.N. and Banville, D. Parathyroid hormone-like peptide: Molecular characterization and biological properties. T.E.M. Sept/Oct, 39-44, 1989.

15. Kukreja, S.C., Shevrin, D.H., Wimbiscus, S.A., Ebeling, P.R., Danks, J.A., Rodda, C.P., Wood, W I. and Martin, T.J. Antibodies to parathyroid hormone-related protein lower serum calcium in athymic mouse models of malignancy-associated hypercalcemia due to human tumors. J. Clin. Invest. 82: 1782-1802, 1988.

16. Henderson, J., Bernier, S., D'Amour, P. and Goltzman, D. Effects of passive immunization against parathyroid hormone (PTH)-like peptide and PTH in hypercalcemic tumor-bearing rats and normocalcemic controls. Endocrinology, 127: 1310-1318, 1990.

17. Stewart, A.F., Insogna, K.L., Goltzman, D. and Broadus, A E. Identification of adenylate cyclase-stimulating activity and glucose-6-phosphate dehydrogenase-stimulating activity in extracts of tumors from patients with humoral hypercalcemia of malignancy. Proc. Natl. Acad. Sci. (USA), 80: 1454-1458, 1983.

18. Rabbani, S.A., Mitchell, J., Roy, D.R., Kremer, R., Bennett, H.P.J. and Goltzman, D. Purification of peptides with parathyroid hormone-like bioactivity from human and rat malignancies associated with hypercalcemia. Endocrinology, 118: 1200-1210, 1986.

19. Merendino, J.J., Insogna, K.L., Milstone, L.M., Broadus, A.E. and Stewart, A.F. A parathyroid hormone-like protein from cultured human keratinocytes. Science, 231: 388-390, 1986.

20. Kremer, R., Karaplis, A.C., Henderson, J., Gulliver, W., Banville, D., Hendy, G.H. and Goltzman, D. Regulation of parathyroid hormone-like peptide in cultured normal human keratinocytes. Effect of growth factors and 1,25 dihydroxyvitamin D_3 on gene expression and secretion. J. Clin. Invest. 122: 884-893, 1991.

21. Drucker, D.J., Asa, S.L., Henderson, J. and Goltzman, D. The parathyroid hormone-like peptide gene is expressed in the normal and neoplastic human endocrine pancreas. Mol. Endocrinol. 3: 1589-1595, 1989.

22. Ikeda, K., Weir, E.C., Sakaguchi, K., Burtis, W.J., Zimering, M., Mangin, M., Dreyer, B.E., Brandi, M.L., Aurbach, G. and Broadus, A.E. Clonal rat parathyroid cell line expresses a parathyroid hormone-related peptide but not parathyroid hormone itself. Biochem. Biophys. Res. Comm. 162: 108-115, 1989.

23. Deftos, L.J., Gazdar, A.F., Ikeda, K. and Broadus, A.E. The parathyroid hormone-related protein associated with malignancy is secreted by neuroendocrine tumors. Mol. Endocrinol. 3: 503-508, 1989.

24. Ikeda, K., Lu, C., Weir, E.C., Mangin, M. and Broadus, A.E. Regulation of parathyroid hormone-related peptide gene expression by cycloheximide. J. Biol. Chem. 265: 5398-5402, 1990.

25. Deftos, L.J., Hogue-Angeletti, R., Chalberg, C. and Tu, S. PTHrP secretion is stimulated by CT and inhibited by CgA peptides. Endocrinol., 125: 563-565, 1989.

26. Rodan, S.B., Wesolowski, G., Ianacone, J., Thiede, M.A. and Rodan, G.A. Production of parathyroid hormone-like peptide in a human osteosarcoma cell line: stimulation by phorbol esters and epidermal growth factor. J. of Endocrinol. 122: 219-227, 1989.

27. Lu, C., Ikeda, K., Deftos, L.J., Gazdar, A.F., Mangin, M. and Broadus, A.E. Glucocorticoid regulation of parathyroid hormone-related peptide gene transcription in a human neuroendocrine cell line. Mol. Endocrinol., 3: 2034-2040, 1989.

28. Rizzoli, R. and Bonjour, J.P. High extracellular calcium increases the production of a parathyroid hormone-like activity by cultured Leydig tumor cells associated with humoral hypercalcemia. J. Bone Min. Res. 4: 839-844, 1989.

29. Durst, M. Gallahan, D., Jay, G. and Rhim, J.S. Glucocorticoid-enhanced neoplastic transformation of human keratinocytes by human papillomavirus Type 16 and an activated ras oncogene. Virology, 173: 767-771, 1989.

30. Boyce, T.S. and Ham, R.G. Cultivation, frozen storage and clonal growth of normal human epidermal keratinocytes in serum-free media. J. Tissue Cult. Methods 9:83-92, 1985.

31. Dürst, M., Dzarlieva-Petrusevska, R.T., Boukamp, P., Fusenig, N.E., and Gissmann, L. Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. Oncogene 1: 251-256, 1987.

32. Allen-Hoffman, B.L., Sheibani, N., Hatfield, J.S., and Rhim, J.S. Use of immortalized human keratinocytes for the study of squamous differentiation and mutagenesis. In: Neoplastic Transformation in Human Cell Systems in vitro: Mechanisms of Carcinogenesis. Ed. J. Rhim and A. Dritschilio. In press.

33. Adams, J.C. and Watt, F.M. Fibronectin inhibits the terminal differentiation of human keratinocytes. Nature 340:307-309, 1989.

34. Rabbani, S.A., Mitchell, J., Roy, D.R., Hendy, G.N. and Goltzman, D. Influence of the amino-terminus on in vitro and in vivo biological activity of synthetic parathyroid hormone-like peptides of malignancy. Endocrinology, 123: 2709-2716, 1988.

35. Salomon, Y., Londos, C. and Rodbell, M. A highly sensitive adenylate cyclase assay. Anal. Biochem. 58: 541-548, 1974.

36. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rulter, W.J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry, 18: 5294-5299, 1979.

37. Kremer, R., Bolivar, I., Goltzman, D. and Hendy, G.N. Influence of calcium and 1,25 dihydroxycholecalciferol on proliferation and proto-oncogene expression in primary cultures of bovine parathyroid cells. Endocrinology, 125: 935-941, 1989.

38. Thomas, P.S. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. (USA), 77: 5201-5205, 1980.

39. Thiede, M.A. The mRNA encoding a parathyroid hormone-like peptide is produced in mammary tissue in response to elevations in serum prolactin. Mol Endocrinol. 3: 1443-1447, 1989.

40. Woodgett, J.R. Early gene induction by growth factors. Brit. Med Bull. 45: 529-540, 1989

41. Thiede, M.A , Daifotis, A.G., Weir, E.C., Brines, M.L. Burtis, W.J , Ikeda, K., Dreyer, B.E., Garfield, R.E., and Broadus, A.E. Intrauterine occupancy controls expression of the parathyroid hormone-related peptide gene in preterm rat myometrium. Proc. Natl. Acad. Sci. (USA) 87: 6969-6973, 1990.

42. Yasuda, T., Banville, B., Hendy, G.N. and Goltzman, D. Characterization of the human parathyroid hormone-like peptide gene: functional and evolutionary aspects. J. Biol. Chem. 264: 7720-7725, 1989.

43. Bikle, D., Nemanic, M.K., Gee, E. and Elias, P. 1,25-dihydroxyvitamin D_3 production by human keratinocytes. J. Clin. Invest. 78: 557-566, 1986.

44. Feldman, D., Chen, T., Herst, M., Colston, K., Karasek, M. and Cone, C. Demonstration of 1,25-dihydroxyvitamin D_3 receptors in human skin biopsies. J. Clin. Endocrinol. Metab. 51: 1463-1465, 1980.

45. Pillai, S., Bickle, D. and Elias, P.M. 1,25-dihydroxyvitamin D_3 production and receptor binding in human keratinocytes varies with differentiation. J. Biol. Chem. 263: 5390-5395, 1988.

46. Smith, E.L., Walworth, N.C. and Holick, M.F. Effect of 1α,25-Dihydroxyvitamin D_3 on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions. J. Invest. Derm. 86: 709-714, 1986.

47. Matsumoto, K., Hashimoto, K., Nishida, Y., Hashiro, M. and Yoshikawa, K. Growth inhibitory effects of 1,25 dihydroxyvitamin D_3 on normal human keratinocytes cultured in serum-free medium. Biochem. Biophys. Res. Comm. 166: 916-923, 1990.

48. Coffey, R.J., Derynck, R., Wilcox, J.N., Bringman, T.S., Goustin, A.S. and Pittelkow, M.R., Production and auto-induction of transforming growth factor-alpha in human keratinocytes. Nature (Lond.), 328: 817-820, 1987.

49. Muffs, R , Nemeth, E.F., Haller-Brem, S. and Fischer, J.A. Regulation of hormone secretion and cytosolic Ca^{2+} by extracellular Ca^{2+} in parathyroid cells and c-cells: role of voltage-sensitive Ca^{2+} channels. Arch. Biochem. Biophys. 265: 128-135, 1988.

Table 1 **Growth and differentiation properties of normal, immortalized and malignant human keratinocytes in culture.**

	Benign NHK	Immortalized HPK1A	Malignant HPK1A-*ras*
Stratification[a]	+++	-	-
Cell envelopes[b]	+++	+	+
Involucrin[b]	+++	++	+
Passages in culture[a,c]	+	+++	+++
Saturation density[c]	+	+	+++
Extracellular matrix[b,d]	+	+	+++
Agar colonies[c]	-	-	+++
Tumor formation[c]	-	-	+++

a. Dürst *et al.* (Reference 31).

b. Allen-Hoffmann *et al.* (Reference 32).

c. Durst *et al.* (Reference 29).

d. Adams *et al.* (Reference 33).

Table 2 Stability of ^{125}I-Tyr^{0}PLP-(1-34) in culture.

	% cpm Protein Precipitable[a]		
	Control mean ± SEM	HPK1A mean ± SEM	HPK1A-ras mean ± SEM
24h	74 ± 4	81 ± 7	80 ± 6
48h	69 ± 3	72 ± 4	73 ± 4
72h	72 ± 4	67 ± 7	70 ± 4

a. ~ 100,000 cpm ^{125}I-Tyr^{0}PLP-(1-34) were added to empty wells (Control) or 50% confluent cultures (HPK1A and HPK1A-ras) at time 0 as described in **Materials and Methods** and aliquots were removed at timed intervals for TCA precipitation. The tracer prior to incubation was 95.6% protein precipitable. Values represent the mean ± SEM of triplicate determinations and are representative of 3 different experiments.

Table 3 **Effect of $1,25(OH)_2D_3$ on EGF stimulated PLP secretion in NHK, HPK1A and HPK1A-ras keratinocytes.**

	NHK[a]		HPK1A[b]		HPK1A-ras[b]	
	ng eq/10^6 cells mean ± SEM	%	ng eq/10^6 cells mean ± SEM	%	ng eq/10^6 cells mean ± SEM	%
Basal	1.05 ± 0.17	100	1.02 ± 0.09	100	2.36 ± 0.23	100
EGF	3.20 ± 0.36	305	2.90 ± 0.30	284	4.30 ± 0.35	182
EGF + $1,25(OH)_2D_3$	1.25 ± 0.32	119[c]	1.80 ± 0.15	176[c,d]	4 09 ± 0.25	175[d]

a. Following 24h in KBM supplemented with insulin, hydrocortisone and 0.15 mM calcium, fresh medium supplemented with 10 ng/ml EGF with or without 10^{-8} M $1,25(OH)_2D_3$, was added to 50% confluent cultures of NHK as described in **Materials and Methods**.

b. Following 24h in DMEM, fresh medium supplemented with EGF 10 ng/ml with or without 10^{-8} M $1,25(OH)_2D_3$ was added to 50% confluent cultures of HPK1A and HPK1A-ras cells. CM removed from all cultures at 24h was assayed for immunoreactive PLP which is expressed as ng eq hPLP-(1-34)/10^6 cells Each value represents the mean ± SEM of 6 determinations and is representative of 2 different experiments.

c. Significant difference from EGF ($p < 0.01$).

d. Significant difference from NHK incubated with EGF + $1,25(OH)_2D_3$ ($p < 0.01$).

Table 4 Effects of 1,25 $(OH)_2D_3$ on unstimulated iPLP secretion in HPK1A and HPK1A ras.

Cell Line		iPLP ng eq/10^6 cells[a] mean±SEM	% Inhibition
HPK1A	Basal	1.22 ± 0.06	
	1,25$(OH)_2D_3$	0.88 ± 0.04[b]	28
HPK1A-ras	Basal	2.08 ± 0.04	
	1,25$(OH)_2D_3$	1.72 ± 0.12	17

a. Following 24h in basal conditions (no growth factors) medium was replaced at time 0 with fresh basal medium with or without 1,25 $(OH)_2D_3$ (10^{-8} M) in 50% confluent cultures of HPK1A and HPK1A ras. Conditioned medium removed at 24h was assayed for immunoreactive PLP (iPLP) which is expressed as ng equivalents (eq) of hPLP (1-34) /10^6 cells. Each value represents the mean ± SEM of 6 determinations and is representative of 3 separate experiments.

b. Significant difference from Basal ($p < 0.01$).

Table 5 Effect of ambient Ca concentration on EGF stimulated iPLP secretion in HPK1A and HPK1A ras.

	Calcium	iPLP ng eq/10^6 cells[a]			
		24 h mean ± SEM	%[b]	72 h mean ± SEM	%[b]
HPK1A	2.0 mM	2.58 ± 0.20		13.60 ± 1.20	
	0.5 mM	1.37 ± 0.08[c]	47	6.82 ± 0 71[c]	50
HPK1A-ras	2.0 mM	3.45 ± 0.21		11.90 ± 1.20	
	0.5 mM	2.47 ± 0.26[c,d]	28	6.88 ± 0.49[c]	42

a. Following 24h in basal conditions (DMEM without growth factors), fresh MEM containing either 0.5 mM Ca or 2.0 mM Ca, supplemented with EGF (10ng/ml), was added to 50% confluent cultures of HPK1A and HPK1A ras at time 0. Conditioned medium was removed at timed intervals and assayed for immunoreactive PLP (iPLP). Values represent the mean ± SEM of 6 determinations and are expressed as ng equivalents (eq) of hPLP(1-34)/10^6 cells. Data is representative of 2 different experiments.

b. Inhibition (%)

c. Significant difference from 2.0 mM Ca at that time point ($p < 0.01$).

d. Significant difference from HPK1A 0.5 mM Ca at 24h ($p < 0.01$).

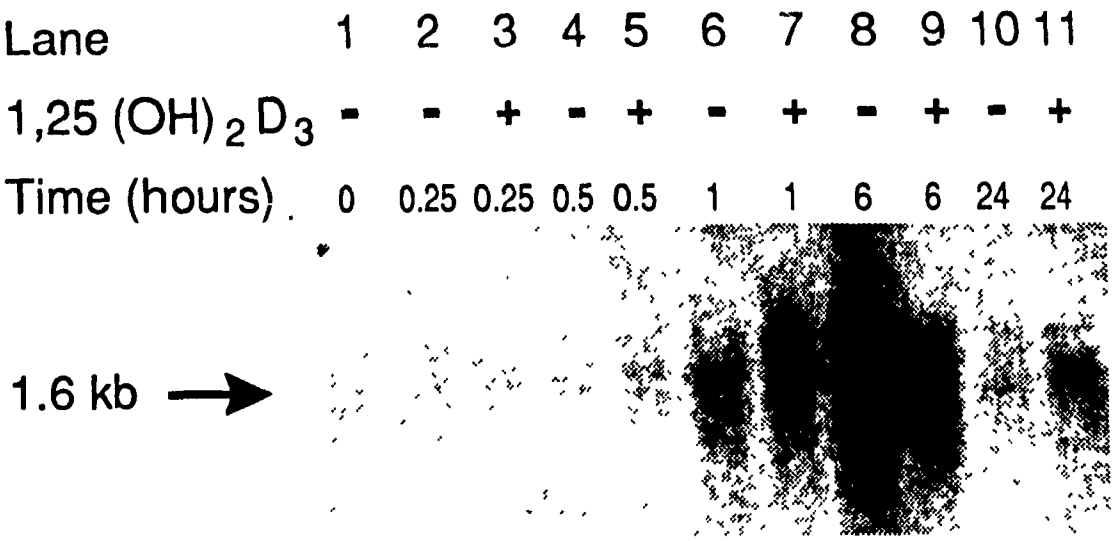

Figure 1. Northern blot analysis of PLP mRNA. Total cellular RNA was prepared as described in **Materials and Methods** from HPK1A-ras cells removed at timed intervals following stimulation with FBS (10%) in the absence (-) or presence (+) of $1,25(OH)_2D_3$ (10^{-7} M). Aliquots of 10 µg/lane were electrophoresed on a 1.1% agarose-formaldehyde gel, blotted onto Nytran filters and probed with a 537-bp restriction fragment encoding exon III of the human PLP gene. Ethidium bromide-stained gels demonstrated equivalent quantities of RNA had been loaded into all lanes. Cyclophilin probed filters showed little change from basal levels of mRNA in all lanes. Data is representative of 2 different experiments.

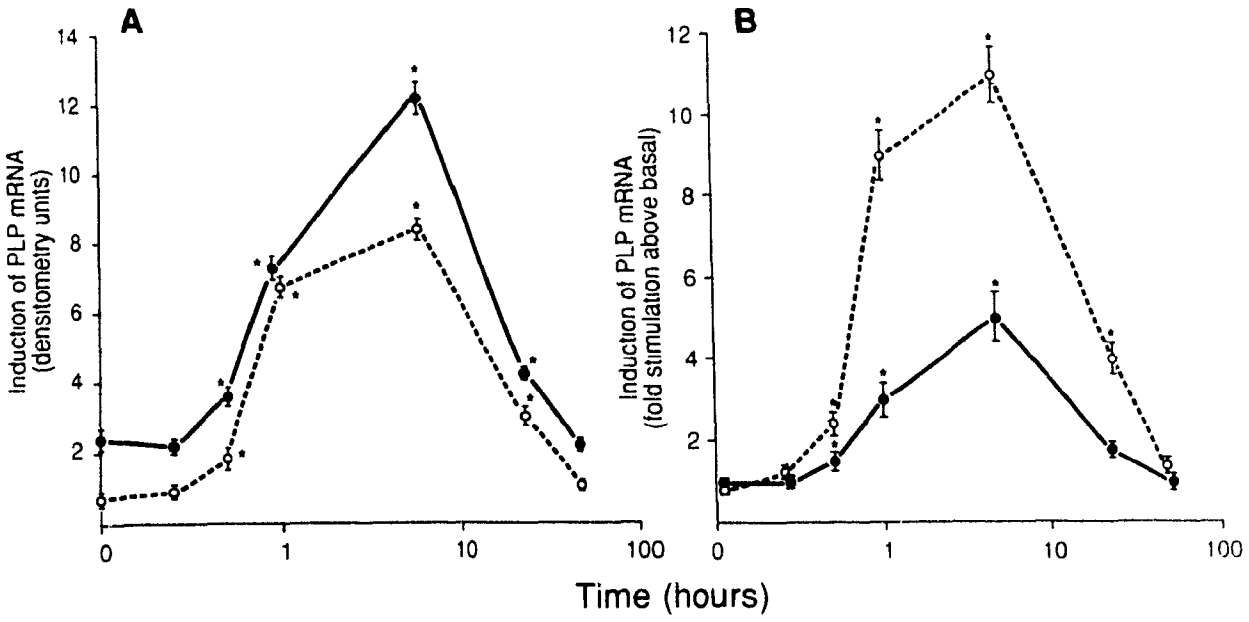

Figure 2. Dot blot analysis of the time course of PLP mRNA induction in HPK1A and HPK1A-ras. GTC extracts of equal numbers of HPK1A (○) and HPK1A-ras (●) cells removed at timed intervals following stimulation with FBS (10%) were subjected to dot blot analysis as described in **Materials and Methods**. Filters were hybridized with a 537 bp restriction fragment encoding exon III of the human PLP gene. Points represents the mean ± SEM of triplicate determinations and are representative of 3 different experiments. mRNA levels are expressed in absolute densitometry units in panel A and as fold stimulation above basal in panel B which in HPK1A-ras was 3 fold greater than in HPK1A cells. The intensity of cyclophilin mRNA showed little change from basal at any time tested. *Significant difference from basal at that time point ($p<0.01$)

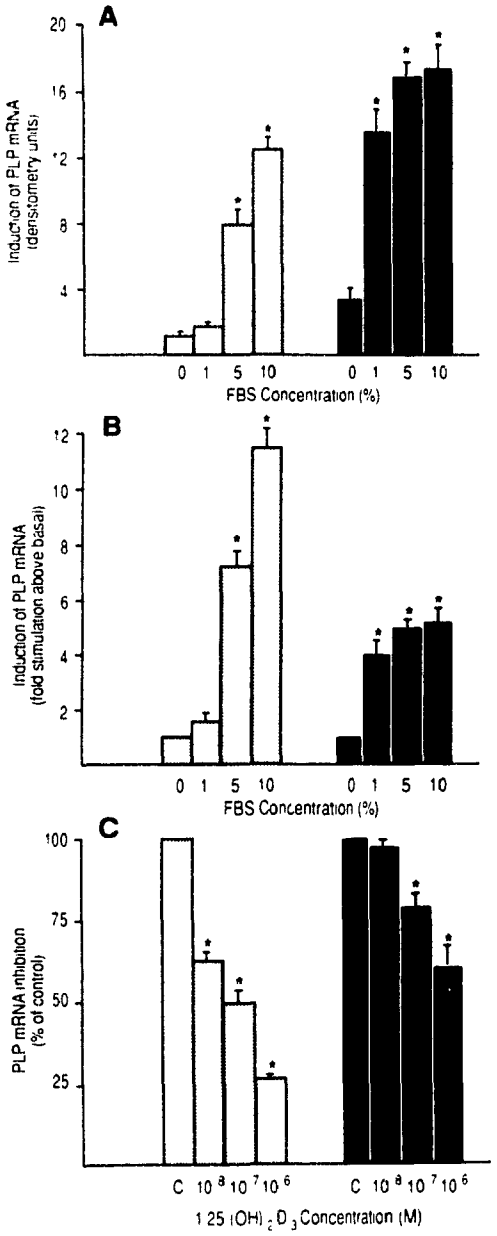

Figure 3. Effect of FBS and $1,25(OH)_2D_3$ on PLP mRNA in HPK1A and HPK1A-ras. GTC extracts of equal numbers of HPK1A (▩) and HPK1A-ras (■) cells removed at 6 h following stimulation with varying concentrations of FBS (panels A and B) or with FBS (10%) in the presence of varying concentrations of $1,25(OH)_2D_3$ (panel C) were subjected to dot blot analysis as described in **Materials and Methods**. Filters were hybridized with a 537 bp restriction fragment encoding exon III of the human PLP gene. Bars represent the mean ± SEM of triplicate determinations. Induction of PLP mRNA is expressed in absolute densitometry units (panel A) and as fold stimulation above basal (0) (panel B) which was 3 fold greater in HPK1A-ras than in HPK1A. Inhibition of PLP mRNA (panel C) is expressed as a % of control (c), which represents the level of PLP mRNA in cells cultured in FBS (10%) in the absence of $1,25(OH)_2D_3$. Data is representative of duplicate experiments. Significant difference from 0 or control *($p<0.01$).

