Stimulation of creatine kinase activity in rat skeletal tissue *in vivo* and *in vitro* by protease-resistant variants of parathyroid hormone fragments

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We have reported that mid-region fragments of human parathyroid hormone (hPTH), exemplified by hPTH-(28–48), stimulated [³H]thymidine incorporation into DNA and increased the specific activity of the brain-type isoenzyme of creatine kinase (CK) in both skeletal-derived cell cultures (ROS 17/2.8 cells) and immature rat epiphyseal cartilage and diaphyseal bone, without stimulating cyclic AMP synthesis which is a prerequisite for bone resorption. In the present study, substitution of amino acids in hPTH-(28–48), which resulted in increased resistance to proteolysis, produced variants that stimulated skeletal systems at two orders of magnitude lower concentration than the wild-type fragment. We modified hPTH-(28–48) at Leu-37 by replacement with Met, Thr or Val. Under conditions in which 20% of the native hPTH-(28–48) resisted proteolysis by cathepsin D for 6 h, approx. 40% of the L37V mutant and 70% of the L37T mutant remained intact. Substitution of Met for Phe-34 in addition to

Thr for Leu-37, or the substitution of Met for Phe-34 alone, produced 100%-resistant fragments. These variants at residue 34 caused maximal stimulation of CK in ROS 17/2.8 cells at 0.24 nM compared with 24 nM for hPTH-(28–48). The double mutant stimulated CK activity significantly in immature rats, at a minimum dose of 12.5 ng/rat, and caused maximal stimulation at 125 ng/rat, a 10-fold lower dose than for hPTH-(28–48). The effect of the double mutant lasted up to 24 h which differs from the stimulation by hPTH-(28–48) in which CK specific activity returns to the control level at 24 h. This same dose also significantly stimulated CK activity in gonadectomized rats. These results show the advantage of using protease-resistant mid-region variants of hPTH-(28–48) to stimulate bone cells, in terms of lower doses and longer duration of effectiveness, both *in vitro* and *in vivo*.

INTRODUCTION

Parathyroid hormone (PTH) acts by different pathways in skeletal tissues to stimulate bone turnover [1,2] leading to either net bone formation [3–6] (for a review see ref. [7]) or net bone resorption [8–10] depending on cell type, dose and schedule of administration. In pathological situations such as osteoporosis (in which the balance of bone turnover is tipped in the direction of increased resorption), treatment with low doses of PTH stimulates bone formation and augments bone mass with minimal side effects [5,11–13]. Most of the agents used to date (oestrogens, bisphosphonates) mainly inhibit resorption. Thus, if PTH is capable of improving bone mass and quality, this peptide will have a potential therapeutic role.

PTH affects mainly the cells of the osteoblast lineage ([14] and references therein). It stimulates osteoblast replication in bone organ culture [15] via an increase in proliferation of osteoprogenitor cells [16,17]. Low concentrations stimulate rat osteoblastic osteosarcoma cells ROS 17/2.8 [18] and UMR106 osteoblasts [19] as well as rat calvaria [20,21] and primary human osteoblasts [16]. Although specific receptors for PTH have been found in osteoblasts [22] and preosteoblast cells [23] as well as in kidney [24,25], they have not been detected in mature osteoclasts. Therefore an indirect mechanism via the increase in cyclic AMP caused by PTH in osteoblasts [26] has been considered to lead to bone resorption [27]. PTH also caused, by a cyclic AMP-independent mechanism [28] involving a Ca²⁺/phosphoinositide pathway [20,29,30], increased cell proliferation in rat osteoblasts and increased DNA synthesis in chicken osteoblasts and chondroblasts [31–33], and in rat calvaria [20]. Moreover, we found

