Mutational Analysis of the Receptor-activating Region of Human Parathyroid Hormone*

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The first 4 residues of parathyroid hormone (PTH) are highly conserved in evolution and are important for biological activity. We randomly mutated codons 1–4 of human PTH (hPTH) with degenerate oligonucleotides and, after expression in COS cells, screened the mutants for receptor binding and cAMP-stimulating activity using ROS 17/2.8 cells. This survey identified Glu4 and Val2 as important determinants of receptor binding and activation, respectively. Positions 1 and 3 were more tolerant of substitutions indicating that these sites are less vital to hormone function. Activities of synthetic hPTH(1–34) analogs further demonstrated the importance of positions 2 and 4. The binding affinity of [Ala4,Tyr34] hPTH(1–34)NH2 was 100-fold reduced relative to [Tyr2,Ala4]hPTH(1–34)NH2 (Kd = 653 ± 270 nM, respectively), and [Arg2,Tyr34]hPTH(1–34)NH2 was a weak partial agonist which bound well to the ROS cell receptor (Kd = 31 ± 10 nM). The Arg2 analog was nearly as potent as PTH(3–34) as an in vitro PTH antagonist in osteoblast derived cells. However, unlike PTH(3–34), [Arg2]PTH was a full agonist in opossum kidney (OK) cells. These observations suggest that the activation domains of the OK and ROS cell PTH receptors are different. Thus, amino-terminal PTH analogs may be useful as probes for distinguishing properties of PTH receptors.

Parathyroid hormone is a principal regulator of calcium and phosphate ion homeostasis. An early step in the PTH response pathway involves the initial binding of the hormone to receptors on the surface of bone and kidney cells. This is followed by an activation step in which the receptor-hormone complex couples to a stimulatory GTP-binding protein/adenylate cyclase signal transduction system. Human parathyroid hormone (hPTH) is synthesized in the parathyroid gland as preproPTH which is processed and secreted as an 84-amino acid polypeptide. The amino-terminal 34 amino acids of the hormone contain all of the information needed for receptor binding and activation; in most assay systems the chemically synthesized 1–34 fragment produces responses identical to those of native human PTH(1–84) (Potts et al., 1971; Tregear et al., 1973; Rosenblatt, 1981).

Phylogenetic comparison of the 1–34 region of the eight currently known PTH and PTH-related peptide (PTHrP) sequences, which include six mammalian and two avian sequences, reveals significant amino acid sequence homology. The first 4 residues are particularly well conserved; position 1 is either serine or alanine, and positions 2, 3, and 4 are, invariably, valine, serine, and glutamic acid, respectively. Studies of PTH analogs with amino-terminal deletions demonstrate the importance of the amino-terminal residues in PTH function. Removal of the first two amino acids reduces cAMP-stimulating potency by as much as three orders of magnitude (Tregear et al., 1973; Rosenblatt, 1981), and deletion of residues 1–6 abolishes agonism entirely (Rosenblatt et al., 1980). Thus, the principal receptor activation determinants lie within residues 1–6. Deletion of these residues does not affect receptor binding as dramatically as it affects activation, since PTH(3–34) and PTH(7–34) display substantial receptor binding affinity (Rosenblatt, 1981; Nussbaum et al., 1980). The weak but specific receptor binding affinity displayed by various carboxyl-terminal fragments, including 14–34, 25–34, and 10–27 (Nussbaum et al., 1980; Rosenblatt et al., 1980), however, leads to the conclusion that the receptor-binding determinants of PTH are dispersed throughout the 1–34 region. The specific residues which are responsible for receptor binding and activation, have not yet been precisely identified.

The use of partially degenerate oligonucleotides as mutagenic primers is an effective means to introduce a wide spectrum of random mutations into a defined region of a protein (Oliphant et al., 1986). By screening the resulting mutants for activity, the functional roles of the target residues can be deduced. This approach has been used to reveal the critical sites in several different proteins, including human growth hormone (Cunningham and Wells, 1989), human immunodeficiency virus protease (Loeb et al., 1989), and a prokaryotic DNA binding protein (Bowie and Sauer, 1989). We have extended this approach to study both the binding and activation roles of the first 4 residues of hPTH(1–84). We screened mutant hormones, which were expressed and secreted by COS cells, for PTH receptor binding and adenylate cyclase-stimulating activity in a rat osteoblast PTH target cell line, ROS 17/2.8. We then studied the effects of selected mutations in more detail by synthesizing [Tyr2]hPTH(1–34)NH2 analogs having corresponding amino acid substitutions and determining their receptor-binding and activation properties. By this approach we found that valine 2 is a key activation residue and that glutamic acid 4 has an important role in receptor binding.

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The abbreviations used are: PTH, parathyroid hormone; hPTH, human PTH; bPTH, bovine PTH; PTHrP, PTH-related peptide; OK, opossum kidney; IRMA, immunoradiometric assay; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
EXPERIMENTAL PROCEDURES

Materials—The following reagents and materials were obtained from the indicated manufacturer: mutagenesis system, Amersham Corp.; intact PTH assay kit, New England Nuclear; cAMP radiomunnoassay kits, Biological Technologies Inc. (Reading, MA); tissue culture media and supplements, Gibco. All other reagents were from Sigma.