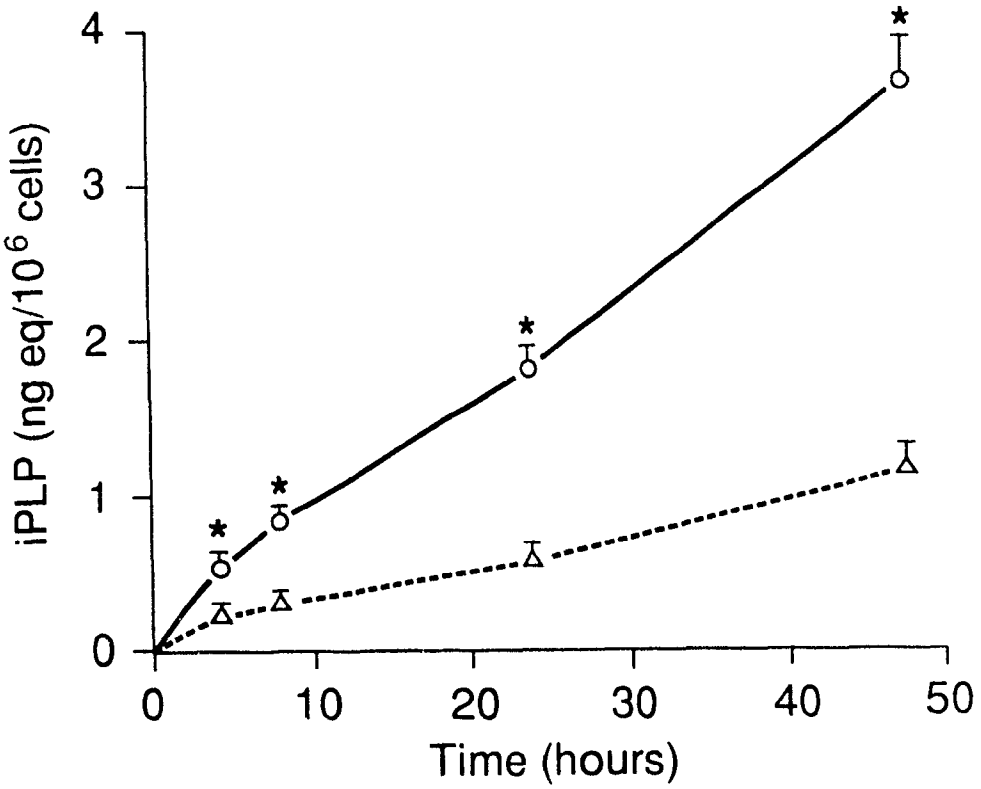

Figure 4. Time course of unstimulated PLP secretion in HPK1A and HPK1A ras. Following 24h in basal conditions (no growth factors) fresh basal medium was added to 50% confluent cultures of HPK1A (Δ) and HPK1A ras (O). Conditioned medium (CM) removed at timed intervals was assayed for immunoreactive PLP (iPLP) as described in **Materials and Methods**. iPLP is expressed as ng equivalents (eq) of hPLP (1-34)/ 10^6 cells. Each value represents the mean ± SEM of triplicate determinations and is representative of 3 separate experiments. *Significant difference from HPK1A at the respective time point ($p < 0.01$).

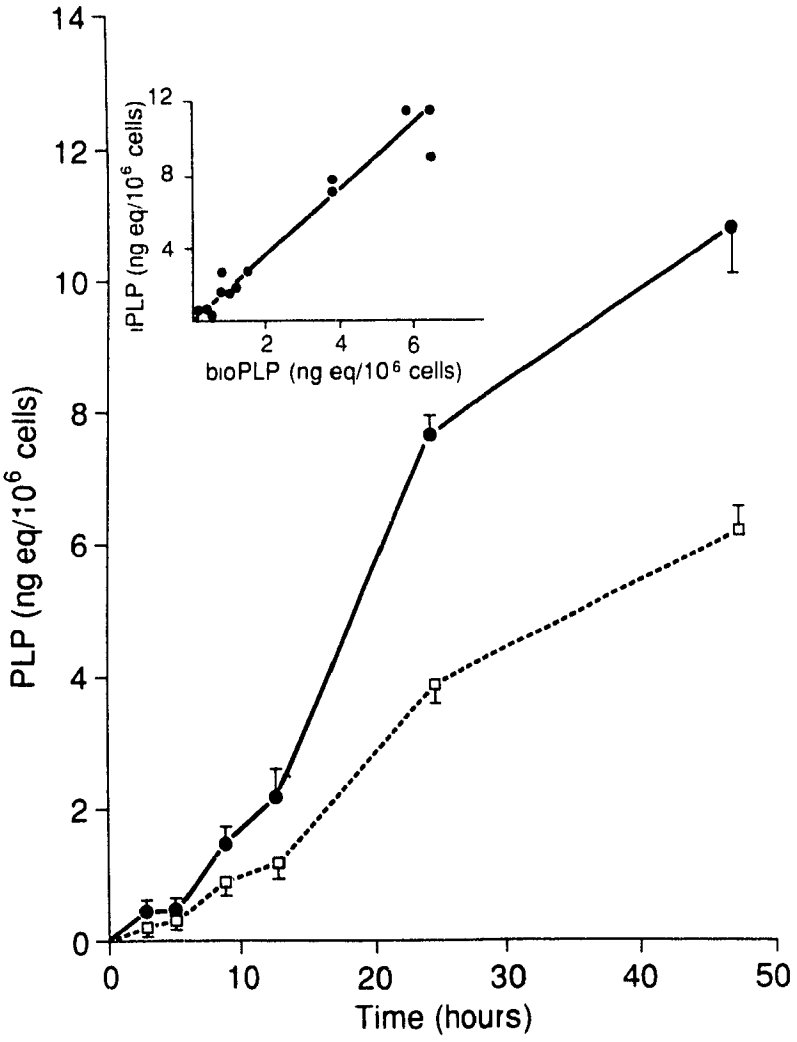

Figure 5. Time course of FBS stimulated immunoreactive (i) and bioactive (bio) PLP in HPK1A ras. Fresh medium containing FBS (10%) was added to 50% confluent cultures of HPK1A ras at time 0. Conditioned medium removed at timed intervals was assayed for immunoreactive (●) and bioactive (□) PLP as described in **Materials and Methods**. Values represent the mean ± SEM of triplicate determinations and are expressed as ng equivalents (eq) of hPLP (1-34) / 10^6 cells. Data is representative of 2 different experiments. Insert represents immunoreactive PLP (iPLP) on the vertical axis plotted against bioactive PLP (bio PLP) on the horizontal axis. Pearson's correlation coefficient equals 0.98 which is significant at $p<0.01$.

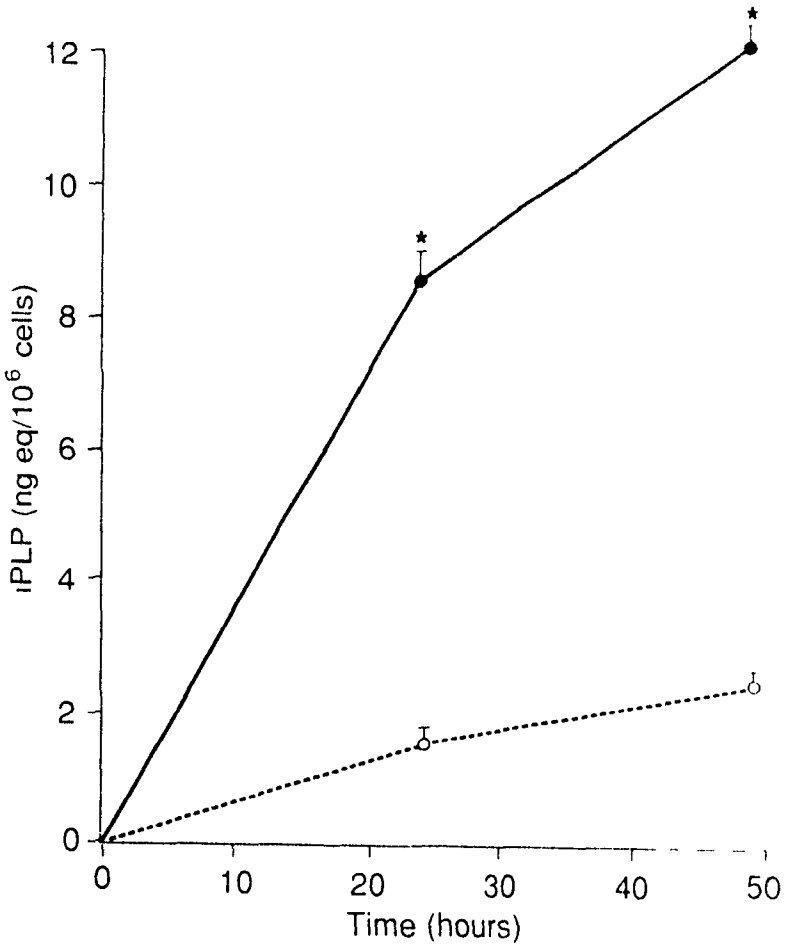

Figure 6. Time course of EGF stimulated PLP secretion in HPK1A ras. Following 24h in basal conditions, fresh medium without (○) or with (●) EGF (10ng/ml) was added to 50% confluent cultures of HPK1A ras Conditioned medium removed at timed intervals was assayed for immunoreactive PLP (iPLP) as described in **Materials and Methods**. Each point represents the mean ± SEM of triplicate determinations and is representative of 3 different experiments. PLP is expressed as ng equivalents (eq) of hPLP (1-34) /10^6 cells. *Significantly different from unstimulated cultures at that time point ($p < 0.01$)

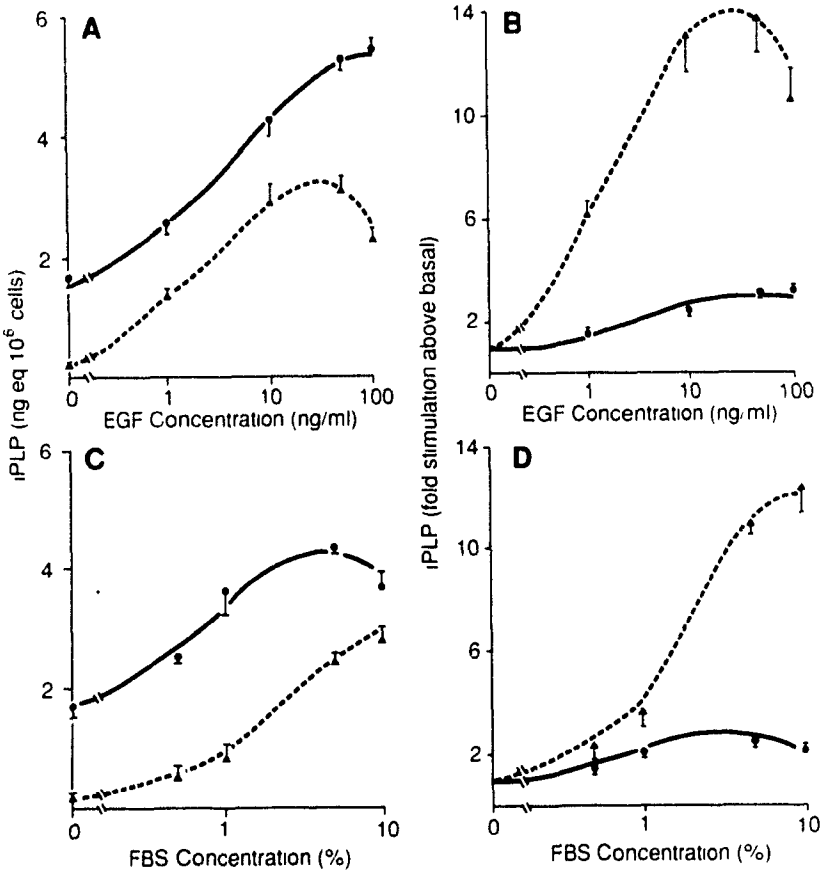

Figure 7. PLP secretion in HPK1A and HPK1A ras in response to varying concentrations of EGF and FBS. Following 24h in basal conditions (no growth factors), fresh medium without or with varying concentrations of either EGF (panels A and B) or FBS (panels C and D) was added to 50% confluent cultures of HPK1A (▲) and HPK1A ras (●) at time 0. Conditioned medium removed 24h later was assayed for immunoreactive PLP (iPLP) as described in Materials and Methods. Each point represents the mean ± SEM of 4-6 determinations and is representative of 3 different experiments iPLP is expressed as ng equivalents (eq) of hPLP (1-34) /10^6 cells in panels A and C, and as fold stimulation above basal activity in panels B and D. Basal activity represents PLP secretion following 48h in basal conditions

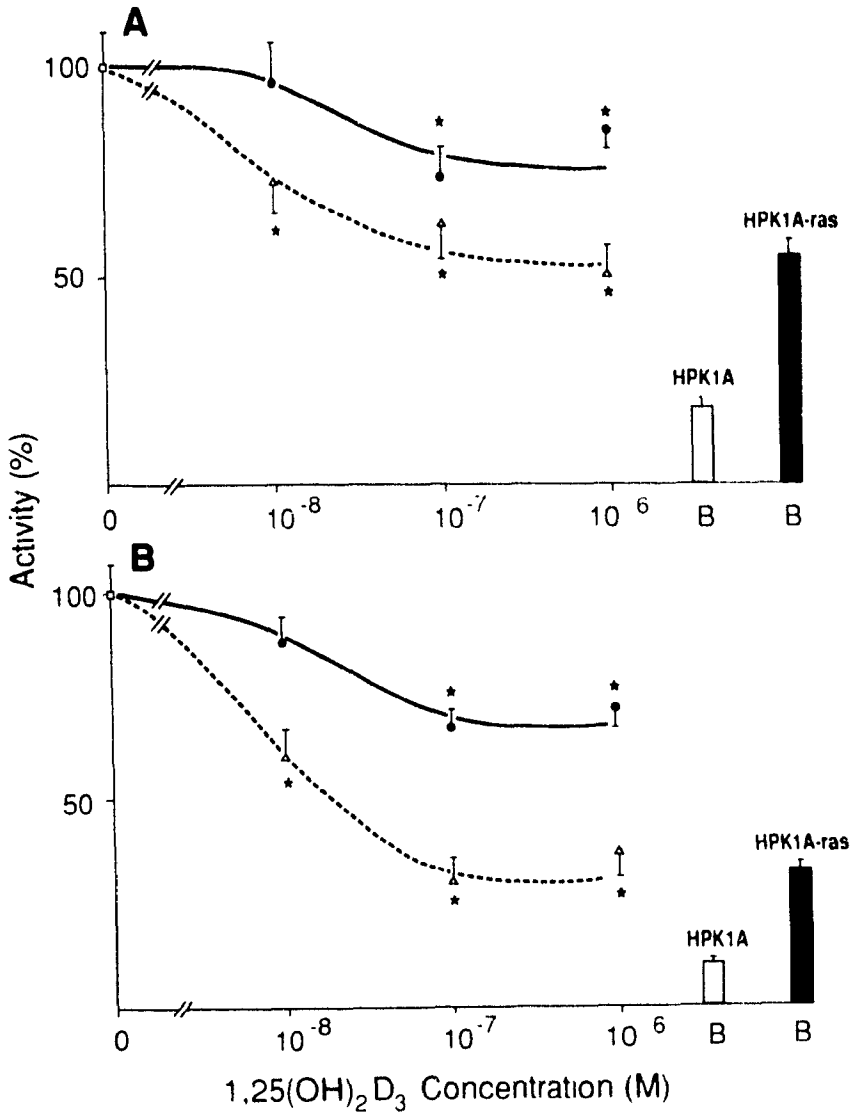

Figure 8. Effect of 1,25(OH)$_2$D$_3$ on EGF and FBS stimulated PLP secretion in HPK1A and HPK1A-*ras* cells. Following 24h in basal conditions, fresh medium containing either EGF (10 ng/ml) (panel A) or FBS (5%) (panel B) with or without varying concentrations of 1,25(OH)$_2$D$_3$, was added to 50% confluent cultures of HPK1A (△) and HPK1A-*ras* cells (●). Basal activity was 0.3 ± 0.02 and 1.1 ± 0.09 ng eq PLP-(1-34)/10^6 cells in panel A and 0.1 ± 0.01 and 0 5 ± 0.03 ng eq PLP-(1-34) /10^6 cells in panel B for HPK1A (△) and PHK1A-*ras* (●) cells respectively 100% activity was 1.56 ± 0.13 and 2.05 ± 0.17 ng eq PLP-(1-34)/10^6 cells in panel A and 1.00 ± 0.09 and 1.46 ± 0.10 ng eq PLP-(1-34)/10^6 cells in panel B for HPK1A and HPK1A-*ras* cells respectively. Each point represents the mean ± SEM of 4-6 determinations and expresses iPLP as a percentage of growth factor stimulated activity. Data is representative of duplicate experiments Significant difference from 100% activity *($p<0.01$).

CHAPTER V IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF ADENYLATE CYCLASE-LINKED RECEPTORS FOR PARATHYROID HORMONE-LIKE PEPTIDES ON IMMORTALIZED HUMAN KERATINOCYTES

Preface

Apart from its localization in neoplastic tissues of widely diverse histological origin, PLP has been shown to be expressed by numerous normal fetal and adult cell types. In addition, PLP was detected in the systemic circulation of normal individuals. Taken together, these observations suggested a potential role for PLP in normal physiology which differed from its parathyroid hormone-like role in malignancy associated hypercalcemia. To explore the possibility that PLP may act in an autocrine manner in a non-classical PLP/PTH target tissue, I examined an established human keratinocyte cell line for evidence of functional PLP receptors.

Abstract

We have identified and characterized receptors for the amino-terminal domains of parathyroid hormone (PTH) and parathyroid hormone-like peptide (PLP) on an immortalized human keratinocyte cell line, RHEK-1 Binding of both PLP-(1-34) and PTH-(1-34) to the RHEK-1 cells was consistent with a two site model, affinities and capacities for each site being similar for the two peptides. Both peptides also stimulated adenylate cyclase activity with an equal ED50 in this cell line. Pertussis toxin pre-treatment enhanced this peptide-mediated enzyme activity suggesting linkage of the receptor to an inhibitory guanyl nucleotide binding protein (Gi). Adenylate cyclase activity was diminished by both homologous [PLP-(1-34)] and heterologous (EGF) effectors. Malignant conversion of the immortalized cells with an activated H-ras oncogene to produce the RHEK-ras cell line was associated with a reduction in binding at both PLP/PTH and EGF receptors as well as a post receptor defect in PLP/PTH stimulated adenylate cyclase activity. The defect in enzyme activity appeared to be due in part to a decrease in the activity of the stimulatory guanyl nucleotide binding protein (Gs) but not due to an increase in Gi activity. Activation of the RHEK-1 amino-terminal PLP/PTH receptor resulted in a small increase in both [^{3}H]thymidine incorporation and cell numbers which was enhanced in the presence of EGF and was markedly reduced when cells were cultured in high calcium medium These studies demonstrate that the amino-terminal region of PLP and PTH activate adenylate cyclase linked receptors which are associated with mitogenesis in RHEK-1 cells and suggest that this cell line represents a suitable model in which to examine the actions of PLP in keratinocytes.

Introduction

Parathyroid hormone-like peptide (PLP) which was originally described as a tumor product, is expressed and secreted by both normal (1, 2) and neoplastic (3, Henderson *et al.* In Press) keratinocytes in culture. In preliminary studies PLP has been implicated as an autocrine modulator of growth and/or differentiation in these cells (4-6), implying the existence of a specific PLP receptor in keratinocytes. PLP and parathyroid hormone (PTH) have been shown to bind with varying affinities to the same receptor not only in the classical PTH target cells of kidney and bone (7-11) but also in a number of other cells, including dermal fibroblasts (12), embryonal carcinoma cells (13) and vascular smooth muscle (14). However, there has been no report to date of a PLP/PTH receptor on keratinocytes. The purpose of this study was therefore to identify and characterize a keratinocyte PLP/PTH receptor Using an immortalized human keratinocyte cell line (RHEK-1) which no longer secretes PLP, we have demonstrated the presence of specific, adenylate cyclase-linked PLP/PTH receptors and have shown that malignant conversion of the established cells is associated with a loss of functional PLP/PTH receptors. In addition, we have examined the capacity of exogenous PLP to modulate the growth characteristics of RHEK-1 cells and to interact with EGF, a known keratinocyte mitogen (15-17).

Materials and Methods

Cell Lines and Culture Conditions

RHEK-1 cells were established as a non-malignant cell-line

from normal, human foreskin keratinocytes in culture following exposure to a hybrid virus (19) adenovirus 12-simian virus 40 (AD12-SV40). Although these cells acquire an indefinite lifespan in culture, they maintain their "flat" epithelial morphology, retain a number of markers associated with epithelial cells, do not form colonies in soft agar and are non-tumorigenic in nude mice (Table 1) (18-21). These immortalized cells were further transformed by stable transfection with an activated human H-ras oncogene, pSV_2-ras (20) into the malignant RHEK-ras cell line. The malignant characteristics acquired by these cells include colony formation in soft agar and the capacity to form invasive squamous cell tumors when injected into nude mice (Table 1). Cells were maintained in Dulbecco's modified eagles medium (DMEM) (Gibco Labs; Grand Is., NY) supplemented with 5% fetal bovine serum (FBS) (Gibco Labs) at 37°C with 5% CO_2, fed routinely twice a week and passaged once a week. After 2-3 months, fresh cells were thawed for experiments.