previously, both *in vitro* and *in vivo*, that mid-region fragments of PTH stimulate DNA synthesis and a marker of bone cell proliferation [the specific activity of the brain isoenzyme of creatine kinase (CK)] without changes in cyclic AMP accumulation [21,34,35]. Thus these mid-region fragments potentially offer improvements over PTH-(1–34) as possible therapeutic agents. From previous experiments, we found that higher doses of human (h) PTH-(28–48) than hPTH-(1–34) or hPTH-(1–84) were needed to stimulate bone cell proliferation maximally, and the effect of hPTH-(28–48) *in vivo* lasted for a shorter time. One reason for the reduced activity of hPTH-(28–48) could be less resistance to circulating and/or cellular proteases. The main PTH-metabolizing activity has been ascribed to the Kupffer cells (macrophages) of the liver, where a cathepsin D-like activity appears to be responsible for the degradation of the hormone [36,37]. Metabolism of PTH has also been shown to occur by cathepsin D action within the endosomes of alveolar macrophages [38]. We therefore decided to synthesize hPTH-(28–48) mutated at the two specific sites known to be attacked by cathepsin D [39] to increase its protease resistance, and to test whether these substitutions would improve the potency and duration of the effects of the fragments *in vivo* and *in vitro*. We have continued to use as the response marker the stimulation of the specific activity of the brain-type isoenzyme of CK which was found to parallel the increase in [³H]thymidine incorporation in bone-derived cells both *in vitro* and *in vivo* [20,21,35] and therefore serves as a sensitive and convenient marker for cell proliferation in skeletal tissue. Preliminary results have shown that, after PTH treatment, the increase in specific activity of CK follows the increase in its mRNA in cultures of ROS 17/2.8 rat osteogenic

Abbreviations used: hPTH, human parathyroid hormone; CK, creatine kinase; i.p., intraperitoneal; TFE, trifluoroethanol.

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osteosarcoma cells and osteoblast-enriched rat calvaria cells, in parallel with the oestrogen stimulation of mRNA for CK b in rat uterus [40,41].

We report here on mutants of the fragment hPTH-(28–48), in particular, a double mutant, F34M/L37T which showed both maximum stimulation *in vivo* at a dose 10 times lower than wild-type hPTH-(28–48) and a longer duration of action.

MATERIALS AND METHODS

Animals

Batches of female Wistar-derived rats, born on the same day and ranging from 20 to 25 days old in particular experiments, were obtained from the Department of Hormone Research Colony at the Weizmann Institute of Science (mean weight $\pm$ S.E.M., 35 ± 1.5 g at 20 days). Male or female rats were gonadectomized at the age of 25 days and used 7–10 days later. Animals were divided randomly into experimental groups and injected intraperitoneally (i.p.) with doses of PTH fragments for time periods as described for each experiment. Rats were fed with pelleted chow *ad libitum* and kept on a 14 h light/10 h dark schedule in air-conditioned rooms maintained at 23 °C. They were killed by cervical dislocation at the times indicated and organs were removed for determination of CK activity.

Peptides

The PTH sequences were assembled with a Milligen 9050 peptide synthesizer on NovaSyn PA 500 resins preloaded with the C-terminal amino acids. Synthesis of the fragments and their purification were carried out as described previously [42]. The correct molecular masses of all peptides have been verified by fast-atom-bombardment MS: wild-type human (h)PTH-(28–48) 2149.0 (exp.), 2148.4 (calc.); L37M 2167.4 (exp.), 2166.0 (calc.); L37T 2137.1 (exp.), 2136.0 (calc.); L37V 2135.2 (exp.), 2134.3 (calc.); F34M/L37T 2121.1 (exp.), 2120.3 (calc.); F34M 2132.7 (exp.), 2131.1 (calc.). PTH-(1–34), used for comparison, was obtained from Sigma, St. Louis, MO, U.S.A.

CD spectroscopy

To examine the global conformational properties of the variant PTH peptides synthesized, CD spectra were recorded using a Jasco J-600 spectropolarimeter (Jasco, Tokyo, Japan). Spectra were recorded in a solution containing 70% trifluoroethanol (TFE) at a peptide concentration of 0.1 mg/ml, between the wavelengths 184 and 260 nm, using a pathlength of 1 mm. Spectra were evaluated for the presence of secondary-structure elements using the program VARSELEC [43].

Cathepsin D cleavage *in vitro*

The limited proteolytic cleavage of the PTH peptides (0.14 mg/ml) was studied at an enzyme/peptide ratio of 1:50 (w/w) in 0.1 M sodium acetate, pH 4.0, at 4 °C. The reaction was stopped by the addition of ice-cold 0.1% trifluoroacetic acid, approx. pH 2. The cleavage products were analysed by analytical HPLC on a C₁₈ nucleosil column (ET 250/8/4, Nucleosil 300-7; Machery Nagel) with a 10–35% acetonitrile gradient in 0.1% trifluoroacetic acid and quantified by measurement of the peak heights.