Tracer Preparation and Binding Studies

For PTH binding, cells were plated at a density of 2×10^5 cells/well in 6 well cluster plates (Becton Dickinson Labware; Lincoln Park, NJ) in DMEM supplemented with 10% FBS and binding studies were carried out 48 h later following a 4 h serum-free period in DMEM ^{125}I-PTH-(1-34) (Bachem, Torrance, CA) was prepared by the lactoperoxidase technique as described previously, using RP-HPLC to separate unlabeled peptide from iodinated ligand (22) Using a ratio of 5 μg PTH-(1-34) per 1 mCi ^{125}I [New England Nuclear (NEN, Boston, MA)] this method yielded a tracer of high specific activity (4.88×10^6 dpm/pmole). Confluent layers

of cells were washed twice with 2 ml Hank's balanced salt solution (HBSS) (Gibco Labs) prior to addition of 120,000 cpm/well ^{125}I-PTH-(1-34) in 0.6 ml minimal essential medium/Hanks salts (MEM/HANKS) (Gibco Labs) supplemented with 15% FBS. Unlabeled hormone was added at varying concentrations in 0.3 ml MEM/HANKS/15% FBS to make a final incubation volume of 0.9 ml

For EGF binding, cells were plated at a density of 5 x 10^4 cells/well in 24 well cluster plates in DMEM supplemented with 10% FBS and binding studies were carried out 48 h later following a 4 h serum-free period in DMEM. ^{125}I-EGF was prepared as described previously (23) using a modification of the chloramine T method, followed by Sephadex G50 purification. This technique yielded a tracer with specific activity of 1.88 x 10^6 dpm/pmole. Confluent layers of cells were washed twice with 0.5 ml HBSS prior to addition of 45,000 cpm/well ^{125}I-EGF in 0.2 ml MEM/HANKS/15% FBS Unlabeled EGF (Sigma Chemical Co., St. Louis, MO) was added at varying concentrations in 0.1 ml MEM/HANKS/15% FBS to make a final incubation volume of 0.3 ml. In some experiments, RHEK-ras cells were washed twice with 1 ml of 150 mM $CaCl_2$ in 0.1% acetic acid containing 1 mg/ml bovine serum albumin (BSA) (Gibco Labs) to remove endogenous ligand immediately prior to binding studies.

For all binding studies, plates were incubated for 2 h at 23° C on a rotational shaker. Following incubation, cells were washed 3 times with HBSS and 2 times with phosphate buffered saline (PBS) before solubilizing in 1N NaOH (1 ml for 6 well plates, 0.5 ml for 24 well plates) and counting in a gamma radiation counter (LKB, Wallac, Finland). Total binding was assessed in the absence of unlabeled peptide and

nonspecific binding in the presence of 10^{-6} M PTH-(1-34) (Bachem), 10^{-6} M PLP-(1-34) (Peninsula Labs; Belmont, CA) or 10^{-7} M EGF (Sigma)

Scatchard analysis of the binding data was accomplished using the LIGAND program. Representative wells from each binding assay were trypsinized and an aliquot was counted on a coulter counter (Coulter Electronics, Beds, UK) to determine cell numbers for the calculation of receptor sites/cell.

Adenylate Cyclase Assays

Cells were plated at a density of 5×10^4 cells/well in 24 well cluster plates in either DMEM supplemented with 10% FBS or in minimal essential medium (MEM) (Gibco Labs) supplemented with 10% FBS and calcium at varying concentrations. Adenylate cyclase assays were performed, as described previously (24), 48 h later following a 4 h serum-free period in the appropriate medium. Briefly, confluent layers of cells were incubated for 2 h with 0.5 μCi/well [^{3}H] adenine (New England Nuclear), washed twice with HBSS and incubated for 10 min at room temperature with the appropriate agonist. The reaction was stopped by aspiration of the medium and immediate addition of 0.5 ml of 10% trichloroacetic acid (TCA) Approximately 2500 cpm [^{14}C]cAMP (New England Nuclear) were added in carrier solution to measure recovery when [^{3}H]cAMP was separated from other cyclic nucleotides by the method of Salomon (25).

Pre-incubation with 50 ng/ml pertussis toxin (List Biological Laboratories Inc.; Campbell, CA), 10^{-8} M PLP-(1-34), 10^{-8} M PLP-(3-34) (Institut Armand Frappier, Montreal, Canada) and 10 ng/well EGF was carried out for 18 h under serum-free conditions. Pre-incubation with 1

μg/ml cholera toxin (List Biological Laboratories Inc.) was carried out for the last 3 hours of the serum-free period, prior to addition of the [^{3}H] adenine.

Additional materials used in the adenylate cyclase assays were forskolin (Calbiochem; Markham, Ontario, Canada), isoproterenol (Sigma Chem. Co.) and PGE_2 (Sigma Chem. Co.).

[^{3}H] Thymidine Incorporation and Cell Numbers

Cells were plated at a density of 2 x 10^4 cells/well in 24 well cluster plates in MEM supplemented with 10% FBS and 0.5 mM, 1 mM or 2 mM calcium. Cultures at 20% confluence were maintained for 18 h in the appropriate serum-free medium. At time 0, fresh MEM with no addition (basal) or containing 10^{-8} M PLP-(1-34) alone, 10 ng/well EGF alone, or 10^{-8} M PLP-(1-34) and 10 ng/well EGF, was added and incubations were continued for 22 h, at which time [^{3}H]thymidine (0.5 μCi/well) (ICN; Costa Mesa, CA) was added and incubations were continued for an additional 2 h. Medium was then aspirated, cells were washed twice with 1 ml of HBSS, treated for 30 min with 0.5 ml 10% TCA and then solubilized in 0.5 ml of 1 N NaOH. Aliquots were counted in a β radiation counter (LKB). Cell numbers were counted in triplicate wells/plate and the cpm [^{3}H]thymidine were corrected for cell numbers, final values being expressed as cpm/10^4 cells.

Cell numbers for the assessment of growth were counted 72 h following commencement of treatment. Additional materials used in the [^{3}H] thymidine assays were $(Bu)_2$cAMP (Sigma Chem. Co.), PLP-(67-86) (Peninsula Labs), PLP-(109-141) (Peninsula Labs) and PTH-(1-34) (Bachem).

Statistical Analysis

All results are expressed as the mean ± SEM of replicate determinations and statistical comparisons based on the Student's t-test Data is representative of at least three experiments.

Results

Characterization of Binding of PTH and EGF to RHEK-1 and RHEK-ras Keratinocytes

Specific binding of ^{125}I-PTH-(1-34) was demonstrated in confluent cultures of RHEK-1 but not RHEK-ras keratinocytes (Fig. 1) Addition of increasing concentrations of either PTH-(1-34) or PLP-(1-34) produced dose-dependent and equivalent inhibition of this binding, whereas addition of ACTH or CT at 10^{-6} M failed to inhibit binding of the radioligand to RHEK-1 cells. Scatchard analysis of the data for both PTH and PLP was consistent with the presence of high affinity (site 1) and low affinity (site 2) binding sites for each agonist, which were respectively of the same order of magnitude (Fig. 1 insert).

Specific binding of ^{125}I-EGF was demonstrated in cultures of both RHEK-1 and RHEK-ras keratinocytes, although total specific binding was much greater in the RHEK-1 cells (Fig. 2). Increasing concentrations of unlabeled EGF produced dose-dependent inhibition of binding of ^{125}I-EGF, whereas addition of 10^{-6} M ACTH or CT failed to inhibit binding of the radioligand to either RHEK-1 or RHEK-ras cells. Scatchard analysis of the data revealed a single class of receptors in both the RHEK-1 and RHEK-ras keratinocytes (Figure 2 insert). Treatment of the RHEK-ras cultures with

dilute acid immediately prior to the binding assay, to remove endogenous ligand, failed to influence the binding characteristics of the RHEK-ras cells (data not shown).

Characterization of Adenylate Cyclase Activity in RHEK-1 and RHEK-ras Keratinocytes

We next examined confluent cultures of RHEK-1 and RHEK-ras cells for evidence of PLP/PTH responsive adenylate cyclase activity. Increasing concentrations of PLP-(1-34) (Fig. 3A) and PTH-(1-34) (Fig. 3B) produced dose-dependent and approximately equivalent increases in adenylate cyclase activity in RHEK-1 cells. Neither PLP-(67-86) nor PLP-(109-141) was capable of stimulating adenylate cyclase activity, at a concentration of 5×10^{-7} M, in these cells (Fig. 3A). Pre-treatment of the RHEK-1 cultures for 18 h with pertussis toxin (50 ng/well) resulted in an approximate 5 fold enhancement of both PLP-(1-34)- and PTH-(1-34)-stimulated adenylate cyclase activity in cultures of RHEK-1 cells. No increase above basal activity was seen at any concentration of PLP-(1-34), PTH-(1-34), PLP-(67-86) or PLP-(109-141) tested in either untreated or pertussis treated cultures of RHEK-ras cells (Fig. 3A and 3B). Basal activity was no different in pertussis treated RHEK-1 or RHEK-ras cultures compared with untreated cultures.

[^{3}H]cAMP accumulation in response to other adenylate cyclase agonists was then examined in the two keratinocyte cell lines. Increasing concentrations of forskolin (Fig. 4A) produced dose-dependent and equivalent stimulation of adenylate cyclase activity in RHEK-1 and RHEK-ras cells A 35 ± 2 fold increase above basal activity was observed in

both cell lines with the highest concentration used. Increasing concentrations of isoproterenol (Fig. 4B) produced dose-dependent increases in [^{3}H]cAMP accumulation which reached a maximum of 37.0 ± 1 0 and 32.7 ± 1.0 fold stimulation above basal activity in cultures of RHEK-1 and RHEK-ras cells respectively. Half maximal stimulation occurred at 1.6 x 10^{-6} M for RHEK-ras and 4.1 x 10^{-6} M for RHEK-1 cells. Unlike the response to forskolin and isoproterenol, the adenylate cyclase response to PGE_2 was greatly attenuated in cultures of RHEK-ras cells, compared with that in RHEK-1 cells (Fig. 4C). PGE_2 at the highest concentration used produced a 34.4 ± 0.7 fold stimulation over basal activity in RHEK-1 cells but only 2.8 ± 0.1 fold stimulation in RHEK-ras cells.

Comparison of adenylate cyclase activity elicited by pre-treatment with cholera toxin (1 μg/well) with that elicited by treatment with forskolin (10^{-4} M) revealed a ratio of cholera toxin/forskolin stimulated activity of 0.73 for RHEK-1 and 0.32 for RHEK-ras (Table 2). The reduced ratio in the RHEK-ras keratinocytes was a function of a reduction in cholera toxin stimulated activity in those cells.

Pre-treatment of cultures with pertussis toxin was shown to significantly increase PGE_2-stimulated, as well as PLP and PTH-stimulated adenylate cyclase activity in RHEK-1 cells but had no effect in RHEK-ras cells. Pre-treatment of cultures with pertussis toxin had no effect on isoproterenol stimulated activity in either cell line (Table 3).

Homologous and Heterologous Desensitization of Adenylate Cyclase Activity in RHEK-1 Keratinocytes

Pre-incubation of RHEK-1 cultures for 18 h with 10^{-7} M PLP-(1-34) or

co-incubation with a 100 fold excess of the PLP receptor antagonist, PLP-(3-34) was shown to abolish the adenylate cyclase activity stimulated by 10^{-7} M PLP-(1-34), whereas pre-incubation with 10^{-7} M PLP-(3-34) or 10 ng EGF reduced activity by 34% and 24% respectively (Table 4). Basal activity, on the other hand, remained unaltered by any of the treatment protocols.

Modulation of Adenylate Cyclase Activity in RHEK-1 Keratinocytes by Extracellular Calcium Concentration

Dose-dependent increases in PLP-(1-34)-stimulated adenylate cyclase activity were progressively reduced in cultures of RHEK-1 keratinocytes in which ambient calcium concentrations were progressively increased (Fig. 5). Maximally stimulating doses of PLP produced 15.0 ± 0.7 - fold, 9.4 ± 0.3 - fold and 6.0 ± 0.3 - fold stimulation above basal when medium calcium concentrations were 0.5 mM, 1.0 mM and 2.0 mM respectively.

[^{3}H]thymidine Incorporation and Cell Growth of RHEK-1 Keratinocytes in Response to PLP and EGF

To determine whether activation of the PLP receptor was associated with any modification in the growth characteristics of RHEK-1 cells, we exarined [^{3}H]thymidine incorporation in response to treatment with 10^{-8} M PLP-(1-34), 10 ng/well EGF or the combination of PLP and EGF in the presence of increasing extracellular calcium concentrations (Fig. 6). At low (0.5 mM) or medium (1.0 mM) calcium concentrations, PLP-(1-34) stimulated a significant increase in [^{3}H]thymidine incorporation which was inhibited when the ambient calcium concentration was raised to 2.0 mM.

EGF stimulated activity, on the other hand, remained unaffected by the extracellular calcium concentration. Simultaneous treatment of the RHEK-1 cultures with PLP-(1-34) and EGF stimulated [^{3}H]thymidine incorporation to a level which was equivalent to or greater than that which was additive for the individual ligands, at all three calcium concentrations tested. Increases in [^{3}H]thymidine incorporation noted at 24 h in response to PLP-(1-34), EGF and the combination of PLP-(1-34) and EGF were associated with approximately equivalent increases in cell numbers, assessed at 72 h (Table 5). In addition, both PTH-(1-34) and $(Bu)_2$cAMP treatment enhanced [^{3}H]thymidine incorporation and cell numbers in a manner analogous to PLP-(1-34), whereas treatment with PLP-(3-34), PLP-(67-86) or PLP-(109-141) had no effect on either parameter. Co-incubation with PLP-(1-34) and PLP-(3-34) substantially reduced both the [^{3}H]thymidine response and the increase in cell numbers noted when cultures were incubated with PLP-(1-34) alone.

Discussion

These studies demonstrate the presence of functional receptors for parathyroid hormone-like peptides on immortalized human keratinocytes and describe several of their properties. As PLP and PTH have been shown to bind to the same receptor on other cells (7-14), we used as a radioligand ^{125}I-PTH-(1-34), previously identified as an efficacious probe for the amino-terminal PTH binding site in traditional target tissues (26). Consistent with previous reports documenting PTH receptor heterogeneity (12, 26, 27), we have identified two classes of PLP/PTH binding sites. The apparent kd of ~ 2 nM for the higher affinity site was the same as

that obtained in UMR 106 cells using identical radioiodinated ligand and binding conditions (28), although fewer sites/cell were evident in the keratinocytes compared with the osseous cells. The inability to demonstrate receptor binding of PLP/PTH on the H-ras transformed cells could reflect the absence of PLP/PTH receptors or the functional alteration of PLP/PTH receptors by the p21 product of the ras oncogene which is known to be overexpressed in this cell line (29). A single class of EGF binding site was demonstrated on both established (RHEK-1) and transformed (RHEK-ras) keratinocytes, although receptors were apparently greatly reduced in number in the transformed cells. Saturation of EGF receptors by endogenous TGFα (18) did not appear to account for this reduction as a mild acid wash prior to binding, which has been shown to remove bound TGFα in other systems (30), failed to alter the EGF binding characteristics. These findings are consistent with other reports demonstrating a loss of EGF (TGFα) receptors in the progression of malignant disease (31,32).

Consistent with the binding data was the observation that PLP-(1-34) and PTH-(1-34) were equipotent in stimulating adenylate cyclase activity in RHEK-1 keratinocytes. In addition, studies with pertussis toxin indicated that these cells possess an inhibitory guanyl nucleotide binding protein (Gi). However, failure of pertussis toxin treatment to stimulate cAMP accumulation in the RHEK-ras cells indicated that over-expression of Gi was not the primary cause of the defective adenylate cyclase activity in those cells. Forskolin produced equivalent stimulation of cAMP accumulation in both cell lines. Cholera toxin, on the other hand, was only about 50% as effective in the RHEK-ras cells compared with the

RHEK-1 cells, suggesting that a defect in G_s might contribute to the altered PLP/PTH-stimulated activity. PGE_2-mediated activity was also severely attenuated in the RHEK-ras cells whereas the β adrenergic receptor retained full activity. In keeping with the current findings, a reduction in agonist stimulated cAMP accumulation has been noted previously in H-ras transformed cells (33, 34) by as yet undetermined mechanisms. Further examination of the PLP/PTH responsive adenylate cyclase activity in the RHEK-1 cells revealed a number of characteristics shared with classical PTH target cells. Cells maintained in a low or medium calcium environment elicited a better cAMP response to PLP/PTH stimulation, perhaps reflecting an interaction between cAMP and calcium messenger systems which has been documented in other cells (35-38) Prolonged exposure to EGF resulted in heterologous desensitization of PLP stimulated adenylate cyclase activity which could have been attributed to altered Gs or receptor downregulation (39, 40). However, the decreased sensitivity of RHEK-ras to EGF would make EGF-induced downregulation an unlikely explanation for the lack of demonstrable PLP/PTH receptors on RHEK-ras cells. RHEK-1 cells demonstrated a greater sensitivity to homologous desensitization than that noted previously for UMR 106 cells (41). As normal keratinocytes in culture are known to secrete substantial quantities of PLP (1), receptor downregulation or occupancy by endogenous hormone may complicate the ability to demonstrate PTH receptor binding in those cells. In this respect, the RHEK cell line, which fails to produce PLP, offers a major advantage for characterizing PTH receptors in a keratinocyte model

PLP has been invoked as a potential modulator of growth in a number

of cell types (42,43), and in preliminary reports, in normal keratinocytes (4-6). In the current studies, activation of the keratinocyte receptor for the amino-terminal region of PLP/PTH was associated with a modest increase in [^{3}H]thymidine uptake and cell proliferation. This activity was mimicked by Bu_2cAMP and noted only when cells were maintained in a low or intermediate extracellular calcium environment, conditions under which PLP stimulation of adenylate cyclase activity was optimized. Taken together, these observations implicate receptor mediated activation of the cAMP pathway in the proliferative response to PLP. Mitogenic activity in response to raised intracellular cAMP levels has been observed in a number of different cell types, including those of epithelial origin (44-46). The mitogenic response to amino-terminal PLP/PTH fragments was small in the RHEK-1 cells, compared with that elicited by EGF, but appeared to be enhanced when cells were co-incubated with both peptides. This is consistent with recent evidence which suggests that the initiation of DNA synthesis is elicited through the synergistic action of growth promoting factors binding to their specific receptors and activating complementary intracellular signals culminating in the proliferative response (44,47). As EGF and PLP/PTH appear to mediate their activity through different effector pathways and each ligand has been shown to modulate activity elicited by the other ligand (39,40,48,49), it is not unreasonable to postulate that enhanced mitogenic effectiveness results from synergistic post-receptor interactions induced by these two peptides.

Our studies therefore demonstrate the presence of adenylate cyclase-linked PLP/PTH receptors on immortalized human keratinocytes and show that malignant transformation of these cells with an H-ras oncogene

is associated with a defect in those receptors. In addition, we have found that activation of this keratinocyte PLP/PTH receptor appears to modulate the growth characteristics of the established RHEK-1 cells. These cell lines therefore appear to provide a suitable model in which to study the action of PLP in keratinocytes.

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References

1. Kremer R, Karaplis AC, Henderson J, Gulliver W, Banville D, Hendy GN, Goltzman D. 1991 Regulation of parathyroid hormone-like peptide in cultured normal human keratinocytes. J Clin Invest 87:884-893

2. Stewart AF, Wu TL, Insogna KL, Milstone LM, Burtis WJ. 1991 Immunoaffinity purification of parathyroid hormone-related protein from bovine milk and human keratinocyte conditioned medium. J Bone Min Res 6:305-311

3. Deftos LJ, Gazdar AF, Ikeda K, Broadus AE. 1989 The parathyroid hormone-related protein associated with malignancy is secreted by neuroendocrine tumors. Mol Endocrinol 3:503-508

4. Milstone L, Fairley J, Insogna K, Stewart A, Reuveni H. 1988 Parathyroid hormone-like peptides increase intracellular calcium and differentiation of keratinocytes. J Cell Biol 107:73A

5. Holick MF, Nussbaum S, Bensons KS. 1988 PTH-like humoral hypercalcemia factor (HHF) of malignancy may be an epidermal differentiation factor: synthetic hHHF(1-34)NH_2 inhibits proliferation and induces terminal differentiation of cultured human keratinocytes. J Bone Min Res 3:S214

6. Ernst M, Rodan GA, Thiede MA. 1989 Rapid induction of parathyroid hormone-like peptide (PTH-LP) in the proliferative response to epidermal growth factor. J Bone Min Res 3:S309

7. Nissenson RA, Diep D, Strewler GJ. 1988 Synthetic peptides comprising the amino-terminal sequence of a parathyroid hormone-like protein from human malignancies; binding to parathyroid hormone receptors and activation of adenylate cyclase in bone cells and kidney. J Biol Chem 263:12866-12871

8. Shigeno C, Yamamoto I, Kitamura N, Noda T, Lee K, Sone T, Shiomi K, Ohtaka A, Fujii N, Yajima H, Konishi J. 1988 Interaction of human parathyroid hormone-related peptide with parathyroid hormone receptors in clonal rat osteosarcoma cells. J Biol Chem 263:18369-18377

9. Juppner H, Abou-Samra A, Uneno S, Gu W, Potts JT, Segre GV. 1988 The parathyroid hormone-like peptide associated with humoral hypercalcemia of malignancy and parathyroid hormone bind to the same receptor on the plasma membrane of ROS 17/2.8 cells. J Biol Chem 263:8557-8560

10. Abou-Samra A, Uneno S, Jueppner H, Potts JT, Segre GV, Nussbaum SR. 1989 Non-homologous sequences of parathyroid hormone and the parathyroid hormone-related peptide bind to a common receptor on ROS 17/2.8 cells. Endocrinology 125·2215-2217

11. Caulfield MP, McKee RL, Goldman ME, Duong LT, Fisher JE, Gay CT, DeHaven PA, Levy JJ, Roubini E, Nutt RF, Chorev M, Rosenblatt M. 1990 The bovine renal parathyroid hormone (PTH) receptor has equal affinity for two different amino acid sequences: the receptor binding domains of PTH and PTH-related protein are located within the 14-34 region. Endocrinology 127:83-87

12. Pun KK, Arnaud CC, Nissenson RA. 1988 Parathyroid hormone receptors in human dermal fibroblasts: structural and functional characterization. J Bone Min Res 3·453-460

13. Chan SDH, Strewler GJ, King KL, Nissenson RA 1990 Expression of a parathyroid hormone-like protein and its receptor during differentiation of embryonal carcinoma cells Mol Endocrinol 4:638-646

14. Nickols GA, Nickols MA, Helwig JJ. 1990 Binding of parathyroid hormone and parathyroid hormone-related protein to vascular smooth muscle of rabbit renal microvessels. Endocrinology 126:721-727

15. Rheinwald JG and Green H. 1977 Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. Nature 265:421-424

16. Krane KF, Murphy DP, Carter DM, Kreuger JG. 1991 Synergistic effects of epidermal growth factor (EGF) and insulin-like growth factor 1/somatomedin C (IGF-1) on keratinocyte proliferation may be mediated by IGF-1 transmodulation of the EGF receptor J Invest Derm 96:419-424

17. Marchese C, Rubin J, Ron D, Faggioni A, Torrisi MR, Messina A, Frati L, Aaronson SA. 1990 Human keratinocyte growth factor activity on proliferation and differentiation of human keratinocytes: differentiation response distinguishes KGF from EGF family. J Cell Physiol 144:326-332

18. Allen-Hoffmann BL, Sheibani N, Hatfield JS, Rhim JS. 1991 In: Neoplastic transformation in human cell systems *in vitro*: mechanisms of carcinogenesis. Ed. J Rhim and A Dritschilo In Press

19. Rhim JS, Jay G, Arnstein P, Price FM, Sanford KK, Aaronson SA. 1985 Neoplastic transformation of human epidermal keratinocytes by AD12-SV40 and kirsten sarcoma viruses. Science 227.1250-1252

20. Rhim JS, Park JB, Jay G 1989 Neoplastic transformation of human keratinocytes by polybrene-induced DNA-mediated transfer of an activated oncogene Oncogene 4:1403-1409

21. Adams JC and Watt FM. 1989 Fibronectin inhibits the terminal differentiation of human keratinocytes. Nature 340:307-309

22. Kremer R, Bennet HPJ, Mitchell J, Goltzman D. 1982 Characterization of the rabbit renal receptor for native parathyroid hormone employing a radioligand purified by reverse-phase liquid chromatography. J Biol Chem 257:14048-14054

23. Lai WH, Guyda HJ, Bergeron JJM. 1986 Binding and internalization of epidermal growth factor in human term placental cells in culture Endocrinology 118:413-423

24. Rabbani SA, Mitchell J, Roy DR, Hendy GN, Goltzman D. 1988 Influence of the amino-terminus on *in vitro* and *in vivo* biological activity of synthetic parathyroid hormone-like peptides of malignancy. Endocrinology 123:2709-2718

25. Salomon Y, Londos C, Rodbell M. 1974 A highly sensitive adenylate cyclase assay. Anal Biochem 58:541-548

26. Demay M, Mitchell J, Goltzman D. 1985 Comparison of renal and osseous binding of parathyroid hormone and hormonal fragments. Am J Physiol 249.E437-446

27. Seitz PK, Nickols GA, Nickols MA, McPherson MB, Cooper CW. 1990 Radioiodinated rat parathyroid hormone-(1-34) binds to its receptor on rat osteosarcoma cells in a manner consistent with two classes of binding sites. J Bone Min Res 5:353-359

28. Mitchell J, Rouleau MF, Goltzman D. 1990 Biochemical and morphological characterization of parathyroid hormone receptor binding to the rat osteosarcoma cell line UMR-106. Endocrinology 126:2327-2335

29. Rhim JS 1989 Neoplastic transformation of human epithelial cells *in vitro*. Anticancer Res 9:1345-1365

30. Coffey RJ, Goustin AS, Soderquist AM, Shipley GD, Wolfshohl J, Carpenter G, Moses HL. 1987 Transforming growth factor alpha and beta expression in human colon cancer lines: implications for an autocrine model. Cancer Res 47:4590-4594

31. Todaro GJ, DeLarco JE, Cohen S. 1976 Transformation by murine and feline sarcoma viruses specificallly blocks binding of epidermal growth factor to cells. Nature (London) 264·26-31

32. Lee LS and Weinstein IB 1978 Tumor-promoting phorbol esters inhibit binding of epidermal growth factor to cellular receptors. Science 202:313-315

33. Tarpley WG, Hopkins NK, Gorman RR. 1986 Reduced hormone-stimulated adenylate cyclase activity in NIH-3T3 cells expressing the EJ human bladder *ras* gene. Proc Natl Acad Sci (USA) 83:3703-3707

34. Derigs HG, Klingberg D, Tricot GJ, Boswell HS. 1989 Effector function for *ras* oncogene in interleukin-3-dependent myeloid cells involves diminished efficacy of prostaglandin E1-mediated inhibition of proliferation Blood 74 1942-1951

35. Yee JA, Sutton JK, Shew RL, Olansky L. 1986 Parathyroid hormone stimulation of alkaline phosphatase activity in cultured neonatal mouse calvarial bone cells· involvement of cAMP and calcium. J Cell Physiol 128:246-250

36. Civitelli R, Hruska KA, Jeffrey JJ, Khan AJ, Avioli LV, Partridge NC. 1989 Second messenger signaling in the regulation of collagenase production by osteogenic sarcoma cells. Endocrinology 124:2928-2934

37. Rasmussen H. 1986 The calcium messenger system. N Engl J Med 314:1164-1170

38. Brown EM. 1991 Extracellular Ca^{2+} sensing, regulation of parathyroid cell function, and role of Ca^{2+} and other ions as extracellular (first) messengers Physiol Rev 71.371-411

39. Bernier SM, Rouleau MF, Goltzman D 1991 Biochemical and morphological analysis of the interaction of epidermal growth factor and parathyroid hormone with UMR 106 osteosarcoma cells. Endocrinology 128·2752-2760

40. Gutierrez GE, Mundy GR, Derynck R, Hewlett EL, Katz MS 1987 Inhibition of parathyroid hormone-resposive adenylate cyclase in clonal osteoblast-like cells by transforming growth factor alpha and epidermal growth factor J Biol Chem 262:15845-15850

41. Mitchell J and Goltzman D. 1990 Mechanisms of homologous and heterologous regulation of parathyroid hormone receptors in the rat osteosarcoma cell line UMR-106. Endocrinology 126:2650-2660

42. Burton PBJ, Moniz C, Knight DE. 1990 Parathyroid hormone related peptide can function as an autocrine growth factor in human renal cell carcinoma. Biochem Biophys Res Comm 167:1134-1138

43. Adachi N, Yamaguchi K. Miyaki Y, Honda S, Nagasaki K, Akiyama Y, Adachi I, Abe K. 1990 Parathyroid hormone-related protein is a possible autocrine growth inhibitor for lymphocytes. Biochem Biophys Res Comm 166: 1088-1094

44. Rozengurt E 1989 Signal transduction pathways in mitogenesis Brit Med Bull 45:515-528

45. Raff MC, Hornby-Smith A, Brockers JP. 1978 Cyclic AMP as a mitogenic signal for cultured rat Schwann cells. Nature 273:672-673

46. Green H. 1978 Cyclic AMP in relation to proliferation of the epidermal cell: a new view. Cell 15:801-811

47. Rozengurt E. 1986 Early signals in the mitogenic response. Science 234:161-166

48. Ohta S, Shigeno C, Yamamoto I, Okumura H, Lee K, Uneno S, Konishi J, Yamamuro T. 1989 Parathyroid hormone down-regulates the epidermal growth factor receptors in clonal osteoblastic mouse calvarial cells, MC3T3-E1: possible mediation by adenosine 3',5'-cyclic monophosphate. Endocrinology 124: 2419-2426

49. Iwashita S, Mitsui K, Shoji-Kasai Y, Senshu-Miyaike M 1990 cAMP-mediated modulation of signal transduction of epidermal growth factor (EGF) receptor systems in human epidermoid carcinoma A431 cells. J Biol Chem 265:10702-10708.

Table 1 **Growth and differentiation properties of normal, immortalized and malignant human keratinocytes in culture.**

	Benign NHK	Immortalized RHEK-1	Malignant RHEK-*ras*
Cell envelopes[a]	+++	-	-
Involucrin[a]	+++	+	+
Passages in culture[b,c]	+	+++	+++
Saturation density[c]	+	+	+++
Extracellular matrix[a,d]	+	+	+++
Agar colonies[b,c]	-	-	+++
Tumor formation[b,c]	-	-	+++

a. Allen-Hoffmann *et al.* (18).

b. Rhim *et al.* (19)

c. Rhim *et al.* (20)

d. Adams *et al.* (21).

Table 2 **Comparison of cholera toxin (C.Tox) stimulated and forskolin stimulated adenylate cyclase activity in RHEK-1 and RHEK-ras keratinocytes.**

Agonist	[^{3}H]cAMP (cpm/well)	
	RHEK-1 mean ± SEM	RHEK-ras mean ± SEM
C. Tox	5786 ± 992	2608 ± 194
Forskolin	7879 ± 438	8267 ± 128
C. Tox/Forskolin	0.73	0.32

a. [^{3}H]cAMP accumulation was measured in confluent cultures of RHEK-1 and RHEK-ras keratinocytes following a 3 h pre-incubation with c. tox (1 μg/well) or in response to 10^{-4} M forskolin as described in **Materials and Methods**. Values represent the mean ± SEM of 6 determinations and are representative of 3 different experiments.

Table 3 **Effect of pertussis toxin (P.Tox) pre-treatment on agonist stimulated adenylate cyclase activity.**

Agonist	P.Tox	Adenylate cyclase (% of basal)[a]	
		RHEK-1 mean ± SEM	RHEK-ras mean ± SEM
PLP-(1-34)[b]	-	267 ± 15	101 ± 10
	+	383 ± 24[c]	102 ± 13
PTH-(1-34)[b]	-	396 ± 30	93 ± 11
	+	506 ± 14[c]	103 ± 20
Isoproterenol[b]	-	2629 ± 119	3225 ± 163
	+	2505 ± 74	2587 ± 148
PGE_2[b]	-	1261 ± 83	200 ± 5
	+	2625 ± 200[c]	207 ± 15

a. Adenylate cyclase activity was measured in confluent layers of RHEK-1 and RHEK-ras keratinocytes which had received no pre-treatment (-) or 18 h of pre-treatment with 50 ng/ml pertussis toxin (P.Tox) (+) as described in **Materials and Methods**. Results are expressed as a % of basal activity which varied between 91 ± 10 and 206 ± 13 cpm/well for RHEK-1 and between 98 ± 12 and 201 ± 25 cpm/well for RHEK-ras. Each value represents the mean ± SEM of triplicate determinations and is representative of 3 different experiments

b. PLP-(1-34) and PTH-(1-34) were added at 5×10^{-8} M, isoproterenol at 5×10^{-6} M and PGE_2 at 10^{-6} M.

c. Significant difference from -P.Tox ($p<0.05$).

Table 4 **Effect of homologous and heterologous peptides on PLP-stimulated adenylate cyclase activity in RHEK-1 keratinocytes.**

Treatment	[^{3}H]cAMP (cpm/well)[a]	
	-PLP mean±SEM	+PLP mean±SEM
Nil	166 ± 7	710 ± 27
18 h PLP-(1-34)	163 ± 20	156 ± 20[b]
18 h PLP-(3-34)	158 ± 13	470 ± 12[b]
Co-incubation PLP-(3-34)	172 ± 6	174 ± 32[b]
18 h EGF	171 ± 18	542 ± 17[b]

a. [^{3}H]cAMP accumulation was measured in the absence (-PLP) or in the presence (+PLP) of 10^{-7} M PLP-(1-34) in confluent cultures of RHEK-1 keratinocytes following 18 h of pre-incubation with 10^{-7} M PLP-(1-34), 10^{-7} M PLP-(3-34) or 10 ng/well EGF or upon co-incubation with 10^{-5} M PLP-(3-34) as described in Materials and Methods. Each value represents the mean ± SEM of triplicate determinations and is representative of 3 different experiments

b Significant difference from Nil ($p<0.01$).

Table 5 **Stimulation of [^{3}H]thymidine incorporation and cell growth in RHEK-1 keratinocytes.**

Treatment	[^{3}H]thymidine (% of basal)[a] mean ± SEM	Cells (% of basal)[b] mean ± SEM
Nil	100 ± 3	100 ± 2
PLP-(1-34)	119 ± 1[c]	139 ± 2[c]
PLP-(3-34)	99 ± 1	99 ± 1
PLP-(1-34) + PLP-(3-34)	106 ± 4	108 ± 2[d]
PLP-(67-86)	92 ± 2	104 ± 2
PLP-(109-141)	102 ± 3	105 ± 2
PTH-(1-34)	119 ± 2[c]	114 ± 2[c]
$(Bu)_2$cAMP	118 ± 2[c]	129 ± 2[c]
EGF	136 ± 2[c]	147 ± 4[c]
EGF + PLP-(1-34)	188 ± 3[c]	166 ± 3[c]

a Incorporation of [^{3}H]thymidine into 30% confluent cultures of RHEK-1 cells was measured as described in Materials and Methods following 24 h of treatment with 10^{-8} M PLP-(1-34), 10^{-8} M PLP-(3-34) or a combination of PLP-(1-34) and PLP-(3-34), 10^{-8} M PLP-(67-86), 10^{-8} M PLP-(109-141), 10^{-8} M PTH-(1-34), 10^{-5} M $(Bu)_2$cAMP, 10 ng/well EGF or a combination of EGF and PLP-(1-34) Values expressed as % of basal activity, represent the mean ± SEM of 6 determinations and are representative of those from 3 separate experiments.

b Cell numbers were counted after 72 h of treatment and are expressed as a % of cell numbers counted after 72 h with no treatment (basal) Each value represents the mean ± SEM of 6 determinations and is representative of 3 separate experiments

c. Significant difference from Nil ($p<0.001$)

d. Significant difference from Nil ($p<0.01$)

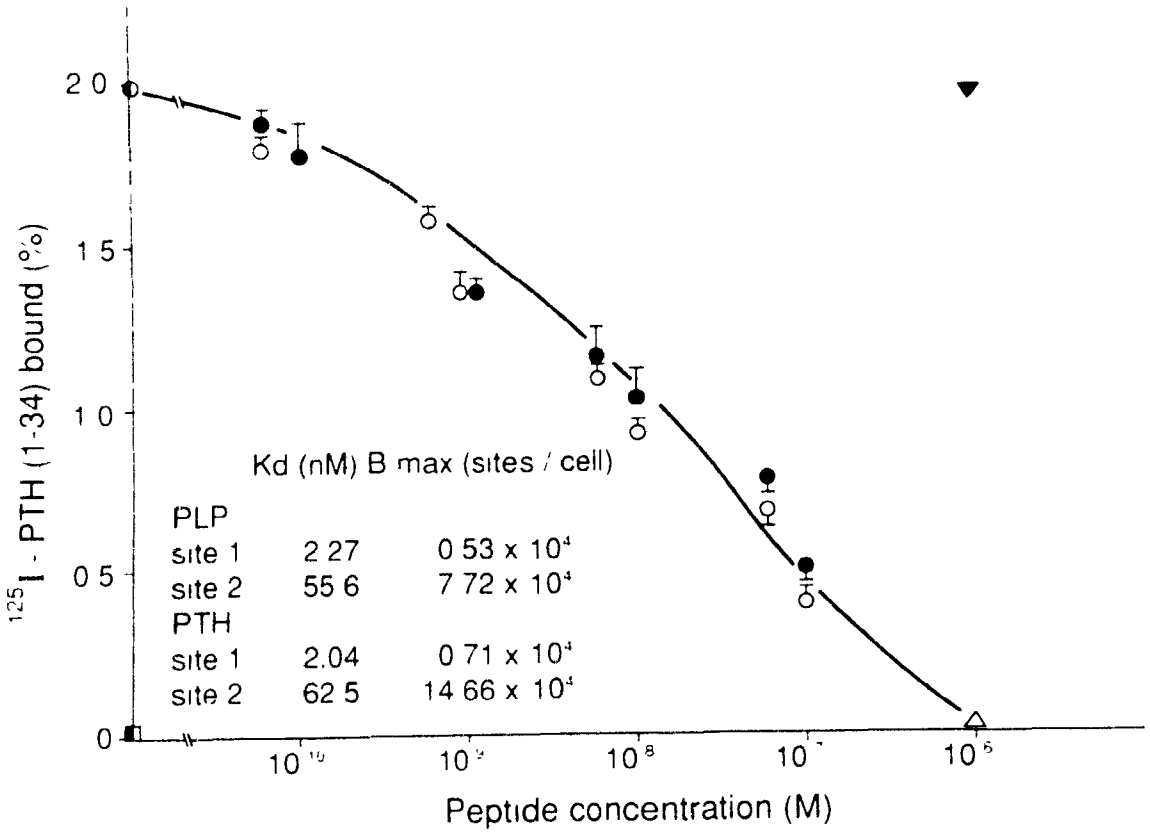

Figure 1. Binding of ^{125}I-PTH-(1-34) to RHEK-1 and RHEK-ras keratinocytes. Binding of ^{125}I-PTH-(1-34) to confluent layers of RHEK-1 (circles) cells was carried out at 23° c for 2 h with 120,000 cpm ^{125}I-PTH-(1-34) in the presence of increasing concentrations of either PLP-(1-34) (O) or PTH-(1-34) (●) or with 10^{-6} M ACTH or CT (▼) as described in Materials and Methods Binding is expressed as % specific binding of total radioligand added A single symbol for RHEK-ras cells (squares) is placed at 0% binding as no specific binding was detected. Non-specific binding (△), assessed in the presence of 10^{-6} M unlabeled hormone was 0.7 ± 0.01% for PTH and 0 6 ± 0.01% for PLP All data points represent the mean ± SEM of triplicate determinations Binding parameters, assessed by Scatchard analysis, are represented in the insert.

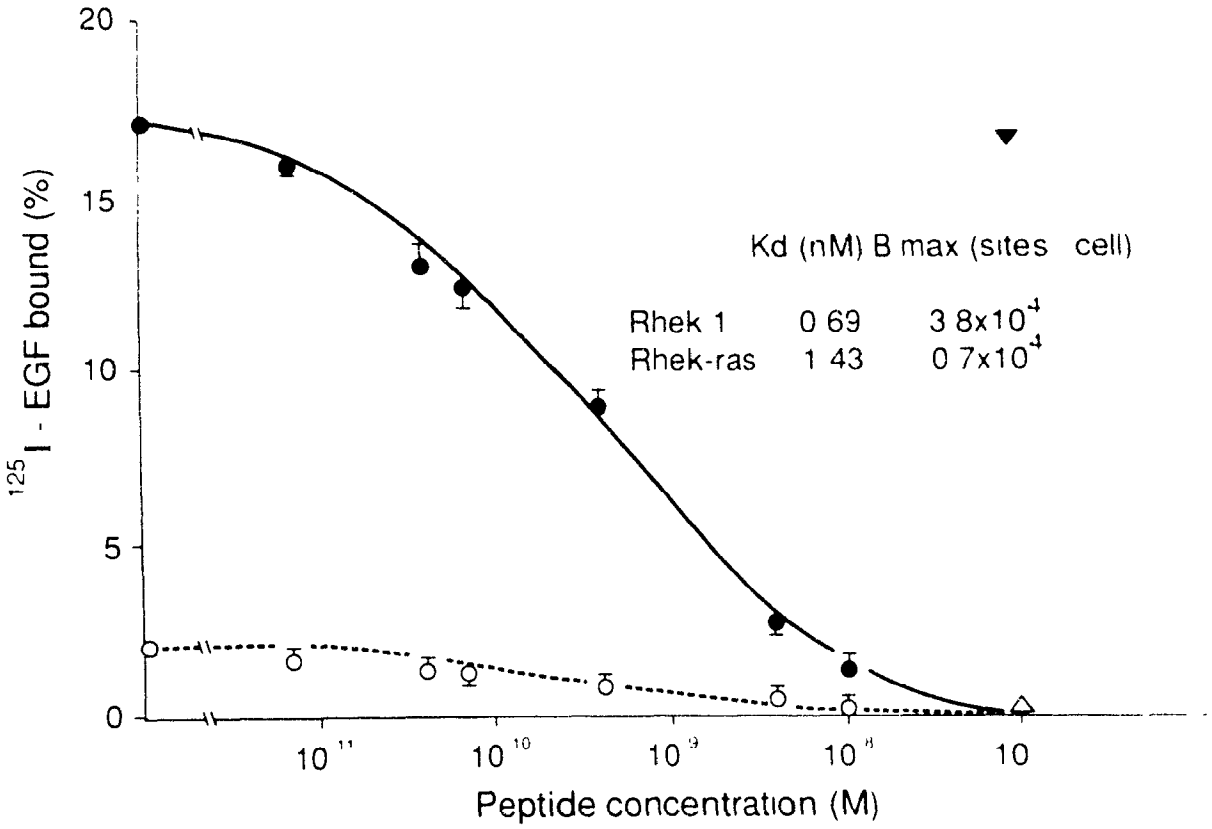

Figure 2 Binding of ^{125}I-EGF to RHEK-1 and RHEK-ras keratinocytes Binding of ^{125}I-EGF to confluent layers of RHEK-1 (•) and RHEK-ras (o) cells was carried out at 23 c for 2 h with 42,000 cpm ^{125}I-EGF in the presence of increasing concentrations of EGF (circles) or with 10^{-6} M ACTH or CT (▼) as described in Materials and Methods Binding is expressed as % specific binding of total radioligand added. Non-specific binding (△), assessed in the presence of 10^{-7} M unlabeled EGF, was 2 0 ± 0 02% for RHEK-1 and 0 2 ± 0.005 for RHEK-ras. All data points represent the mean ± SEM of triplicate determinations. Binding parameters, assessed by Scatchard analysis, are represented in the insert.

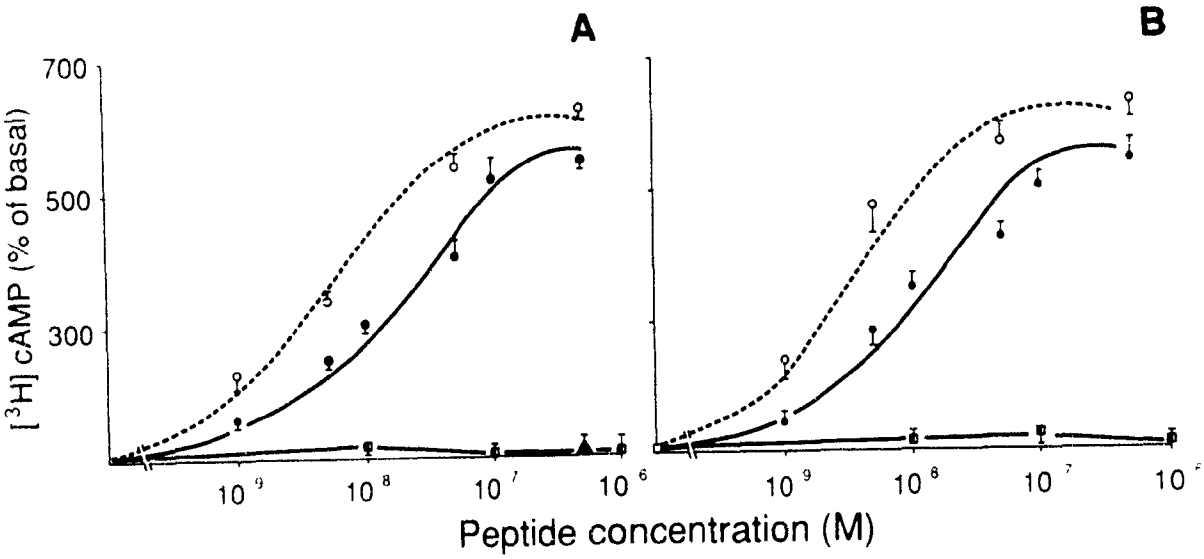

Figure 3. PLP and PTH responsive adenylate cyclase activity in RHEK-1 and RHEK-*ras* keratinocytes. Adenylate cyclase activity was determined in confluent cultures of RHEK-1 (circles) and RHEK-ras (squares) cells in response to increasing concentrations of PLP-(1-34) (panel A) or PTH-(1-34) (panel B) following no pre-treatment (solid symbols) or following 18 h pre-treatment with 50 ng/well pertussis toxin (open symbols) as described in Materials and Methods PLP-(67-86) and PLP-(109-141), each at 5×10^{-7} M (▲), produced no increase in adenylate cyclase activity in either RHEK-1 cells or RHEK-ras cells, as shown in panel A. Adenylate cyclase activity is expressed as a % of basal activity which for RHEK-1 cells was 100 ± 6 cpm [^{3}H]cAMP/well and 91 ± 17 cpm [^{3}H]cAMP/well for untreated and treated cells respectively and in RHEK-ras cells was 130 ± 20 cpm [^{3}H]cAMP/well and 125 ± 12 cpm [^{3}H]cAMP/well for untreated and treated cells respectively. Each data point represents the mean ± SEM of triplicate determinations and figures are representative of 3 different experiments.