Cell cultures

ROS 17/2.8 cells, a subclone of a rat osteogenic osteosarcoma line [18], were cultured in 35 mm-diameter culture dishes in 2 ml

of Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1) [44] containing 10% fetal calf serum [45] at 37 °C in 8% CO₂. Cells were used when subconfluent.

CK assay

After treatment, cells were washed with PBS and homogenized by repeated freezing and thawing in isotonic homogenization buffer (50 mM Tris/HCl, pH 6.8, 250 mM sucrose, 5 mM magnesium acetate, 0.4 mM EDTA and 2.5 mM dithiothreitol). Supernatant extracts were obtained by centrifugation in an Eppendorf Microfuge at 14000 g for 5 min at 4 °C [20].

Rat kidney and diaphysis and epiphyses of the tibia were collected and chilled on ice; the bones were split and the marrow scraped out. Organs were homogenized in the same ice-cold homogenization buffer using a Polytron homogenizer (Kinematica AG). Extracts were prepared as for the cellular extracts. CK activity was assayed at 30 °C in a Gilford 250 automatic recording spectrophotometer using an assay described previously [21,35] in which the ATP formed was coupled via the action of hexokinase and glucose-6-phosphate dehydrogenase to the formation of NADH. Protein concentrations were determined by the Coomassie Brilliant Blue dye-binding method of Bradford [46] with BSA as standard.

Reagents

All reagents used were of analytical grade. Biochemicals were obtained from Sigma.

Statistical significance

The significance of differences between experimental and control values was evaluated by unpaired two-tailed Student's *t* tests.

RESULTS AND DISCUSSION

Properties of mid-region variants of mitogenic PTH fragments

In order to increase the half-life *in vivo* of a mitogenic mid-region fragment of hPTH, variant forms of hPTH were synthesized by replacing either one or two potentially critical amino acids. The sites of substitutions in the hPTH-(28–48) fragment were based on data showing that the major cleavage sites of hPTH-(1–84) are between residues 33 and 34 and residues 37 and 38 [47]. Therefore the leucine residue at position 37 was replaced by methionine, threonine or valine, or the phenylalanine residue at position 34 was replaced by methionine. In addition, a double mutant was synthesized with substitutions at both these sensitive sites (F34M/L37T) and referred to for convenience as m4.

We first compared the global folding properties of the synthetic PTH variants with those of the wild-type hPTH-(28–48) fragment to evaluate the possibility that gross changes in their structure might modify their interaction with the PTH receptor. Previous

	28	30			34			37			40			48							
hPTH-(28-48)	L	Q	D	V	H	N	F	V	A	L	G	A	P	L	A	P	R	D	A	G	S
m1	-	-	-	-	-	-	-	-	-	M	-	-	-	-	-	-	-	-	-	-	-
m2	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-
m3	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-
m4	-	-	-	-	-	-	M	-	-	T	-	-	-	-	-	-	-	-	-	-	-
m5	-	-	-	-	-	-	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 1 Variants of hPTH-(28–48)

Figure 2 Resistance of variants of hPTH-(28-48) to degradation by cathepsin D

Wild-type hPTH-(28-48) and five variants (Figure 1) were incubated with cathepsin D at an enzyme/peptide ratio of 1:50 (w/w) in 0.1 M sodium acetate, pH 4.0, at 37 °C and the residual fragments were quantified by HPLC analysis. Details are described in the Materials and methods section. □, 28-48 [wild-type hPTH-(28-48)]; ●, m1 (L37/M); ▲, m2 (L37T); ▼, m3 (L37V); ◆, m4 (F34M/L37T); ■, m5 (F34M).

Figure 3 Concentration-dependent stimulation of activity in ROS 17/2.8 cells of CK by m4, m5 and hPTH-(28-48)

Cell cultures at subconfluence were incubated for 24 h in increasing concentrations of PTH fragments, m4 (■), m5 (△) or 28-48 (●). At the end of the incubation, CK was extracted and assayed for specific activity as described in the Materials and methods section. The results are means $\pm$ S.E.M. ($n \geq 5$). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.005$, compared with activity in the absence of PTH fragment.

structural investigations with hPTH and PTH-derived peptides showed that they tend to be largely unstructured in aqueous solution [42]. However, they can adopt significant α -helical conformation when the hydrophobicity of the solution is in-