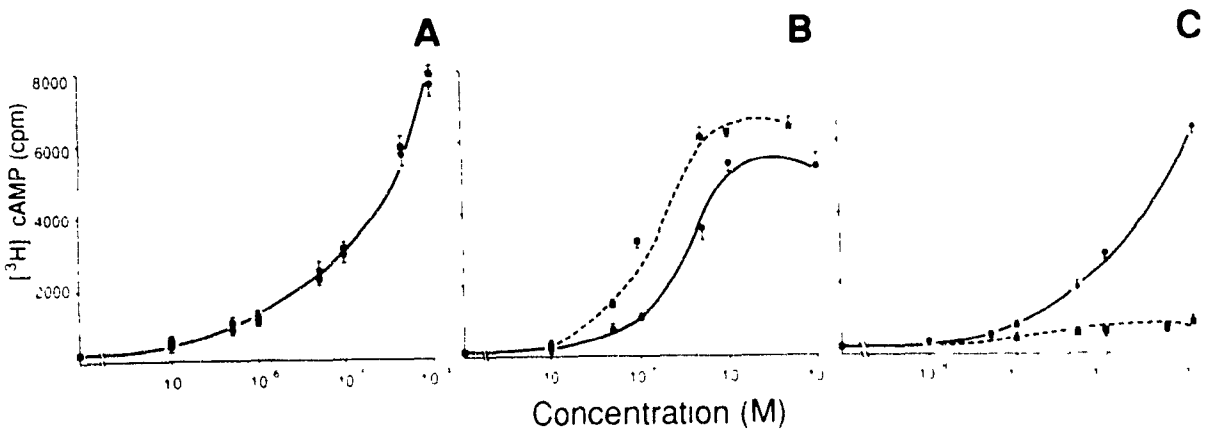

Figure 4. Forskolin, isoproterenol and PGE_2 responsive adenylate cyclase activity in RHEK-1 and RHEK-ras keratinocytes. Adenylate cyclase activity, expressed as cpm [^{3}H]cAMP accumulation/well, was determined in confluent layers of RHEK-1 (●——●) and RHEK-ras (■----■) cells in response to increasing concentrations of forskolin (panel A), isoproterenol (panel B) and PGE_2 (panel C) as described in Materials and Methods. Basal levels of activity for RHEK and RHEK-ras cells were 227 ± 35 and 207 ± 79 cpm [^{3}H]cAMP/well for forskolin, 147 ± 18 and 190 ± 5 cpm ^{3}[H]cAMP/well for isoproterenol and 190 ± 24 and 230 ± 15 cpm ^{3}[H]cAMP/well for PGE_2. All data points represent the mean ± SEM of triplicate determinations and are representative of 3 different experiments

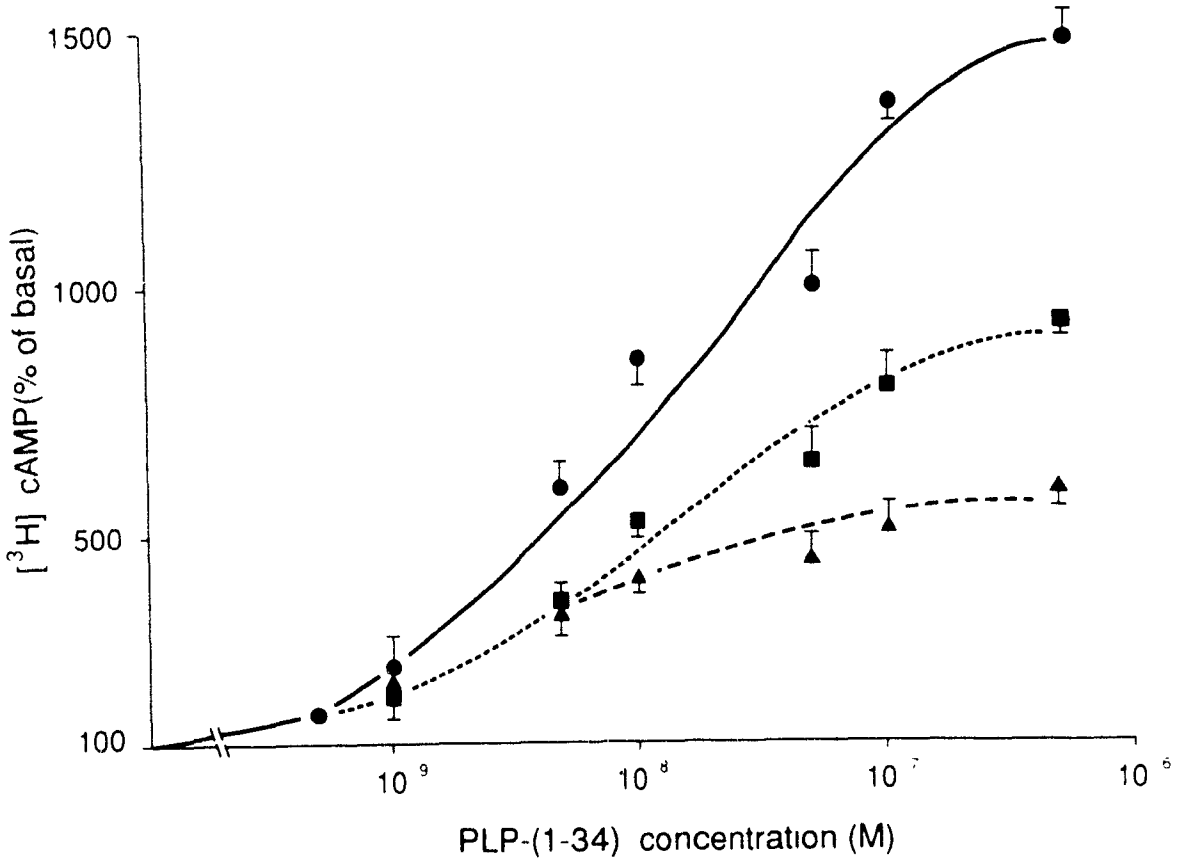

Figure 5. Modulation of PLP-(1-34) stimulated adenylate cyclase activity in RHEK-1 keratinocytes by extracellular calcium concentration. PLP-(1-34) responsive adenylate cyclase activity, expressed as a percent of basal [^{3}H]cAMP accumulation/well, was determined in confluent layers of RHEK-1 cells 48 h after plating in MEM supplemented with 0.5 mM Ca (●——●), 1 mM Ca (■----■) or 2 mM Ca (▲----▲) as described in Materials and Methods. Basal levels of activity were 109 ± 20 cpm [^{3}H]cAMP/well for 0 5 mM Ca, 143 ± 35 cpm [^{3}H]cAMP/well for 1 mM Ca and 89 ± 23 cpm [^{3}H]cAMP/well for 2 mM Ca. All data points represent the mean ± SEM of triplicate determinations and are representative of 3 different experiments.

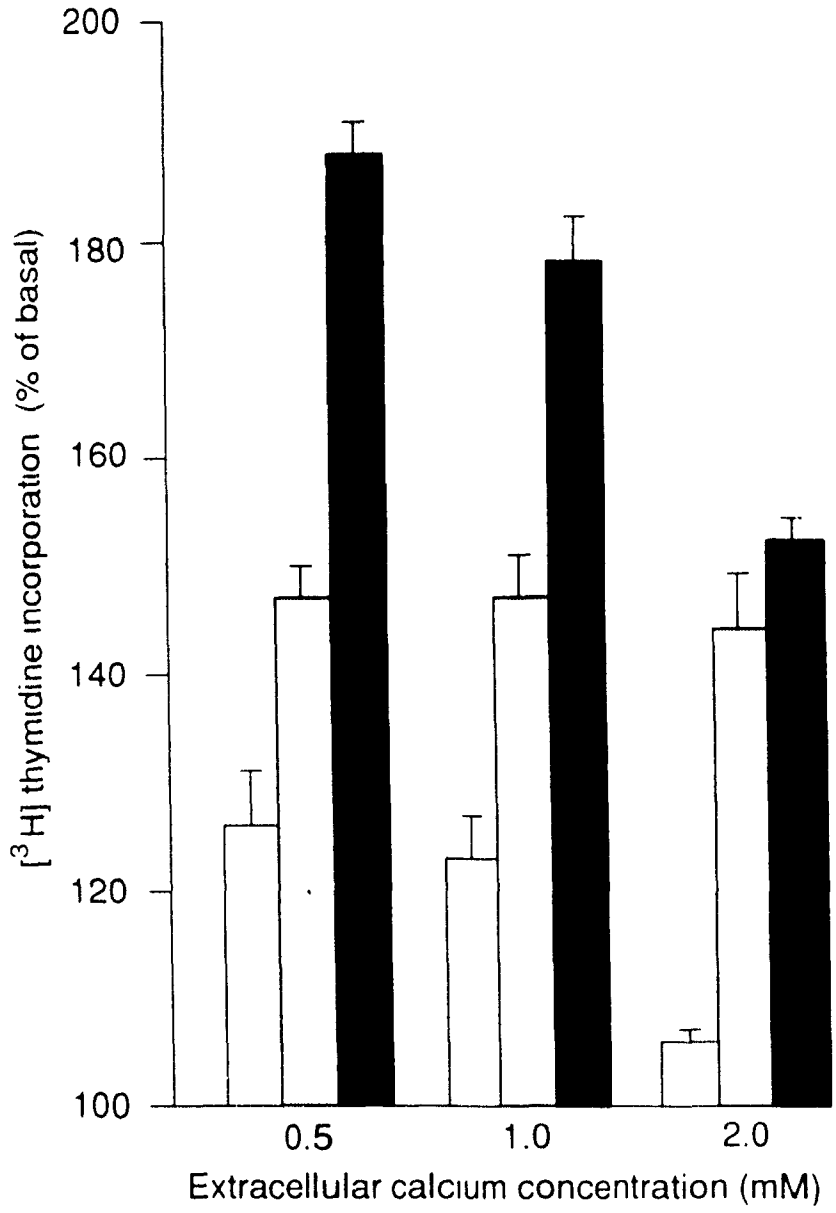

Figure 6. Modulation of PLP and EGF-stimulated [^{3}H]thymidine incorporation into RHEK-1 keratinocytes by extracellular calcium concentration. [^{3}H]thymidine incorporation was determined in 30% confluent cultures of RHEK-1 cells, plated in MEM supplemented with 0 5 mM Ca, 1 mM Ca or 2 mM Ca following treatment for 24 h with 10^{-8} M PLP-(1-34) (□), 10 ng/well EGF (▨) or 10^{-8} M PLP-(1-34) plus 10 ng/well EGF (■) as described in Materials and Methods. [^{3}H]thymidine incorporation, measured as cpm/well, was corrected for cell number and expressed as a percentage of basal activity which was measured in the absence of added peptide and was 4191 ± 112 cpm [^{3}H]thymidine/10^4 cells for 0.5 mM Ca, 5289 ± 91 cpm [^{3}H]thymidine/10^4 cells for 1 mM Ca and 4401 ± 64 cpm [^{3}H]thymidine/10^4 cells for 2 mM Ca. Each bar represents the mean ± SEM of 4-6 determinations and is representative of 3 different experiments.

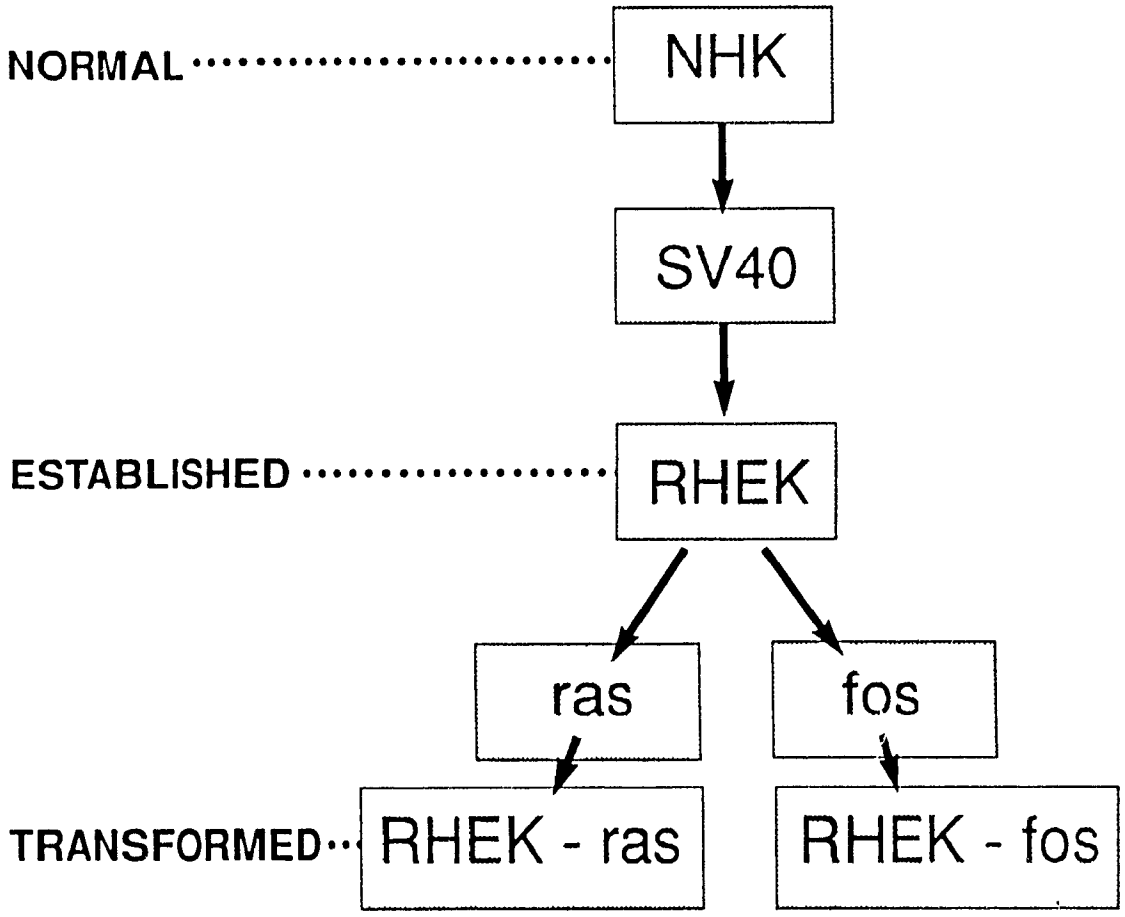

Figure 1 **Derivation of non-PLP secreting keratinocyte cell lines.** RHEK-1 keratinocytes were established as a non-malignant cell line from normal human keratinocytes (NHK) following exposure to a hybrid virus Adenovirus 12-Simian Virus 40 (SV40). Transformation into the malignant RHEK-ras and RHEK-fos cell lines was accomplished following transfection with activated ras and fos oncogenes respectively.

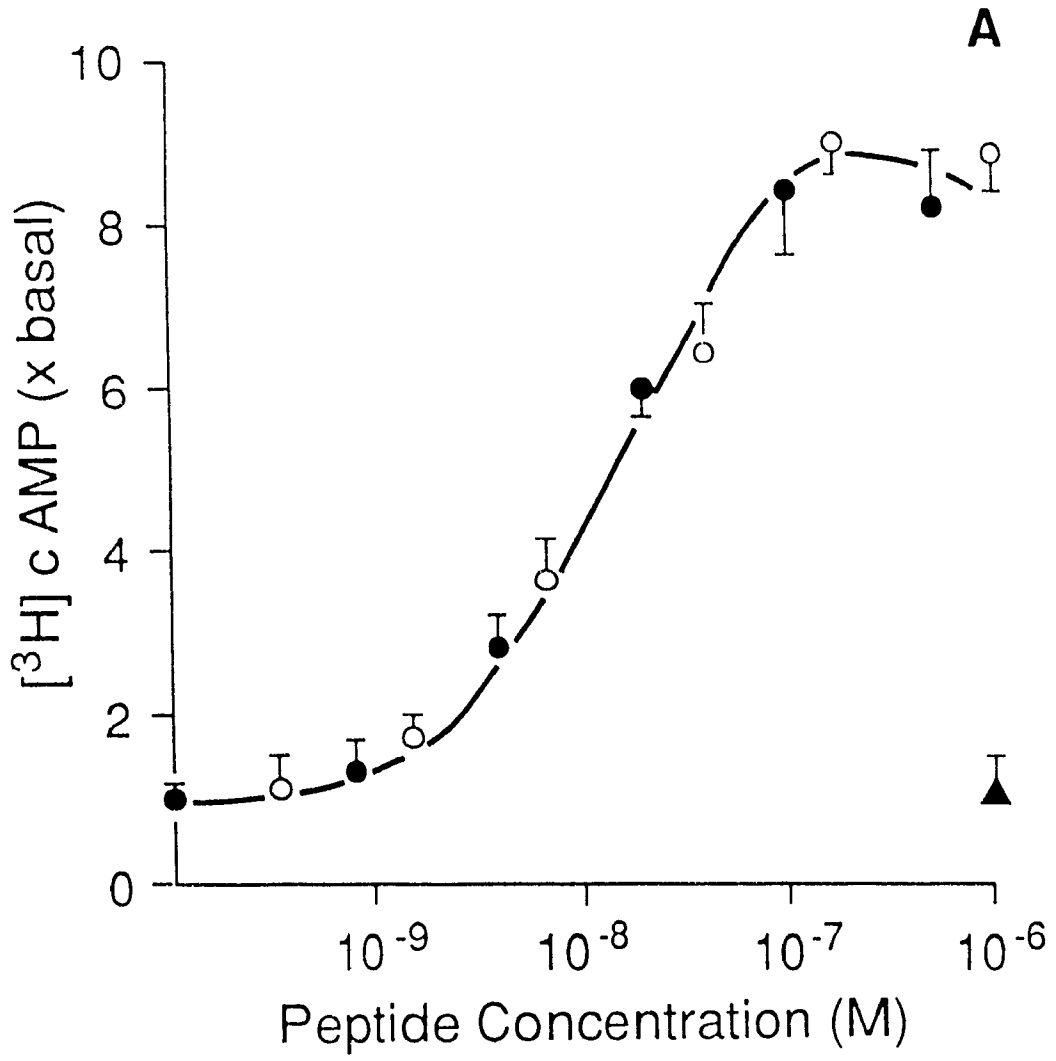

Figure 2 **Stimulation of adenylate cyclase activity in RHEK-1 keratinocytes by amino-terminal and full length PLP.** Increasing concentrations of hPLP-1-34 (●) and recombinant (r) PLP-1-141 (○) produced dose dependent and equivalent stimulation of adenylate cyclase activity in RHEK-1 cells Addition of hPLP-3-34, hPLP-38-64, hPLP-67-86 and hPLP-109-141 at 10^{-6}M (▲) failed to produce any increase in [^{3}H]cAMP accumulation above the basal level which was 83±12 cpm/well. All points represent the mean±SEM of triplicate determinations.

CHAPTER VI GENERAL DISCUSSION

Preface

The aims of the studies undertaken in the preceding chapters were to examine the production and biological act'on of a parathyroid hormone-like peptide, PLP, which was originally associated with the hypercalcemia of malignancy. The data examining circulating concentrations of PLP provided strong evidence for a role for this peptide in the pathogenesis of malignancy associated hypercalcemia. To pursue this issue I examined the effects of neutralization of endogenous PLP on the biochemical indices of the syndrome in a well characterized rat model of the human condition These studies demonstrated that PLP was both necessary and sufficient for the development and maintenance of hypercalcemia in this animal model.

In view of the fact that I had shown that cancers of squamous cell origin were one of the largest groups associated with both hypercalcemia and elevated circulating levels of PLP, my next project examined the dysregulation of PLP expression in a keratinocyte model of tumor progression. The conclusions drawn from these studies were that several regulatory mechanisms for PLP, which were present in normal keratinocytes, were altered in the progression to the malignant phenotype. These findings could help to explain the overproduction of PLP by squamous cancers IN VIVO.

Apart from its PTH-like role in the pathogenesis of hypercalcemia there is a growing body of evidence pointing to an autocrine/paracrine role for PLP in normal physiology. My final work therefore looked for evidence of PLP receptor-mediated activity in keratinocytes, a non-classical PTH/PLP target tissue known to produce PLP. Adenylate cyclase-linked receptors for PLP were identified in a human keratinocyte cell line In addition, activation of those receptors by NH_2-terminal PLP was associated with increased mitogenesis suggesting an autocrine/paracrine role for PLP in keratinocyte cell growth.

2 Circulating concentrations of PLP in patients with malignancy

My study examining circulating concentrations of PLP in cancer patients and in patients with hyperparathyroidism is one of 4 similar works published to date (1-4). Widely disparate immunoassay conditions and specificities of the individual antisera most probably account for the differences in absolute values between the reports This hypothesis is supported by the fact that the Burtis assay (3) recognised only about 60% of the bioactive material present in rat tissue extracts (5) whereas my own assay appeared to overestimate the bioactive material present in keratinocyte conditioned medium (6). In addition, full-length recombinant PLP 1-141 was recognised at about 1/4 the potentcy of PLP 1-34 in the Budayr RIA (1) whereas both peptides were recognised equally well in my own RIA (p 68). These discrepancies could account for the relatively high absolute values in my own work compared with those of others.

Despite these differences several important observations were shared by the four groups. First, the highest mean circulating levels of PLP occurred in patients with malignancy associated hypercalcemia (p 61). Reduction of tumor mass, either by chemotherapy (2-4) or by surgical excision (3) resulted in reductions in both plasma Ca and plasma PLP In addition, in two patients studied prior to surgical removal of tumor tissue (lung and carcinoid), a PLP gradient was measured between tumor venous effluent and peripheral plasma (30.0/7.6 and 550/44 pmol/L respectively). These observations provide support for the tumor-derived origin of the circulating peptide and also establish its importance in the pathogenesis of hypercalcemia associated with malignancy.

The second observation shared by all four groups was that elevated

levels of PLP were detected in hypercalcemic patients harboring a wide variety of histological types of tumors including those of breast and hematologic origin, whose hypercalcemia has traditionally been associated with the presence of focal osteolytic lesions. My own study (2) indicated that about 40% of breast cancer patients had elevated circulating PLP levels (p 60) while those of Budayr (1), Burtis (3) and Kao (4) indicated high PLP levels in about 50%, 40% and 75% of breast cancer patients respectively. In all cases hypercalcemia correlated better with the presence of increased circulating PLP than with the presence of osteolytic lesions. Further evidence in support of PLP induced hypercalcemia in some patients with breast cancer comes from immunohistochemical studies showing positive staining for PLP in 60% of primary breast tumors (7) and 48% of breast cancer metastases (8).

Three of the four studies (2-4) also identified lymphoma patients with elevated PLP levels. The association of PLP overexpression with HTLV-1 infected lymphocytes in adult T-cell lymphoma has prompted examination of the interaction between the HTLV-1 gene product, TAX, and the PLP gene (9). Preliminary data has located a TAX responsive element in the PLP promoter region and identified TAX as a positive transactivator of PLP gene expression.

Notable amongst reports of PLP overexpression associated with neoplasia are those documenting tumors of endocrine origin (10-14). Since one of the highest levels of NH_2-terminal PLP was measured in a hypercalcemic patient with pancreatic cancer in my own studies, it was interesting to note an independent report on a pancreatic cancer patient having a 10 fold elevation above control levels of circulating PLP,

assessed with a mid-region (56-86) assay (14).

The third consensus observation was that low levels of PLP could be detected in the circulation of some normal individuals (p 65). Although numerous normal tissues have been shown to express PLP mRNA (6,10,15,16) and protein (6,10,12,17-21), the IN VIVO source(s) of the circulating material in normal human adults remain to be defined.

A fourth observation shared by 3 of the 4 groups (1-3) was the presence of PLP levels above the mean control level in some normocalcemic cancer patients. The values tended to be lower than those detected in hypercalcemic individuals (p 61,65), perhaps reflecting a smaller tumor volume which may have produced insufficient PLP to cause hypercalcemia. In a study correlating PLP immunoreactivity, tumor mass and indices of malignancy associated hypercalcemia (22), patients with the hypercalcemic syndrome had a larger tumor mass i.e. more PLP, than the normocalcemic cancer patients. An alternative explanation for the failure to develop hypercalcemia in patients with elevated levels of PLP could be the existence of multiple immunoreactive forms differing in biopotency

In my own studies, gel filtration analysis of plasma taken from two hypercalcemic breast cancer patients revealed both large (>15 Kd) and small (6-7 Kd) molecular weight immunoreactive species (p 67) This observation of PLP heterogeneity was in agreement with previous work identifying high (17 Kd) and low (6-9 Kd) molecular weight adenylate cyclase stimulating activity in tumors associated with hypercalcemia (23) as well as work documenting a major 6 Kd and minor 3.4, 8 and 15 Kd species of PLP in medium conditioned by renal carcinoma cells in the presence of protease inhibitors (24). In addition, studies using a

sensitive renal CBA (25), identified multiple species of PTH-like bioactivity in both the plasma and extracts of tumor tissue removed from hypercalcemic cancer patients . Burtis et al, using an immunoradiometric (IRMA) assay recognising PLP 1-74 and a carboxy-terminal RIA which recognises PLP 109-138, have suggested that PLP 1-74 and PLP 109-138 circulate independently and that intact PLP 1-141 does not circulate (3). These conclusions were drawn when PLP 1-74 immunoreactivity was completely abolished from the plasma of hypercalcemic cancer patients following immunoextraction on an anti-PLP 1-36 affinity column whereas PLP 109-138 immunoreactivity remained quantitatively unaffected. The presence of multiple potential cleavage sites in the region encompassing amino acids 88-108 was given as a possible explanation for the existence of the separate NH_2-terminal and carboxy-terminal fragments Preliminary data from this group (26) characterizing circulating NH_2-terminal PLP in the plasma of hypercalcemic cancer patients suggests the presence of a single species migrating with an apparent molecular weight of 25-30 Kd. This value is well in excess of the predicted 10-13 Kd species resulting from cleavage in the 88-108 region as suggested. When added to the weight of the COOH-terminal fragment (12 Kd) the sum exceeds by far the MW of any predicted full length PLP (maximum 21 Kd for PLP 1-173). In keeping with my earlier suggestion (p 52), this may indicate the presence of protein-bound moieties in the circulation. The inability of these latter studies to detect the smaller fragment (6-7 Kd) identified in my own work most likely rests in the "sandwich" nature of their assay being unable to identify fragments of less than 74 amino acids in length. Further characterization, using combinations of gel filtration, Western analysis and gel

electrophoresis, as well as the development of sensitive immunoassays which recognise intact PLP, will be required to precisely define the circulating forms of PLP.

Although synthetic PLP 1-36, PLP 1-74 and recombinant PLP 1-141 appeared to have approximately the same potency in stimulating hypercalcemia when infused into rats for 2 days a continuous rise in serum calcium was noted in animals infused with PLP 1-36 over a 5d period whereas serum calcium levels appeared to stabilize at a lower level in the animals infused with PLP 1-74 (27,28). Whether this represents a real difference in biopotency or was a consequence of assay conditions requires further investigation. Nevertheless, characterization of the bioactivity of different circulating forms, once they have been defined, will be required in order to determine the relative contributions being made by these peptides to the syndrome of hypercalcemia of malignancy.

Despite a number of independent reports documenting overexpression of PLP at both mRNA (15,29,30) and protein (12,30,31) levels in both adenomatous and hyperplastic parathyroid glands, my assay appears to be the only one which detects elevations of PLP in a significant number of patients with either primary or secondary hyperparathyroidism (p 65). PTH 1-34 added at a concentration of >2ug/tube was not recognised by the antibody in my RIA suggesting that the elevated levels detected in hyperparathyroid patients were not a function of cross-reactivity with high endogenous levels of PTH. Furthermore, preliminary data using commercially available RIAs (Peninsula, Incstar) suggests PLP is detectable in the medium conditioned by bovine parathyroid cells (32) and is elevated in the plasma of some patients with hyperparathyroidism

(Dr.G.N.Hendy and D.Drucker M.D., personal communication). In addition, antiserum R14 directed against PLP 1-74 and used in the Burtis IRMA assay (3), detected substantial quantities of immunoreactive PLP in medium conditioned by a clonal rat parathyroid cell line. This indication that several different antisera, raised by independent investigators, recognise PLP as a secretory product of parathyroid cells provides supporting evidence that PLP could be released into the circulation of some patients with hyperparathyroidism.

Overall, subsequent studies have therefore substantiated and extended my own observations regarding circulating concentrations of NH_2-terminal PLP in the plasma of patients with malignancy. However, discrepancies regarding PLP levels in patients with hyperparathyroidism await further clarification as does identification and characterization of the circulating molecular forms of PLP.

3 Passive immunization against PLP in an animal model of HHM

Despite indirect evidence (33-38) that PLP is a pathogenetic agent in the hypercalcemia associated with the Rice-500 Leydig cell tumor, direct measurement of circulating concentrations of PLP in this animal model were only recently reported (39). Due to the striking homology between rat and human peptides in the 1-111 region of PLP, an IRMA assay developed to measure human PLP 1-74 (3) was modified to measure rat PLP. Whereas 50% of normal human subjects had detectable PLP levels using this assay, PLP was undetectable in all control rats suggesting the peptide does not circulate under normal conditions in these animals. However, in keeping with my own studies (40) and those of others (41-43), tumor

bearing rats in the Gaich study developed hypercalcemia about 10 days following tumor transplantation. A concomitant rise in plasma PLP accompanied the hypercalcemia and a strong positive correlation was demonstrated between circulating levels of PLP and calcium as well as between plasma PLP and tumor size. In addition, tumor excision resulted in a reduction in plasma calcium to control levels and a return of plasma PLP to undetectable within 24h.

In my own passive immunization studies, hypercalcemic tumor-bearing rats treated with a bolus dose of antiserum directed against NH_2-terminal PLP demonstrated a similar rapid decline in plasma calcium (onset at 5h), with levels dropping into the normal range by 24h (40). Therefore, studies measuring plasma PLP levels in hypercalcemic rats complemented my own passive immunization studies which demonstrated the pathogenetic role played by PLP in the hypercalcemia associated with the Rice 500 Leydig cell tumor.

As discussed earlier (p 22), infusion of PLP into rats elicited biological responses similar to those observed in the hypercalcemic rat bearing the Leydig cell tumor i.e. hypercalcemia, hypophosphatemia, renal phosphate wasting, increased excretion of cAMP, mild metabolic alkalosis and increased circulating $1,25(OH)_2D_3$. A large body of data accumulated from IN VITRO experiments using kidney cell, tissue and organ culture, points to a central role being played by the kidney in the development and maintenance of these biochemical abnormalities. Using an isolated, perfused rat kidney, physiological doses of PLP 1-34 and PTH 1-34 were shown to be equipotent in a dose-dependent manner in increasing cAMP and phosphate excretion while decreasing that of calcium (44). However, in

other studies, only PLP was shown to influence hydrogen and bicarbonate ion excretion. Perfusion of a rat kidney with PLP 1-34 decreased urinary pH (45) while PLP 1-141 was shown to decrease bicarbonate excretion (46). On the other hand, perfusion with PTH 1-34 had no effect on either hydrogen or bicarbonate ion transport. These apparently PLP-specific effects on acid-base balance may help to explain the relative alkalosis associated with hypercalcemia and malignancy compared with the metabolic acidosis associated with hyperparathyroidism. The apparent specificity for PLP may reflect different receptor classes (47), preferential use of alternative second messengers (48) or, in the case of PLP 1-141, a different receptor for a sequence outside of the 1-34 region of the molecule. (46). In a further attempt to explain acid-base differences manifested in malignancy and hyperparathyroidism, the sodium-hydrogen antiporter was examined in opossum kidney (OK) cells.(49) The NH_2-terminal fragments of PLP and PTH were, however, equipotent in inhibiting amiloride-sensitive sodium transport making it unlikely that this was the source of the difference.

The PTH responsive opossum OK cell line, which has morphological and transport properties characteristic of proximal tubular cells, was used to study the effects of PLP on sodium dependent phosphate transport Synthetic PLP 1-36 and PTH 1-34 inhibited sodium-phosphate co-transport in OK cells in an equivalent manner (50). The inhibitory activity was shown by others to be mimicked by forskolin and Bu_2cAMP and to be inhibited by the antagonist PTH 3-34 (51) suggesting mediation through a PTH receptor by a cAMP dependent mechanism. An extension of these studies was undertaken, aimed at defining the mechanism responsible for altered

phosphate metabolism in the Leydig tumor bearing rat. Sodium-dependent phosphate transport was examined in renal cortical brush border membrane vesicles prepared from kidneys removed from animals at timed intervals following tumor implantation (43). A specific, persistent impairment of sodium-dependent phosphate uptake was demonstrated as early as 5d post implant, well before overt hypercalcemia and hypophosphatemia occured. The impairment was shown to increase over time but to be independent of the plasma calcium level of the rat from which the kidney was removed.

In my own studies using tumor-bearing rats, the effects of PLP immunoneutralization appeared to be greater on calcium metabolism than on phosphate metabolism (40). Plasma and urinary calcium changes were rapid, marked and prolonged compared with the modest and transient changes in plasma and urinary phosphate (P 91,92). Perhaps this reflected the persistence of impaired renal phosphate transport, even in the absence of endogenous PLP, as suggested by the Sartori study (43).

The earliest effect (5h) of PLP immunoneutralization noted in my study was an augmentation of urinary calcium and magnesium excretion, accompanied by a decrease in plasma calcium (P 93,94). Administration of an inhibitor of renal calcium reabsorption to hypercalcemic rats bearing the Walker 256 carcinosarcoma resulted in the same early alteration in the renal handling of magnesium and calcium (52). This study not only supports my own data indicating an early renal response to neutralization of endogenous PLP but re- emphasises the distal tubular transport mechanism shared by calcium and magnesium.

In the rat model of malignancy associated hypercalcemia, circulating $1,25(OH)_2D_3$ is above normal as it is in hyperparathyroidism.

In contrast, $1,25(OH)_2D_3$ levels are either normal or depressed in the human syndrome of malignancy associated hypercalcemia. Infusion of mice with amino terminal fragments of either PLP or PTH elicited equipotent time and dose dependent increases in 25 hydroxyvitamin D 1a-hydroxylase activity in kidneys removed following the infusion (53). Plasma calcium and phosphate levels showed the expected rise and fall respectively in response to both peptides. As no data was given for circulating $1,25(OH)_2D_3$ in these latter studies the results regarding enzyme activity are more difficult to interpret. However, the results are consistent with IN VIVO data and suggest increased 25 hydroxyvitamin D 1α-hydroxylase activity, stimulated by endogenous PLP, is responsible for the increased levels of circulating $1,25(OH)_2D_3$ in the tumor-bearing rat Although small sample volume prevented direct measurement of $1,25(OH)_2D_3$ levels in my own study, a reduction in the level of this steroid following PLP immunoneutralization most probably occurred and could have contributed to the prolonged period of normocalcemia following antibody treatment.

The action of PLP in the kidney therefore appeared to be making a major contribution to the biochemical abnormalities present in the Leydig tumor-bearing rats and passive immunization against NH_2-terminal PLP resulted in early and pronounced changes in those abnormalities.

In addition to the contributions made by the kidney, the action of PLP in bone has been shown to be an important factor in the development and maintenance of malignancy associated hypercalcemia. Quantitative histomorphometry performed on bone sections of hypercalcemic humans (54) rats (42) and mice (55) bearing tumors revealed a characteristic dissociation of resorption and formation (increased resorption, decreased

formation) compared with the coupled increases in both parameters seen in hyperparathyroidism The catabolic action of PLP in bone has been well documented both IN VIVO (p 23) and IN VITRO. Despite conflicting reports on the relative potencies of NH_2-terminal PLP and PTH in activating second messenger systems (p 26) in bone cells the two peptides appear to be qualitatively and quantitatively similar in their capacity to stimulate bone resorption. Thus, using the fetal rat long bone ^{45}Ca release assay as an index of activity, PLP 1-34 and PTH 1-34 demonstrated equivalent, dose-dependent activity which was qualitatively similar with respect to inhibition by calcitonin and glucocorticoids (56). Similar results were obtained in a neonatal mouse calvarial assay where PLP and PTH appeared to have approximately equal potency in stimulating ^{45}Ca release (57).

The mechamism(s) by which PTH/PLP stimulate osteoclastic bone resorption remain undefined although there is general agreement amongst investigators that resorption is mediated by osteogenic cells. Several reports have documented PLP/PTH stimulated release of soluble " resorption factors ", varying in apparent size from 2-110 Kd, by osteosarcoma cells in culture (58-60). Conditioned medium removed from SAOS-2 cells which had been incubated in the presence of PLP 1-74 stimulated ^{45}Ca release from rat bones in culture but failed to stimulate adenylate cyclase activity in the ROS bioassay The latter observation suggested that the resorptive activity (^{45}Ca release) was not a function of residual bioactive PLP 1-74 in the medium. As the size of the partially purified "resorptive factor" was 9 kD, the same size as PLP 1-74, further characterization and sequencing will be required to determine if it is indeed a novel protein, a fragment of some existing cytokine or residual PLP which was

biologically inactive. An alternative means by which PTH-like peptides could mediate bone resorption would be by local stimulation of cytokines.

Both rat osteosarcoma cells and primary cultures of osteoblast-like cells derived from fetal rat calvaria have been shown to secrete Interleukin-6 (IL-6) into the culture medium in response to stimulation with either PLP or PTH (61). In addition, recombinant IL 6 stimulated bone resorption in metacarpal explants containing only osteoclast precursors but not in explants of radii which contained mature osteoclasts It was suggested that IL-6 could be involved in the recruitment of osteoclast precursors from bone marrow rather than acting directly on mature osteoclasts.

The sustained reduction in urinary calcium excretion in association with normal plasma calcium levels following passive immunization with PLP antiserum in my own studies was most probably a function of a reduced filtered load of calcium consequent to diminished efflux of calcium from bone (p 93). As discussed earlier, a reduction in circulating $1,25(OH)_2D_3$ could also have contributed to this period of normocalcemia. An additional indication that bone healing may have been taking place following neutralization of endogenous PLP was the progressive rise in plasma alkaline phosphatase levels during this period (p 91) In support of this observation are studies documenting PLP inhibition of cell growth and alkaline phosphatase activity in ROS 17/2.8 cells (62) and inhibition of ^3H proline incorporation into collagenase-digestible protein of fetal rat calvaria following three days of treatment (63). These inhibitory effects of NH_2-terminal PLP on bone cell growth and collagen production were, however, mimicked by NH_2-terminal PTH and therefore would not appear to

explain the apparent discrepancy between " coupled " and " uncoupled" activity in hyperparathyroidism and malignancy associated hypercalcemia respectively Continued identification (64,65) and characterization (66,67) of PLP/PTH responsive osseous target cells as well as development and evaluation of PLP/PTH sensitive osteogenic cell lines (68,69) from the multiplicity of cell types present in skeletal tissue should help to explain the process of bone resorption in general as well as anomalies in bone turnover associated with hyperparathyroidism and hypercalcemia of malignancy.

The lack of significant biochemical changes in control animals treated with antiserum against PLP in my studies (p 93-96) is not surprising in view of the inability to detect circulating levels of the peptide in normal rats (39). This latter finding therefore supports the conclusion I arrived at regarding PLP playing no role in the mineral metabolism of the normal post-natal rat (p 82)

4 **Dysregulation of PLP expression in a model of tumor progression**

Little is known regarding the regulation of PLP production in either normal or neoplastic cells or tissues. Human keratinocytes in culture were the first normal cells shown to express PLP mRNA (70) and to secrete PLP into the surrounding medium (6). It is not known if these cells are the source of the peptide in the systemic circulation of normal individuals although affinity purification of keratinocyte conditioned medium (71) yielded 3 distinct PLP species with estimated weights of 8-17 kd which approximated those identified in my gel filtration analysis of human plasma (2) I have extended recent studies examining the regulatory

effects of growth factors and $1,25(OH)_2D_3$ on PLP expression and secretion in normal human keratinocytes (6) by examining the dysregulation of PLP production in a keratinocyte model of tumor progression (72) The established cell line, HPK1A, used in this work retains many features characteristic of normal keratinocytes (p 119) including those of regulated PLP production. In addition, their indefinite lifespan in culture offers an advantage over normal keratinocytes, which senesce after several passages, requiring constant renewal from sources which may differ in PLP expression The well characterized malignant nature of the ras-transformed cells, HPK1A-ras (p 119), coupled with their overproduction of PLP make them an ideal source in which to study the mechanisms of dysregulation of PLP expression.

In the former studies (6), normal keratinocytes were shown to respond to serum and growth factor stimulation with a rapid and transient increase in PLP mRNA. Chimeric constructs, containing different size fragments of the 5' flanking region of the PLP gene linked to a growth hormone reporter gene, demonstrated both serum and growth factor enhanced PLP promoter activity when transfected into normal human keratinocytes As no characteristic serum responsive sequence has been identified to date in the 5' flanking region of about 1 kb of the rat gene (73) and more than 5 kb of the human gene (74), the elements conferring responsivenes in the latter studies are either novel or lie further upstream of the known sequence. Using the same technique, an inhibitory, cis-acting element responsive to $1,25(OH)_2D_3$ was identified in the promoter region of the PLP gene. Whether or not the resistance to serum and $1,25(OH)_2D_3$ noted in my own studies in the malignant HPK1A-ras cells (p 126,130,131) lies at the

level of gene transcription remains to be determined. Preliminary data indicates that the resistance to $1,25(OH)_2D_3$ lies somewhere beyond the process of binding to its cytosolic receptor (75). Both established and malignant keratinocytes demonstrated $1,25(OH)_2D_3$ binding characteristics which were similar to those found in normal keratinocytes. These included receptor number, affinity and migration pattern of the receptor/ligand complex on a sucrose density gradient.

In addition to growth factors, serum and $1,25(OH)_2D_3$, 17ß estradiol (E_2) has been shown to regulate PLP mRNA expression in rat myometrial tissue. Elevated levels of PLP mRNA had been noted in the myometrium of rat uteri as early as 8d of gestation (5). Levels remained elevated above those of non-gravid uteri for about 10d then increased sharply to term and declined rapidly following parturition. Although the latter sharp increase was thought to be stretch induced and was attributed directly to increasing size of the feto-placental unit, the early increase was not adequately explained Subsequent studies, therefore examined the effects of exogenous 17ß estradiol on uterine PLP expression in ovariectomized rats (76). A rapid and transient increase in PLP mRNA was seen in uterine tissue removed from E_2 treated animals compared with control animals treated with vehicle demonstrating the E_2 inducibility of the PLP gene.

While peak levels of activity occurred at 2h after commencement of treatment in the latter study, maximum levels of PLP mRNA induction were noted at 1h in serum stimulated normal keratinocytes (6), at 6h in serum stimulated established and malignant keratinocytes (72) and at 2h in rat islet cells treated with sodium butyrate (77). Despite variability regarding the time of maximum activity, all of these studies indicate a

rapid onset of PLP mRNA induction subsequent to stimulation with a wide variety of agents. In addition, two reports (77,78) have documented superinduction of PLP mRNA following cycloheximide treatment. Taken together, these observations give credence to the hypothesis that PLP may belong to a family of early response genes such as those which have been implicated in cell growth (79).

Unlike the rapid and transient inducibility of PLP mRNA outlined above, E_2 induced activity in early passage cultures of primary rat myometrial cells appeared to peak at 24h and was maintained over a 5d period in association with secretion of PLP into the surrounding medium (80). However, a significant reduction in PLP expression and secretion after two passages suggests a requirement for further characterization before these cells can be considered a suitable model in which to study PLP gene expression. In contrast, the characteristics of PLP expression and secretion remained constant over more than 50 passages in the HPK1A and HPK1A-ras cells used in my own studies (72).

In light of the E_2 inducibility of PLP mRNA, transient tansfection analysis of fragments of the PLP gene promoter have been initiated in an attempt to identify an E_2 responsive element (81). Co-transfection of fusion constructs and an estrogen receptor expression plasmid conferred E_2 responsiveness to a receptor naive monkey kidney cell line. As was the case with serum and $1,25(OH)_2D_3$ in our own studies (6), no consensus E_2 responsive sequence was detected in any of the fragments shown to enhance reporter gene activity. However, treatment with dexamethasone completely abolished the E_2 enhanced activity of even the smallest (0.2kb) construct suggesting the presence of a negative glucocorticoid responsive element in

this region. The existence of such an element has been alluded to in other studies demonstrating glucocorticoid-dependent decreases in PLP mRNA in human neuroendocrine cell lines (82,83) as well as in the rat islet cell line (77).

Overall, these observations regarding transcriptional regulation of the PLP gene suggest the involvement of a complex and interdependent network of factors. The precise genetic localization and identity of the responsive elements, as well as characterization of their interactions with one another, will require extensive investigation.