Figure 4 Time course of stimulation of CK activity *in vitro* by m4

ROS 17/2.8 cells at subconfluence were incubated with 0.24 nM m4 for increasing periods of time. At the end of the incubation, CK was extracted and assayed as described in the Materials and methods section. The results are expressed as means $\pm$ S.E.M. ($n \geq 5$). * $P \leq 0.05$, ** $P \leq 0.01$, compared with initial activity.

creased by adding TFE, which may be supposed to mimic the situation when the hormone molecules are approaching their surface receptor [42,48]. We therefore monitored the α -helical content of hPTH-(28-48) in the presence of increasing TFE concentrations by CD spectroscopy and found it to reach a plateau when 70 % TFE is exceeded. Comparison of the global folding properties of the synthetic hPTH variants with those of the wild-type hPTH-(28-48) fragments revealed no significant differences.

We next studied the degradation *in vitro* of the mid-regional PTH peptide variants (Figure 1) by the intracellular protease cathepsin D (Figure 2). Under the conditions of the incubation, the wild-type hPTH-(28-48) exhibited a half-life of less than an hour. Substitutions at Leu-37 or Phe-34, the two possible target sites for cathepsin D [39], led to an increase in the half-life of the fragment to more than 3.5 h in all cases. A differentially enhanced resistance against cathepsin D was observed in the order wild-type < L37V (m3) < L37M (m1) < L37T (m2) < F34M (m5) = F34M/L37T (m4), the latter two being completely insensitive for more than 4 h under conditions in which wild-type hPTH-(28-48) is degraded by 75 % in 2 h.

Cathepsin D is an aspartyl protease which cleaves preferentially at the C-terminal sides of hydrophobic amino acid residues which are unbranched in the β position [39]. In contrast, branched residues are cleaved much less readily, which coincides with our observations on the L37T and L37V variants. The methionine substitutions obviously also do not match the cathepsin D requirements and result in a particularly high resistance against the protease when introduced in position 34, indicating that the primary determinant for cleavage of hPTH-(28-48) by cathepsin D is the phenylalanine residue at position 34. However, modifications at position 37 also enhance the resistance against this proteolytic activity. In the light of the degradation data, we

Figure 5 Minimal time needed for maximal stimulation of CK by hPTH fragments in ROS 17/2.8 cells

Cells were untreated (○) or treated with m4 at 0.24 nM (■), hPTH-(28–48) at 24 nM (▲) or hPTH-(1–34) at 2.4 nM (●). At the time points indicated, the medium was changed to one without added hormone and incubated until completion of the 24 h period from the beginning of the experiment; cells were then extracted and CK was assayed as described in the Materials and methods section. The results are expressed as means $\pm$ S.E.M. ($n \geq 5$). * $P \leq 0.05$, ** $P \leq 0.01$, compared with untreated cells.

therefore decided to compare wild-type hPTH-(28–48) with the two Phe-34 variants, m4 and m5.

Comparison of the dose-dependence of stimulation by PTH variants in ROS 17/2.8 cells

ROS 17/2.8 cells were incubated for 4 h with increasing doses of hPTH-(28–48) and the mutants m4 and m5 (Figure 3). Significant stimulation of CK specific activity with the wild-type fragment hPTH-(28–48) was seen at a minimal concentration of 2.4 nM; a maximal effect required 24 nM. In comparison, the double mutant showed a significant increase at 0.024 nM (24 pM) and a maximal effect at 0.24 nM, and the single mutant showed a significant and maximal stimulation at 0.24 nM (Figure 3). These results revealed the mutants to be able to cause maximal stimulation *in vitro* at one to two orders of magnitude lower concentrations than the wild-type fragment. As m4 showed a significant stimulation of CK specific activity at a concentration an order of magnitude less than the single mutant (m5), we concentrated on m4 as the most potent of the protease-resistant mutants.

Comparison of the minimal time needed for maximal stimulation

When m4 was tested to determine the time-dependence of its action in ROS 17/2.8 cells, it was found to cause a significant (> 2-fold) increase in activity after 4 h (Figure 4); the 50% increase at 2 h was not significant in this *in vitro* experiment,

Figure 6 Dose-dependent stimulation by m4 of CK specific activity in rat organs

Female prepubertal rats were injected i.p. with m4. After 4 h, the rats were killed and extracts were prepared and assayed as described in the Materials and methods section. ■, Epiphysis; ●, diaphysis; ▲, kidney. The results are expressed as means $\pm$ S.E.M. ($n \geq 5$). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.005$, compared with activity in the absence of m4.

whereas previously we showed that hPTH-(1–34), hPTH-(1–84) and hPTH-(28–48) all stimulate significant increases *in vivo* after 1–2 h [35].