Before the development of specific immunoassays to measure PLP directly, early studies looking at PLP secretion used the adenylate cyclase bioassay as an index of the amount of PLP present in conditioned medium. One of these studies examined the regulation of PLP secretion from human osteosarcoma cells using serum, EGF and 12-O-tetradecanoylphorbol-13-acetate (TPA) as secretagogues (84). Combinations of 10% serum and EGF or 10% serum and TPA were shown to increase bioactivity in conditioned medium and to stimulate PLP mRNA expression in the cells. The relative contributions made by the individual agents to the stimulatory response could not be assessed as no attempt was made to measure either basal secretion or secretion in response to single agents. A similar study examining PLP production by cultured normal keratinocytes reported increased bioactivity in serum-stimulated cells but no significant difference in activity from control in cells treated with calcium, EGF or $1,25(OH)_2D_3$ (85). These observations differ considerably from our own which showed increased PLP secretion in response to a high extracellular calcium environment as well as a dose dependent decrease in PLP production in

response to $1,25(OH)_2D_3$ in normal keratinocytes (6). In addition, both established and malignant keratinocytes demonstrated dose dependent responses to serum, EGF and $1,25(OH)_2D_3$ at the level of mRNA (p 126) and secreted protein (p 130,131). Although bioactive PLP was shown to correlate well with immunoreactive PLP in the latter studies, the radioimmunoassay may be a more sensitive index of altered secretion than the bioassay used in the former studies. Variations in culture conditions, length of the stimulation period or the use of a resting (serum-free) period prior to stimulation could also account in part for the observed differences between the two studies.

The remaining reports, with the exception of two documenting the regulation of PLP secretion in neuroendocrine tumors (86,87), examine the role played by calcium in the PLP secretory response. Various investigators have shown that raising the extracellular calcium to 2.5mM or more had no effect (32), minimal effect (88) or a significant effect (89) in reducing the rate of PLP secretion from bovine and rat parathyroid cells. On the other hand, human keratinocytes (6,90) and rat Leydig tumor cells (91) cultured in a high calcium environment produced more than 100% more PLP than the same cell type cultured in a low calcium environment. In addition, treatment of lung cancer cells in culture with the calcium ionophore, ionomycin, resulted in an increase in both intracellular calcium and PLP secretion within minutes (92). Addition of the phorbol ester phorbol myristate acetate (PMA) or phospholipase-C to the culture medium resulted in the same rapid onset of PLP secretion suggesting involvement of the protein kinase C pathway in this response. As a positive correlation is known to exist between extracellular and

intracellular calcium levels (93), these preliminary experiments implicating intracellular calcium transients and the calcium messenger system in the PLP secretory response lend support to my own data (90) as well as that of others (91) demonstrating the modulation of PLP secretion by extracellular calcium.

Treatment of the Leydig tumor cells with cycloheximide abolished the calcium-enhanced PLP response whereas treatment with transcriptional inhibitors did not. The calcium induced changes in PLP secretion from the lung tumor cells remained unaffected by either transcriptional or protein synthesis inhibitors. However, as the latter experiments were conducted over a very short (15min) time course an effect of either agent occurring at a later time point could have been missed. The failure of transcriptional inhibitors to alter the calcium-induced increment in PLP secretion is in keeping with our own transient transfection studies in which no calcium responsive element could be identified in the PLP gene promoter (6). Further investigation will therefore be required to identify the mechanisms involved in the regulation of PLP production by extracellular calcium. These studies could have implications IN VIVO where elevated calcium levels could exacerbate the hypercalcemic syndrome in malignancy by promoting the secretion of PLP from tumor tissue.

The regulation of PLP gene expression and secretion has also been studied in the lactating mammary gland IN VIVO. Unlike keratinocytes, in which PLP is a constitutive secretory product, it appears to be expressed and secreted in mammary epithelia only during pregnancy and lactation. Because PLP was initially characterized as a calciotropic hormone attempts were made to identify it as the unknown factor responsible for the

mobilization and transfer of large quantities of calcium into milk during lactation. Thus, examination of the mammary tissue of rats revealed a single 1.5 kb PLP transcript appearing at the onset of lactation and reaching a maximum level of expression at 24h postpartum (94) PLP continued to be expressed at a high level over the next 20 days in the continued presence of the litter. Removal of the pups, however, resulted in a rapid decline in PLP mRNA to undetectable levels by 4h. Re-introduction of the litter at this point resulted in a rapid induction of PLP mRNA whereas re-introduction of the litter following cessation of lactation failed to induce the message, demonstrating a requirement for lactation not just suckling. Translation of PLP mRNA was verified when extracts of lactating mammary gland were shown to stimulate adenylate cyclase activity in the rat osteosarcoma bioassay.

The time course for the suckling-induced appearance of PLP mRNA in this model closely resembled that reported for the rise in serum prolactin following the onset of suckling in the rat (95). Further examination of the lactating rat model (96) revealed a close temporal relationship between the rise in serum prolactin and the induction of mammary PLP mRNA. A 20 fold increase in serum prolactin which was induced by suckling was followed several hours later by a 20 fold increase in PLP mRNA in the mammary tissue. Given the 10 fold increase in serum prolactin that occurs 4-6h prior to parturition in the rat (95) and the fact that induction of PLP mRNA by prolactin is well established by 2h, the lack of demonstrable PLP mRNA in mammary tissue at the time of parturition in the earlier study (94) was puzzling. However, a later report using a more sensitive method for detecting low abundance mRNA not only confirmed high levels of

expression during lactation but also documented the presence of PLP mRNA in non-lactating mammary glands during late pregnancy (97).

Earlier studies had implicated prolactin in the mobilization of maternal calcium and phosphorus during milk production (98,99). As prolactin had been shown to have no direct effect on mineral mobilization, these latter studies raised the possibility that PLP might be the agent through which prolactin was working. To determine whether this locally produced PLP was released into the maternal circulation, immunoreactive PLP levels were measured in the serum of lactating and hyperprolactinemic women. Despite up to 15 fold elevations above the upper limit of normal for prolactin , circulating levels of PLP in pre-partum and post-partum women, as well as women with hyperprolactinemia, remained within the normal range (100,101).On the other hand, up to 10,000 fold elevations above normal circulating levels of amino-terminal PLP were detected in unprocessed human, bovine and rat milk as well as in samples of commercial milk products including infant formulas. Therefore, although PLP produced locally in the breast in response to elevations in serum prolactin did not appear to gain access to the maternal circulation it was released in large quantities into milk, perhaps to play a role in neonatal calcium homeostasis. Further evidence that PLP produced locally in lactating mammary tissue does not enter the maternal circulation came from passive immunization experiments in lactating mice (102). Immunoneutralization of PLP in lactating mice had no effect on maternal serum calcium, milk calcium and phosphorus concentrations or on maternal bone calcium content suggesting PLP was not involved in the mobilization of calcium and phosphorus from the mother for milk production. Using the goat as a model

of lactation a small PLP gradient was demonstrated between the systemic circulation and mammary venous effluent (103). However the PLP values reported for the maternal circulation were approximately 5000 fold less than those reported for the milk suggesting a relatively unimportant role, if any, being played by the peptide in maternal mineral mobilization.

The possibility remained that PLP could be responsible for the transfer of calcium from blood to milk. Although a recent study showed a reasonable correlation between the plasma level of PLP and the duration of lactation in cows (r=0.67), the data correlating plasma PLP with milk calcium content was less convincing (r=0.35)(104).It would also be difficult to justify the requirement for levels in excess of $5x10^3$ times normal circulating levels of PLP for the transfer of calcium into milk IN VIVO. A more likely explanation for the high levels of PLP in milk is that it plays some physiological role in the neonate.

A wide variety of cell types have therefore been shown to express and secrete PLP in a regulated manner both IN VITRO and IN VIVO although the function of the secreted protein under normal circumstances remains undetermined.

5 Adenylate-cyclase linked PLP receptors on human keratinocytes.

The SV40-transformed keratinocyte cell lines (p 164) were specifically selected for PLP receptor studies because they do not secrete PLP. Neither immunoreactive nor bioactive PLP was detected in the conditioned medium (concentrated up to 20 times) which was removed from cultures of RHEK-1, RHEK-ras or RHEK-fos cells (unpublished observations)

Characterization of the binding of ^{125}I- PTH and activation of adenylate cyclase in the RHEK-fos cells revealed activity similar to that obtained for the RHEK-1 keratinocytes (105). I was, therefore, able to demonstrate specific binding of ^{125}I-PTH and activation of adenylate cyclase in both established (106) and malignant (105) keratinocytes which no longer secreted PLP. The ability to demonstrate PLP receptor binding and activation only in the absence of endogenous ligand is not unprecedented. SAOS-2/B-10 cells, which were sub-cloned from the parent SAOS-2 line, expressed and secreted PLP during early passages but showed no evidence of the PLP responsive adenylate cyclase activity which was characteristic of the parent cells (84). In later passages however, the cells ceased to express and secrete PLP but developed a strong adenylate cyclase response to PLP. In addition, mouse embryonal carcinoma cells were shown to express PLP mRNA and PLP receptors during the process of differentiation to parietal endoderm (107). However, no immunoreactive PLP could be detected in the medium conditioned by differentiated cells despite the presence of a strong signal on Northern analysis. Therefore, no study to date has demonstrated the presence of functional PLP/PTH receptors on a PLP secreting cell, perhaps reflecting a desensitization and/or downregulation of receptors by endogenous ligand. Supporting evidence comes from the observations made in my own study (106) that not only were PLP receptors present at a low concentration on the RHEK-1 keratinocytes (p 158) but also showed a much greater sensitivity to homologous downregulation than had been previously demonstrated in other cell types (p 156). A preliminary report has identified a low affinity binding protein for PLP on several malignant keratinocyte cell lines which

also secreted PLP (108). However, as binding of ^{125}I-PLP could not be associated with activation of any transduction mechanism the authors suggested it did not represent a true receptor. The same report also documented stimulation of intracellular calcium transients in both normal and malignant squamous cells, similar to those which have been observed in other cells in response to PLP/PTH stimulation The EC_{50} of 0.08 nM suggested the presence of a high affinity receptor for PLP on those cells. Therefore, in support of my own observations identifying both high and low affinity binding sites for PLP on human keratinocytes (p 158) these investigators had apparently identified a low affinity site and suggested the existence of a high affinity site. The inability to detect a cAMP response is puzzling although not entirely inconsistent with my own data which failed to identify either a receptor or a cAMP response in the ras transformed keratinocytes. I attributed this anomaly in part to overexpression of the p21 product of the ras oncogene. Although this protein is located on the inner side of the plasma membrane and binds GTP and GDP in a manner analagous to the guanyl nucleotide regulatory proteins (109), there is no evidence to date that it regulates adenylate cyclase activity by mimicking either Gs or Gi (110). However, an overabundance of the protein could interfere with the normal functioning of the regulatory G proteins. This hypothesis is supported in my own studies documenting a defect in both PLP (p 160) and PGE_2 (p 161) stimulated adenylate cyclase activity in ras transformed cells compared with established and fos transformed cells (105,106). However, overexpression of p21 seems an unlikely explanation for the lack of PLP responsive adenylate cyclase activity in several different squamous cancer cell lines as well as in

normal keratinocytes in the Orloff study. These discrepancies therefore warrant further investigation.

The rat pancreatic islet cell may well prove to be an alternative model in which to study a potential autocrine/paracrine role of PLP. Normal rat islet cells in culture have been shown to express PLP mRNA which is translated into peptide and secreted into the surrounding medium (10). In an unrelated study, PTH was shown to stimulate glucose-induced insulin release from rat pancreatic islets (111). As PLP and PTH are known to interact with the same receptor the possibility is raised that PTH receptors on the rat islet cell could be activated by endogenously produced PLP. In the latter studies, at low concentration, PTH stimulated an increase in both cAMP and intracellular calcium as well as augmenting phorbol ester and glucose-induced insulin release. In addition, these actions were shown to be modulated by the extracellular calcium concentration The action of PTH in stimulating insulin release from pancreatic islet cells therefore appeared to involve more than one transduction mechanism. Although these latter studies were carried out using NH_2-terminal PTH the observations are in keeping with my own studies in keratinocytes using NH_2-terminal PLP (106). PLP stimulated cAMP accumulation and mitogenesis in the established RHEK-1 cells were both modulated by the extracellular calcium concentration (p 162,163). In addition, PLP stimulated mitogenesis appeared to be enhanced in the presence of EGF suggesting an interaction between the transduction mechanisms of these two peptides (p 163).

Most studies to date have examined the binding, signal transduction and biological action of amino-terminal PLP. There are, however,

several indications that the region beyond PLP 1-34 may have physiological relevance. These include the potential for variable processing of the human gene giving rise to 3 proteins with different carboxy termini, the extensive conservation in amino acids 35-111 between rat and human peptides and the presence of multiple potential cleavage sites between positions 88 and 108. Using recombinant (r) PLP 1-141 (112) I have initiated studies comparing the actions of the full length peptide with that of NH_2-terminal and mid-region fragments on RHEK-1 keratinocytes Preliminary data indicates that unlike NH_2-terminal PLP and PTH which stimulate growth in these cells, (r) PLP-1-141 appears to inhibit growth in RHEK-1 cells (113). Supporting evidence for an inhibitory role for PLP in keratinocyte growth comes from preliminary studies in which PLP-producing HPK1A keratinocytes were infected with a retroviral vector expressing PLP in an antisense orientation (114). Inhibition of translation of endogenous PLP mRNA in infected cells resulted in a dramatic reduction in secreted PLP accompanied by an increase in cell growth.

Although PLP 1-141 and PLP 1-34 were equipotent in stimulating adenylate cyclase activity in RHEK-1 cells (p 165), co-incubation of PLP 1-141 and PLP 3-34 failed to prevent the inhibition of RHEK-1 cell growth This suggested that the effect was mediated outside of the sequence required for adenylate cyclase activation. Incubation of RHEK-1 cells with different fragments of PLP which encompassed almost the entire 141 amino acids failed to alter mitogenic activity indicating the presence of the full length recombinant molecule was required for the inhibitory effect. In a few other instances, biological activity has been attributed to

sequences of PLP and PTH outside of the 1-34 region. PLP 75-86 has been implicated as an active fragment in the transfer of calcium and magnesium across the ovine placenta (115) and PLP 1-141 was shown to decrease bicarbonate excretion via a domain outside of that required for amino-terminal binding (46). In addition PLP 107-139 (116) and PTH 53-84 (117,118) have been shown to play a role in the regulation of bone cell biology. Taken together with my own data demonstrating the inhibitory effect of PLP 1-141 on mitogenesis in keratinocytes, these findings suggest that both PLP and PTH could contain biologically relevant sequences outside of those required for activation of the receptor for their amino-terminal sequences.

The molecular structure of the classical PTH receptor, which has been cloned from both OK and ROS 17/2.8 cells, will be disclosed within a short time (119-121). The answers to questions regarding receptor subclasses, alternative transduction mechanisms and alternative receptors can then, hopefully, be more easily pursued.

REFERENCES

1. Budayr A.A, Nissenson R.A, Klein R F, Pun K.K, Clark O H, Diep D, Arnaud C.D and Strewler G.J. 1989 Increased serum levels of a parathyroid hormone-like protein in malignancy-associated hypercalcemia. Ann Int Med 111 807-812.

2. Henderson J.E, Shustik C, Kremer R, Rabbani S A, Hendy G,N and Goltzman D. 1990 Circulating concentrations of parathyroid hormone-like peptide in malignancy and in hyperparathyroidism J Bone Min Res 5 105-113

3. Burtis W J, Brady T.G, Orloff J.J, Ersbak J B, Warrell R P, Olson B.R, Wu T.L, Mitnick M.E, Broadus A E and Stewart A.F 1990 Immunochemical characterization of circulating parathyroid hormone-related protein in patients with humoral hypercalcemia of cancer. New Engl J Med 322 1106-1112

4. Kao P.C, Klee G.G, Taylor R L and Heath H 1990 Parathyroid hormone-related peptide in plasma of patients with hypercalcemia and malignant lesions. Mayo Clin Proc 65 1399-1407.

5. Thiede M.A, Daifotis A G, Weir E.C, Brines M L, Burtis W J, Ikeda K, Dreyer B.E, Garfield R.E and Broadus A.E. 1990 Intrauterine occupancy controls expression of the parathyroid hormone-related peptide gene in preterm rat myometrium Proc Natl Acad Sci (USA) 87 6969-6973.

6. Kremer R, Karaplis A.C, Henderson J, Gulliver W, Banville D, Hendy G.N and Goltzman D. 1991 Regulation of parathyroid hormone-like peptide in cultured normal human keratinocytes. J Clin Invest 87 884-893

7. Southby J, Kissin M.W, Danks J.A, Hayman J.A, Moseley J M, Henderson M.A, Bennett R.C and Martin T.J. 1990 Immunohistochemical localization of parathyroid hormone-related protein in human breast cancer. Cancer Res 50 7710-7716.

8. Powell G.J, Southby J, Danks J A, Stillwell R.G, Hayman J.A, Henderson M.A, Bennett R C and Martin T,J. 1991 Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared with other sites Cancer Res 51 3059-3061.

9. Zakalik D, Hooks M.A, Nissenson R.A and Strewler G.J. 1991 Regulation of parathyroid hormone-related protein by the HTLV-1 transactivator Tax J Bone Min Res 6S1 S234.

10. Drucker D.J, Asa S.L, Henderson J and Goltzman D. 1989 The parathyroid hormone-like peptide gene is expressed in the normal and neoplastic human endocrine pancreas. Mol Endocrinol 3 1589-1595

11. Rizzoli R, Sappino A.P and Bonjour J.P. 1990 Parathyroid hormone-related protein and hypercalcemia in pancreatic neuro-endocrine tumors. Int J Cancer 46 394-398.

12. Asa S.L, Henderson J, Goltzman D and Drucker D,J. 1990 Parathyroid hormone-like peptide in normal and neoplastic human endocrine tissues. J Clin Endocrinol Metab 71 1112-1118.

13. Kimura S, Nishimura Y, Yamaguchi K, Nagasaki K, Shimada K and Uchida H. 1990 A case of pheochromocytoma producing parathyroid hormone-related protein and presenting with hypercalcemia. J Clin Endocrinol Metab 70 1559-1563.

14. Mitlak B.H, Hutchison J.S, Kaufman S.D and Nussbaum S.R. 1991 Parathyroid hormone-related peptide mediates hypercalcemia in an islet cell tumor of the pancreas. Horm Metab Res 23 344-346.

15. Ikeda K, Weir E.C, Mangin M, Dannies P.S, Kinder B, Deftos L, Brown E.M and Broadus A E. 1988 Expression of messenger ribonucleic acids encoding a parathyroid hormone-like peptide in normal human and animal tissues with abnormal expression in human parathyroid adenomas. Mol Endocrinol 2 1230-1236.

16. Weir E.C, Brines M.L, Ikeda K, Burtis W.J, Broadus A.E and Robbins R.J. 1990 Parathyroid hormone-related peptide gene is expressed in the mammalian central nervous system. Proc Natl Acad Sci (USA) 87 108-112.

17. Hayman J.A, Danks J.A, Ebeling P.R, Moseley J.M, Kemp B.E and Martin T.J. 1989 Expression of parathyroid hormone related protein in normal skin and in tumors of skin and skin appendages. J Pathol 158 293-296.

18. McAuley P, Asa S L, Chiu B, Henderson J, Goltzman D and Drucker D.J. 1990 Parathyroid hormone-like peptide in normal and neoplastic mesothelial cells. Cancer 66 1975-1979.

19. Kitazawa S, Fukase M, Kitazawa R, Takenaka A, Gotoh A, Fujita T and Maeda S. 1990 Immunohistologic evaluation of parathyroid hormone-related protein in human lung cancer and normal tissue with newly developed monoclonal antibody. Cancer 67 984-989.

20. Atillasoy E.J, Burtis W.J and Milstone L.M. 1991 Immunohistochemical localization of parathyroid hormone-related protein (PTHRP) in normal human skin. J Invest Derm 96 277-280.

21. Kramer S, Reynolds F.H, Castillo M, Valenzuela D.M Thorikay M and Sorvillo J.M. 1991 Immunological identification and distribution of parathyroid hormone-like protein polypeptides in normal and malignant tissues. Endocrinology 128 1927-1936.

22. Ralston S.H, Danks J, Hayman J, Fraser W.D, Stewart C.S and Martin T.J. 1991 Parathyroid hormone-related protein of malignancy: immunohistochemical and biochemical studies in normocalcemic and hypercalcemic patients. J Clin Pathol 44 472-476.

23. Stewart A.F, Burtis W.J, Wu T, Goumas D and Broadus A.E. 1987 Two forms of parathyroid hormone-like adenylate cyclase-stimulating protein derived from tumors associated with humoral hypercalcemia of malignancy. J Bone Min Res 2 587-593.

24. Strewler G.J, Stern P.H, Jacobs J.W, Eveloff J, Klein R.F, Leung S.C, Rosenblatt M and Nissenson R.A. 1987 Parathyroid hormonelike protein from human renal carcinoma cells: structural and functional homology with parathyroid hormone. J Clin Invest 80 1803-07

25. Goltzman D, Bennett H.P.J, Koutsilieris M, Mitchell J, Rabbani S.A and Roueau M. 1986 Studies of the multiple molecular forms of bioactive parathyroid hormone and parathyroid hormone-like substances. Recent Prog Horm Res 42 665-703

26. Burtis W, Gaich G, Fodero J and Stewart A 1991 Characterization of circulating species of PTH-related protein in HHM. J Bone Min Res 6S1 S228.

27. Stewart A.F, Eliot J, Burtis W.J, Wu T and Insogna K.L. 1989 Synthetic parathyroid hormone-like protein-(1-74): biochemical and physiological characterization. Endocrinology 124 642-648.

28. Thorikay M, Kramer S, Reynolds F.H, Sorvillo J.M, Doescher L, Wu T, Morris C.A, Burtis W.J, Insogna K.L, Valenzuela D M and Stewart A.F 1989 Synthesis of a gene encoding parathyroid hormone-like protein-(1-141): purification and biological characterization of the expressed protein. Endocrinology 124 111-118.

29. Ikeda K, Arnold A, Mangin M, Kinder B, Vydelingum N.A, Brennan M.F and Broadus A.E. 1989 Expression of transcripts encoding a parathyroid hormone-related peptide in abnormal human parathyroid tissues. J Clin Edocrinol Metab 69 1240-1247

30. Docherty H.M, Ratcliffe W.A, Heath D A and Docherty K 1991 Expression of parathyroid hormone-related protein in abnormal human parathyroids. J Endocrinol 129 431-438

31. Danks J.A, Ebeling P.R, Hayman J.A, Diefenbach-Jagger H, Collier F, Grill V, Southby J, Moseley J.M, Chou S T and Martin T.J. 1990 Immunohistochemical localization of parathyroid hormone-related protein in parathyroid adenoma and hyperplasia. J Pathol 161 27-33.

32. Connor C.S, Drees B.M, Thurston A, Forte L, Hermreck A.S and Hamilton J.W. 1989 Bovine parathyroid tissue: a model to compare the biosynthesis and secretion of parathyroid hormone and parathyroid hormone-related protein. Surgery 106 1057-1062.

33. Yasuda T, Banville D, Rabbani S.A, Hendy G.N and Goltzman D. 1989 Rat parathyroid hormone-like peptide:comparison with the human homologue and expression in malignant and normal tissue. Mol Endocrinol 3 518-524.

34. Horiuchi N, Caulfield M.P, Fisher J.E, Goldman M.E, McKee R.L, Reagan J.E, Levy J.J, Nutt R.F, Rodan S.B, Schofield T.I, Clemens T.I and Rosenblatt M. 1987 Similarity of synthetic peptide from human tumor to parathyroid hormone IN VIVO and IN VITRO. Science 238 1566-1568.

35. Rabbani S.A, Mitchell J, Roy D.R, Hendy G.N and Goltzman D 1988 Influence of the amino-terminus on IN VITRO and IN VIVO biological activity of synthetic parathyroid hormone-like peptides of malignancy. Endocrinology 123 2709-2716.

36. Yates A.J.P, Gutierrez G.E, Smolens P, Travis P.S, Katz M.S, Aufdemorte T.B, Boyce B.F, Hymer T.K, Poser J.W and Mundy G.R. 1988 Effects of a synthetic peptide of a parathyroid-hormone related protein on calcium homeostasis, renal calcium reabsorption and bone metabolism IN VIVO and IN VITRO in rodents. J. Clin. Invest. 81 932-938.

37 Zhou H, Leaver D.D, Moseley J.M, Kemp B, Ebeling P.R and Martin T.J. 1989 Actions of parathyroid hormone-related protein on the rat kidney IN VIVO. J. Endocrinol 122 229-235.

38. Kukreja S.C, Shevrin D.H, Wimbiscus S.A, Ebeling P.R, Danks J.A, Rodda C.P, Wood W.I and Martin T.J. 1988 Antibodies to parathyroid hormone-related protein lower serum calcium in athymic mouse models of malignancy associated hypercalcemia due to human tumors. J Clin Invest 82 1798-1802.

39. Gaich G and Burtis W.J. 1990 Measurement of circulating parathyroid hormone-related protein in rats with humoral hypercalcemia of malignancy using a two-site immunoradiometric assay. Endocrinology 127 1444-1449.

40. Henderson J, Bernier S, D'Amour P and Goltzman D. 1990 Effects of passive immunization against parathyroid hormone (PTH)-like peptide and PTH in hypercalcemic tumor-bearing rats and normocalcemic controls. Endocrinology 127 1310-1318.

41. Sica D.A, Martodam R.R, Aronow J and Mundy G.R. 1983 The hypercalcemic rat leydig cell tumor- A model of the humoral hypercalcemia of malignancy. Calcif Tiss Int 35 287-293.

42. Insogna K.L, Stewart A.F, Vignery A.M.C, Weir E, Namnum P.A, Baron R.E, Kirkwood J.M, Deftos L.M and Broadus A.E. 1984 Biochemical and histomorphometric characterization of a rat model for humoral hypercalcemia of malignancy. Endocrinology 114 888-896.

43. Sartori L, Insogna K.L and Barrett P.Q. 1988 Renal phosphate transport in humoral hypercalcemia of malignancy. Amer J Physiol F1078-1084.

44. Scheinman S.J, Mitnick M.E and Stewart A.F. 1990 Quantitative evaluation of anticalciuretic effects of synthetic parathyroid hormonelike peptides. J Bone Min Res 5 653-658.

45. Ebeling P.R, Adam W.R, Moseley J.M and Martin T.J. 1989 Actions of synthetic parathyroid hormone-related protein (1-34) on the isolated rat kidney. J Endocrinol 120 45-50.

46. Ellis A.G, Adam W.R and Martin T.J. 1990 Comparison of the effects of parathyroid hormone (PTH) and recombinant PTH-related protein on bicarbonate excretion by the isolated perfused rat kidney. J Endocrinol 126 403-408.

47. Orloff J.J, Wu T.L and Stewart A.F. 1989 Parathyroid hormone-like proteins: biochemical responses and receptor interactions. Endocrine Rev 10 476-495.

48. Donahue H.J, Fryer M.J and Heath H. 1990 Structure-function relationships for full-length recombinant parathyroid hormone-related peptide and its amino-terminal fragments: effects on cytosolic calcium ion mobilization and adenylate cyclase activation in rat osteoblast-like cells. Endocrinology 126 1471-1477.

49. Caverzasio J, Rizzoli R, Martin T.J and Bonjour J.P 1988 Tumoral synthetic parathyroid hormone related peptide inhibits amiloride-sensitive sodium transport in cultured renal epithelia. Pflugers Arch 413 96-98.

50. Sartori L, Weir E.C, Stewart A.F, Broadus A.E, Mangin M, Barrett P.Q and Insogna K.L. 1988 Synthetic and partially purified adenylate cyclase-stimulating proteins from tumors associated with humoral hypercalcemia of malignancy inhibit phosphate transport in a PTH-responsive renal cell line. J Clin Endocrinol Metab 66 459-461

51. Nakai M, Fukase M, Yamaguchi T, Tsukamoto T, Fujii N and Fujita T. 1990 Human PTH-(3-34) inhibited the effects of human parathyroid hormone-related protein on phosphate uptake in a cultured renal cell line (OK cells). J Bone Min Res 5 995-1002.

52. Hurtado J, Esbrit P and Rapado A. 1991 Relative role of bone and kidney in the hypercalcemia associated with the rat Walker carcinosarcoma 256. Europ J Cancer 27 76-79.

53. Walker A.T, Stewart A.F, Korn E.A, Shiratori T, Mitnick M.A and Carpenter T.O. 1990 Effect of parathyroid hormone-like peptides on 25-hydroxyvitamin D-1a-hydroxylase activity in rodents. Am J Physiol E297-303.

54. Stewart A.F, Vignery A, Silverglate A, Ravin N.D, LiVolsi V, Broadus A.E and Baron R. 1982 Quantitative bone histomorphometry in humoral hypercalcemia of malignancy: uncoupling of bone cell activity. J Clin Endocrinol Metab 55 219-227

55. Kukreja S.C, Rosol T.J, Shevrin D.H and York P.A. 1988 Quantitative bone histomorphometry in nude mice bearing a human squamous cell lung cancer. J Bone Min Res 3 341-346.

56. Raisz L.G, Simmons H.A, Vargas S.J, Kemp B.E and Martin T.J. 1990 Comparison of the effects of amino-terminal synthetic parathyroid hormone-related peptide (PTHrP) of malignancy and parathyroid hormone on resorption of cultured fetal rat long bones. Cal Tissue Int 46 233-238.

57. Fukayama S, Bosma T.J, Goad D.L, Voelkel E.F and Tashjian A.H. 1988 Human parathyroid hormone (PTH)- related protein and human PTH: comparative biological activities on human bone cells and bone resorption. Endocrinology 123 2841-2847.

58. McSheehy P.M.J and Chambers T.J. 1986 Osteoblast-like cells in the presence of parathyroid hormone release soluble factor that stimulates osteoclastic bone resorption. Endocrinology 119 1654-1659.

59. Perry H.M, Skogen W, Chappel J, Kahn A.J, Wilner G and Teitelbaum S.L. 1989 Partial characterization of a parathyroid hormone-stimulated resorption factor(s) from osteoblast-like cells. Endocrinology 125 2075-2082.

60. Morris C.A, Mitnick M.E, Weir E.C, Horowitz M, Kreider B.L and Insogna K.L. 1990 The parathyroid hormone-related protein stimulates human osteoblast-like cells to secrete a 9,000 dalton bone-resorbing protein. Endocrinology 126 1783-1785.

61. Lowik C.W.G.M, van der Pluijm G, Bloys H, Hoekman K, Bijvoet O.L.M, Aarden L.A and Papapoulos S.E. 1989 Parathyroid hormone (PTH) and PTH-like protein (PLP) stimulate Interleukin-6 production by osteogenic cells: a possible role of Interleukin-6 in osteoclastogenesis. Biochem Biophys Res Comm 162 1546-1552.

62. Rodan S.B, Noda M, Wesolowski G, Rosenblatt M and Rodan G.A. 1988 Comparison of postreceptor effects of 1-34 human hypercalcemia factor and human parathyroid hormone in rat osteosarcoma cells. J Clin Invest 81 924-927.

63. Canalis E, McCarthy T.L and Centrella M. 1990 Differential effects of continuous and transient treatment with parathyroid hormone related peptide (PTHrP) on bone collagen synthesis. Endocrinology 126 1806-1812.

64. Rouleau M.F, Warshawsky H and Goltzman D. 1986 Parathyroid hormone binding IN VIVO to renal, hepatic and skeletal tissues of the rat using a radioautographic approach. Endocrinology 118 919-931.

65. Rouleau M.F, Mitchell J and Goltzman D. 1988 IN VIVO distribution of parathyroid hormone receptors in bone: evidence that a predominant osseous target cell is not the mature osteoblast. Endocrinology 123 187-191.

66. Rouleau M.F, Mitchell J and Goltzman D. 1990 Characterization of the major parathyroid hormone target cell in the endosteal metaphysis of rat long bones. J Bone Min Res 5 1043-1053.

67. Bernier S.M, Rouleau M.F and Goltzman D. 1991 Biochemical and morphological analysis of the interaction of epidermal growth factor and parathyroid hormone with UMR 106 osteosarcoma cells. Endocrinology 128 2752-2760.

68. Aubin J.E, Heersche J.N.M, Merrilees M.J and Sodek J. 1982 Isolation of bone cell clones which differ in growth, hormone responses, and extracellular matrix production. J Cell Biol 92 452-461.

69. Bernier S.M, Desjardins J, Sullivan A.K and Goltzman D. 1990 Establishment of an osseous cell line from fetal rat calvaria using an immunocytolytic method of cell selection: characterization of the cell line and of derived clones. J Cellular Physiol 145 274-285.

70. Mangin M, Webb A.C, Dreyer B.E, Posillico J.T, Ikeda K, Weir E.C, Stewart A.F, Bander N.H, Milstone L, Barton D.E, Francke U and Broadus A.E. 1988 Identification of a cDNA encoding a parathyroid hormone-like peptide from a human tumor associated with humoral hypercalcemia of malignancy. Proc Natl Acad Sci (USA) 85 597-601.

71. Stewart A.F, Wu T.L, Insogna K.L, Milstone L.M and Burtis W.J. 1991 Immunoaffinity purification of parathyroid hormone-related protein from bovine milk and human keratinocyte-conditioned medium. J Bone Min Res 6 305-311.

72. Henderson J, Sebag M, Rhim J, Goltzman D and Kremer R. 1991 Dysregulation of parathyroid hormone-like peptide expression and secretion in a keratinocyte model of tumor progression. Cancer Res In Press.

73. Karaplis A.C, Yasuda T, Hendy G.N, Goltzman D and Banville D. 1990 Gene encoding parathyroid hormone-like peptide: nucleotide sequence of the rat gene and comparison with the human homologue. Mol Endocrinol 4 441-445.

74. Mangin M, Ikeda K, Dreyer B.E and Broadus A.E. 1990 Identification of an up-stream promoter of the human parathyroid hormone-related peptide gene. Mol Endocrinol 4 851-858.

75. Sebag M, Henderson J, Rhim J and Kremer R. 1991 Control of cell proliferation by growth factors and $1,25(OH)_2D_3$ in immortalized and transformed keratinocytes. J Bone Min Res 6S1 S292.

76. Thiede M.A, Harm S, Hasson D.A and Gardner R.M. 1991 IN VIVO regulation of parathyroid hormone-related peptide messenger ribonucleic acid in the rat uterus by 17ß estradiol. Endocrinology 128 2317-2323.

77. Streutker C and Drucker D.J. 1991 Rapid induction of parathyroid hormone-like peptide gene expression by sodium hutyrate in a rat islet cell line. Mol Endocrinol 5 703-708.

78. Ikeda K, Lu C, Weir E.C, Mangin M and Broadus A.E. 1990 Regulation of parathyroid hormone-related peptide gene expression by cycloheximide. J Biol Chem 265 5398-5402.

79. Woodget J.R. 1989 Early gene induction by growth factors. Brit Med Bull. 45 529-540.

80. Weir E, Daifotis A, Dreyer B, Burtis W and Broadus A. 1991 Estrogen responsive expression of the parathyroid hormone-related peptide gene by cultured primary myometrial cells. J Bone Min Res. 6S1 S233

81. Suva L, Gillespie M, Center R, Gardner R, Rodan G, Martin T and Thiede M. 1991 A sequence in the human PTHrP gene promoter responsive to estrogen. J Bone Min Res 6S1 S196.

82. Ikeda K, Lu C, Weir E.C, Mangin M and Broadus A.E. 1989 Transcriptional regulation of the parathyroid hormone-related peptide gene by glucocorticoids and vitamin D in a human C-cell line J Biol Chem 264 15743-15745.

83. Lu C, Ikeda K, Deftos L.J, Gazdar A.F, Mangin M and Broadus A.E. 1989 Glucocorticoid regulation of parathyroid hormone-related peptide gene transcription in a human neuroendocrine cell line. Mol Endocrinol 3 2034-2040.

84. Rodan S.B, Weslowski G, Ianacone J, Thiede M.A and Rodan G.A. 1989 Production of parathyroid hormone-like peptide in a human osteosarcoma cell line: stimulation by phorbol esters and epidermal growth factor. J Endocrinol 122 219-227.

85. Insogna K.L, Stewart A.F, Ikeda K, Centrella M and Milstone L.M. 1988 Characterization of a parathyroid hormonelike peptide secreted by human keratinocytes. Ann N Y. Acad Sci 548 146-149

86. Deftos L.J, Gazdar A.F, Ikeda K and Broadus A.E. 1989 The parathyroid hormone-related protein associated with malignancy is secreted by neuroendocrine tumors. Mol Endocrinol 3 503-508.

87. Deftos L.J, Hogue-Angeletti R, Chalberg C and Tu S 1989 PTHrP secretion is stimulated by CT and inhibited by CgA peptides Endocrinology 125 563-565.

88. Ikeda K, Weir E.C, Sakaguchi K, Burtis W.J, Zimering M, Mangin M, Dreyer B.E, Brandi M.L, Aurbach G.D and Broadus A.E. 1989 Clonal rat parathyroid cell line expresses a parathyroid hormone-related peptide but not parathyroid hormone itself. Biochem Biophys Res Comm 162 108-115.

89. Zajac J.D, Callaghan J, Eldridge C, Diefenbach-Jagger H, Suva L.J, Hudson P, Moseley J.M, Michelangeli V.P and Pasquini G. 1989 Production of a parathyroid hormone-related protein by a rat parathyroid cell line. Mol Cell Endocrinol 67 107-112.

90. Henderson J, Sebag M, Rhim J, Goltzman D and Kremer R. 1991 modulation of parathyroid hormone-like peptide release and cell growth by epidermal growth factor and calcium in human keratinocytes in culture. J Bone Min Res 6S1 S231.

91. Rizzoli R and Bonjour J.P. 1989 High extracellular calcium increases the production of a parathyroid hormone-like activity by cultured Leydig tumor cells associated with humoral hypercalcemia. J Bone Min Res 4 839-844.

92. Brandt D.W, Pandol S.J and Deftos L.J. 1991 Calcium-stimulated parathyroid hormone-like protein secretion: potentiation through a protein kinase-C pathway. Endocrinology 128 2999-3004.

93. Brown E.M. 1991 Extracellular Ca^{2+} sensing, regulation of parathyroid cell function, and role of Ca^{2+} and other ions as extracellular (first) messengers. Physiol Rev 71 371-411.

94. Thiede M.A and Rodan G A. 1988 Expression of a calcium-mobilizing parathyroid hormone-like peptide in lactating mammary tissue. Science 242 278-280.

95. Terry L.C, Saunders A, Audet J, Willoughby J.D, Brazeau P and Martin J.B. 1977 Physiologic secretion of growth hormone and prolactin in male and female rats. Clin Endocrinol 6 19s-28s.

96. Thiede M.A. 1989 The mRNA encoding a parathyroid hormone-like peptide is produced in mammary tissue in response to elevations in serum prolactin. Mol Endocrinol 3 1443-1447.

97. Selvanayagam P, Graves K, Cooper C and Rajaraman S. 1991 Expression of the parathyroid hormone-related peptide gene in rat tissues. Lab Invest 64 713-717.

98. Pahuja D.N and DeLuca H.F. 1981 Stimulation of intestinal calcium transport and bone calcium mobilization by prolactin in vitamin D-deficient rats. Science 214 1038-1039.

99. Caraceni M.P, Corghi E, Ortolani S, Casazza S, D'Alberton A and Motta T. 1985 Increased forearm bone mineral content after bromocriptene treatment in hyperprolactinemia. Calcif Tiss Int 37 687-689.

100. Khosla S, Johansen K.L, Ory S.J, O'Brien P.C and Kao P.C. 1990 Parathyroid hormone-related peptide in lactation and in umbilical cord blood. Mayo Clin Proc 65 1408-1414.

101. Budayr A.A, Halloran B.P, King J.C, Diep D, Nissenson R.A and Strewler G.J. 1989b High levels of a parathyroid hormone-like protein in milk. Proc Natl Acad Sci (USA) 86 7183-7185.

102. Melton M.E, D'Anza J.J, Wimbiscus S.A, Grill V, Martin T.J and Kukreja S.C. 1991 Parathyroid hormone-related protein and calcium homeostasis in lactating mice. Am J Physiol E792-796.

103. Ratcliffe W.A, Thompson G.E, Care A.D and Peaker M. 1991 Production of PTH-related protein (PTHrP) by the mammary gland of the goat. J Endocrinol 129S S134.

104. Law F.M.K, Moate P.J, Leaver D.D, Diefenbach-Jagger H, Grill V, Ho P.W.M and Martin T.J. 1991 Parathyroid hormone-related protein in milk and its correlation with milk calcium. J Endocrinol 128 21-27.

105. Henderson J, Kremer R, Rhim J and Goltzman D. 1990 Characteristics of parathyroid hormone and parathyroid hormone-like peptide stimulated adenylate cyclase activity in human keratinocyte cell lines. J Bone Min Res 5S2 S77.

106. Henderson J, Kremer R, Rhim J,S and Goltzman D. 1991 Identification and functional characterization of adenylate cyclase-linked receptors for parathyroid hormone-like peptides on immortalized human keratinocytes. Endocrinology In Press

107. Chan S.D.H, Strewler G.J, King K.L and Nissenson R.A. 1990 Expression of a parathyroid hormone-like protein and its receptor during differentiation of embryonal carcinoma cells. Mol Endocrinol 4 638-646.

108. Orloff J.J, Ganz M.B, Ribaudo A.E, Burtis W.J, Reiss M, Milstone L.M and Stewart A.F. 1991 A binding protein for PTH-related protein (PTHRP) on malignant but not benign squamous cells. J Bone Min Res 6S1 S230.

109. Macara I.G. 1989 Oncogenes and cellular signal transduction. Physiol Rev 69 797-819.

110. Beckner S.K, Hattori S and Shih T.Y. 1985 The ras oncogene product p21 is not a regulatory component of adenylate cyclase. Nature 317 71-72.

111. Fadda G.Z, Akmal M, Lipson L.G and Massry S.G. 1990 Direct effect of parathyroid hormone on insulin secretion from pancreatic islets. Amer J Phsiol E975-984.

112. Rabbani S.A, Wong R, Kronis K.A, Henderson J, Rajwans N, Bozzato R.P, Hendy G,N and Goltzman D. 1991 Expression and characterization of recombinant human parathyroid hormone-like peptide using a bacterial excretion system J Bone Min Res 6S1 S116.

113. Henderson J, Rabbani S.A, Rhim J, Kremer R and Goltzman D. 1991 Parathyroid hormone-like peptide is an inhibitor of DNA synthesis in human keratinocytes in culture by a cAMP independent mechanism. J Bone Min Res 6S1 S231.

114. Kaiser S.M, Laneuville P, Rhim J, Kremer R and Goltzman D. 1991 Expression of antisense RNA for parathyroid hormone-like peptide enhances growth of human keratinocytes in culture. J Bone Min Res 6S1 S195.

115. Care A.D, Abbas S.K, Pickard D.W, Barri M, Drinkhill M, Findlay J.B.C, White I.R and Caple I.W. 1990 Stimulation of ovine placental transport of calcium and magnesium by mid-molecule fragments of human parathyroid hormone-related protein. Exptl Physiol 75 605-608.

116. Fenton A.J, Kemp B.E, Kent G.N, Moseley J.M, Zheng M.H, Rowe D.J, Britto J.M, Martin T.J and Nicholson G.C. 1991 A carboxyl-terminal peptide from the parathyroid hormone-related protein inhibits bone resorption by osteoclasts. Endocrinology 129 1762-1768.

117. Murray T.M, Rao L.G, Muzaffar S.A and Ly H. 1989 Human parathyroid hormone carboxyterminal pwptide (53-84) stimulates alkaline phosphatase activity in dexamethasone-treated rat osteosarcoma cells in vitro. Endocrinology 124 1097-1099.

118. Sutherland M, Rao L.G, Wylie J, Sodek J, Haussler M and Murray T.M. 1991 Human PTH (53-84) has unique biological actions in cultured human bone cells. J Bone Min Res 6S1 S115.

119. Kong X, Jueppner H, Schipani E, Kronenberg H and Segre G. 1991 Characterization of the rat gene encoding the receptor for parathyroid hormone and parathyroid hormone related peptide J Bone Min Res 6S1 S13.

120. Bringhurst F, Jueppner H, Kronenberg H, Abou-Samra A and Segre G. 1991 Stable expression of functional PTH receptors in LLC-PK1 porcine renal epithalial cells. j Bone Min Res 6S1 S19

121. Abou-Samra A, Jueppner H, Alberts D, Kong F and Segre G. 1991 Activation of PTH/PTHRP receptors stably expressed in the mouse corticotroph tumor ATT-20 cells stimulate ACTH secretion. J Bone Min Res 6S1 S11.

122. Gardella T, Jueppner H, Abou-Samra A, Keutman H, Potts J, Segre G, Kronenberg H and Nussbaum S. 1991 [Arg^2]-PTH detects functional differences between the ROS and OK PTH receptors. J Bone Min Res 6S1 S11.

CHAPTER VII CLAIMS TO ORIGINAL RESEARCH

Development and application of one of the first radioimmunoassays for a novel PTH-like peptide

Demonstration of a definitive role being played by this PTH-like peptide in the hypercalcemia associated with malignancy.

Demonstration of dysregulated expression and secretion of PLP in the progression from established to malignant phenotype.

Identification of functional PLP receptors which are linked to the growth cycle of human keratinocytes.