This differential prompted us to examine the minimal time needed for interaction of the hormones with the cells in order to obtain maximal stimulation at later time points. We found (Figure 5) that, whereas hPTH-(1–34) at 2.4 nM needed to be present for 2 h only (before the culture was washed and incubated for a further 22 h before analysis), hPTH-(28–48) at 24 nM or m4 at 0.24 nM needed to be present for at least 4 h. These differences may indicate that the mid-region fragments, as they are shorter and lack sequences that might contribute to stability of binding, need a longer time to achieve productive interaction with the PTH receptor (complex) to activate (all) the pathways needed for stimulation of CK activity.

Stimulation of CK activity in rat organs by m4

We have shown previously [35] that both hPTH-(1–34) and hPTH-(28–48), when injected into prepubertal rats, are maximally effective at a dose of 1.25–2 μ g/rat for stimulation of CK activity in epiphysis, diaphysis and kidney. When m4 was injected into rats of the same age (Figure 6) a maximal increase in enzyme activity was seen at 0.125 μ g/rat in all three organs. We postulate that this higher potency is probably due to decreased degradation of the variant hormone fragment.

In previous experiments, hPTH-(1–34), as well as bovine PTH-(1–84), showed significant stimulation of CK by 1 h and a maximal stimulation by 4 h or earlier after injection; the CK activity remained at the same maximal level up to 24 h after injection [35]. In comparison, hPTH-(28–48) injection, although also showing significant stimulation of CK activity at 1 h with maximal stimulation at 4 h, did not maintain this level and

Figure 7 Time-dependent stimulation by m4 of CK specific activity in rat organs

Prepubertal female rats (20–25 days old) were injected i.p. with m4 (0.125 $\mu\text{g}/\text{rat}$). At the indicated times, rats were killed and extracts were prepared and assayed as described in the Materials and methods section. ■, Diaphysis; ●, epiphysis; ▲, kidney. The results are expressed as means $\pm$ S.E.M. ($n \geq 5$). * $P \leq 0.05$, ** $P \leq 0.01$, compared with initial activity.

Table 1 Comparison of m4 and hPTH-(28–48) for persistence of stimulation of CK specific activity in rat organs

Prepubertal female rats were injected i.p. with either hPTH-(28–48) (1.25 $\mu\text{g}/\text{rat}$) or m4 (0.125 $\mu\text{g}/\text{rat}$) and killed at either 4 or 24 h after injection. CK was extracted and assayed as described in the Materials and methods section. The results are expressed as means $\pm$ S.E.M. ($n \geq 5$). * $P \leq 0.05$, ** $P \leq 0.01$, compared with activity in the absence of PTH fragment.

Organ	Specific activity of CK ($\mu\text{mol}/\text{min}$ per mg of protein)				
	No hPTH	hPTH-(28–48)		m4	
		4 h	24 h	4 h	24 h
Epiphysis	7.0 $\pm$ 1.0	11.6 $\pm$ 1.3*	8.9 $\pm$ 0.4	11.4 $\pm$ 1.3*	13.5 $\pm$ 1.4*
Diaphysis	9.1 $\pm$ 1.3	13.7 $\pm$ 1.6*	12.9 $\pm$ 0.9*	14.1 $\pm$ 1.5*	15.2 $\pm$ 1.5**
Kidney	1.1 $\pm$ 0.2	1.7 $\pm$ 0.1*	1.2 $\pm$ 0.3	1.5 $\pm$ 0.2*	1.5 $\pm$ 0.1*

resulted in a decrease by 24 h to close to control levels [35]. In contrast, when m4 is injected, there is a significant stimulation of CK activity in diaphyseal bone and epiphyseal cartilage only at 2 h (Figure 7); however, this maximal activity does not decline significantly up to 24 h. These results suggest that the more stable compound, m4, is less degraded *in vivo* as well as *in vitro* (Figure 3) and therefore is active for a longer time. When we compared directly the effects of m4 and hPTH-(28–48) on CK activity at 4 and 24 h in epiphysis, diaphysis and kidney (Table 1), we found that the stimulation of CK by m4 remained as high as 24 h as at 4 h in all three organs, whereas injection of hPTH-(28–48) resulted in activity not significantly different from control levels in epiphyseal cartilage at 24 h compared with that at 4 h.

We also compared the CK response of gonadectomized rats to

Table 2 Stimulation by hPTH fragments of CK specific activity in organs of gonadectomized rats

Male and female rats (25 days old) were gonadectomized and 3 weeks later were injected with either hPTH-(1–34) (2 $\mu\text{g}/\text{rat}$), hPTH-(28–48) (1.25 $\mu\text{g}/\text{rat}$) or m4 (0.125 $\mu\text{g}/\text{rat}$). After 4 h, rats were killed, organs were collected and CK was extracted and assayed as described in the Materials and methods section. The results are expressed as means $\pm$ S.E.M., $n = 3$ –6. * $P \leq 0.05$; ** $P \leq 0.01$.

Organ	Creatine kinase specific activity ($\mu\text{mol}/\text{min}$ per mg of protein)			
	Control	hPTH-(1–34)	hPTH-(28–48)	hPTH(m4)
Castrated males				
Epiphysis	5.4 $\pm$ 0.7	7.4 $\pm$ 1.1*	8.2 $\pm$ 2.2*	9.4 $\pm$ 0.7**
Diaphysis	7.4 $\pm$ 1.7	18.5 $\pm$ 2.2**	13.3 $\pm$ 1.7**	10.0 $\pm$ 1.1*
Kidney	0.7 $\pm$ 0.2	1.0 $\pm$ 0.1*	1.2 $\pm$ 0.1**	1.4 $\pm$ 0.1**
Ovariectomized females				
Epiphysis	6.5 $\pm$ 0.7	10.2 $\pm$ 1.1*	11.5 $\pm$ 1.1**	8.8 $\pm$ 0.8*
Diaphysis	6.9 $\pm$ 1.3	12.6 $\pm$ 2.6**	13.7 $\pm$ 1.7**	10.2 $\pm$ 1.1**
Kidney	0.5 $\pm$ 0.1	0.8 $\pm$ 0.2*	0.8 $\pm$ 0.1*	1.5 $\pm$ 0.04**

PTH-(1–34), PTH-(28–48) and m4 at 4 h after injection (Table 2). A tenfold lower dose of m4 significantly stimulated CK specific activity in kidney, diaphysis and epiphysis of both castrated males and ovariectomized females. Full dose–response curves for m4 compared with the wild-type-fragments will be necessary to determine to what extent the apparent differences in response seen at this dose differential are representative of the maximal potency of the fragments. Nevertheless, as m4 is capable of stimulating both diaphyseal bone and epiphyseal cartilage in gonadectomized female rats as well as intact animals, it may be a suitable entry point in this model system for studying restoration of bone loss by variant PTH fragments.

Concluding remarks: therapeutic use of PTH in osteoporosis

Anabolic action of PTH on bone is receiving renewed and extensive attention (see ref. [7] for review). The reports that PTH restores lost bone mass in ovariectomized rats either in combination with oestrogen [49,50] or independently [6] re-emphasizes the possibility of therapeutic use of low doses of PTH for treatment of osteoporosis. There may be an added advantage in using a mid-region fragment of PTH or an N-terminal truncated form of PTH-(1–34) [30] which is incapable of stimulating the cyclic AMP formation which is linked to bone resorption. The present demonstration that modifications of hPTH-(28–48) that increase its resistance to proteolysis by cathepsin indeed permit the use of a much lower dose to achieve maximal anabolic stimulation of bone cells may provide a useful starting point for evaluation of modified fragments and full-length PTH molecules for their therapeutic value in preventing and restoring bone loss.

The results presented in this paper demonstrate that mutant hPTH-(28–48) fragments can be synthesized that are more active than the wild-type fragment. They are active at lower doses both *in vivo* and *in vitro* and for longer times *in vivo*. This correlates with the fact that they undergo less degradation by proteases present in serum or in the cells. However, at the low effective doses, they need a longer period of time to cause stimulation *in vivo* presumably for productive interaction with the receptor in order to achieve the same maximal stimulation and longer lasting effects *in vivo*.

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