PTH Plasmin Construction and Oligonucleotide Mutagenesis—The plasmid used for oligonucleotide mutagenesis and COS cell expression of hPTH(1-84) (pCDM-PTH-1), was kindly provided by Dr. Mason Freeman (Massachusetts General Hospital, Boston MA). In this plasmid, the HindIII-XbaI fragment of pPTH124, containing human preproPTH cDNA (Born et al., 1987), was inserted between the HindIII and XbaI sites of pCDM8 (Seed, 1987) placing the PTH gene under transcriptional control of the CMV IE promoter. Single-stranded plasmid DNA, was mutagenized in vitro by the method of Taylor et al. (1985). A pool of mutagenic primers spanning codons -3 to +1 of PTH was synthesized on an applied model 380A DNA synthesizer (Applied Biosystems, Mountain View, CA). At positions corresponding to codons 1-4, bases were mixed at a ratio of 80% of the correct base to 6.7% each of the other three bases. As predicted by the genetic code and probability theory, this combination will yield the wild-type amino acid sequence at a frequency of 13%, mutants with single and double amino acid substitutions at a frequency of 34% each, and mutants with two single amino acid substitutions at a frequency of 18%. Double-stranded heteroduplex DNA was introduced into the Escherichia coli strain F2g (MC1061/P3/F'lac), and transformants were selected on plates containing ampicillin (12.5 mg/ml) and tetracycline (6 μg/ml). A total of 162 independent transformants was isolated; cloned hPTH(1-84) type-plasmid DNA was identified by hybridizing single-stranded plasmid DNA prepared from each clone to a radiolabeled wild-type oligonucleotide spanning codons -1 to +5 under conditions that demand a perfect match between plasmid and oligonucleotide. By this procedure, 50 wild-type clones were identified. DNA sequence analysis of plasmids from several of these clones revealed the wild-type sequence. Specific mutations carried on the remaining plasmids were identified by DNA sequence analysis.

Tissue Cell Culture—Cells were maintained in a humidified atmosphere in the presence of 5% CO₂. For transfection and bioactivity assays, cells were subcultured into 24-well plates (1.5-cm diameter wells). The clonal rat osteosarcoma cell line, ROS 17/2.8 (Majeska et al., 1980), was cultured in Ham's F-12 medium supplemented with 5% fetal bovine serum, and test plates were maintained at confluence for 7-10 days prior to assay. COS-7 (Gluzman, 1981) and opossum kidney (OK) cells (Teitelbaum and Strewler, 1984) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. OK cells were maintained at confluence for 2 days prior to assay.

PTH Expression—COS cell transfections were carried out by the DEAE-dextran procedure (Seed and Aruffo, 1987) using miniprep plasmid DNA. The medium was changed 3-4 days after transfection. 24 h later the conditioned medium was harvested, new medium was applied, and this was harvested 24 h later and pooled with the first harvest. This cycle was repeated for up to 5 days. PTH in the media was measured using an immunoradiometric assay (IRMA) specific for the full-length hPTH(1-84) (Nichols Institute) using synthetic hPTH(1-84) as standards. This assay demands recognition of hPTH(1-84) by two different affinity-purified polyclonal PTH antisera directed against the amino- and carboxyl-terminal regions of the molecule. PTH concentrations were also quantified by a radioimmunoassay directed against residues in the 44-68 region of hPTH (Nichols). The results of this assay agree closely with the IRMA results; this agreement indicates that mutations in the 1-4 region do not interfere with recognition by the amino-terminal antisera of 145 S, the IRMA. The PTH level in the media was 32 ± 13 nm (mean ± S.E.); media below 0.5 nm were not assayed for receptor binding and cAMP-stimulating activity, since this is near the detection limit of these assays (Gardella et al., 1990).

cAMP Stimulation—Stimulation and quantification of intracellular cAMP were performed as previously described (Gardella et al., 1989; Abou-Samra et al., 1997). Incubations (25 min/37°C) were performed in a volume of 0.3 ml containing 0.2 μl of cAMP assay buffer (Ham's F-12 medium, 2 mM 3-isobutyl-1-methylxanthine, 1 mg/ml bovine serum albumin, 35 mM HEPES/NaOH, pH 7.4, 20 μg/ml ascorbic acid) and 0.1 μl of either hPTH(1-34) peptides in binding buffer or conditioned COS media containing PTH mutants. Intracellular cAMP levels were determined by radiodimmunooassay (Biological Technologies Inc.). For synthetic peptides, data are expressed as the percentage of the maximum cAMP produced by 1 μM [Tyr₃⁴]hPTH(1-34)NH₂. The relative cAMP-stimulating activity of the hPTH(1-84) mutants was determined by dividing the cAMP produced in the presence of the mutant hormone by the concentration of the mutant peptide in the conditioned medium. The resulting value is expressed as the percentage of the wild-type hPTH(1-84) value. Stimulation of cAMP production could be detected with wild-type hPTH(1-84)-containing COS media which had been diluted up to 10-fold; thus, the detection limit of the assay is 2% of the observed binding of hPTH(1-84).

RESULTS

We obtained 53 different PTH mutants (listed in Table I), from a total of 162 independent E. coli transformants resulting from the mutagenesis procedure. The 53 mutants were assayed for receptor binding affinity and cAMP-stimulating activity in ROS 17/2.8 cells. We found that each of the 4 residues in this region appears to be important for interaction of the hormone with the ROS cell receptor. Each mutant containing more than one amino acid substitution was inactive in both receptor binding and cAMP stimulation assays (not shown). This indicates a reduction of at least 10-fold in binding affinity and 50-fold in cAMP-stimulating activity. Fig. 1 shows the activity of the mutants carrying single amino acid substitutions. Only one mutant was fully active; [Ala₂]-hPTH(1-84) displayed a slight enhancement of binding affinity and cAMP-stimulating activity.

As shown in Fig. 1A, the glutamic acid residue at position 4 in the wild-type sequence appears to be important for binding to the ROS cell PTH receptor. All six mutations at position 4, including the conservative Glu→Asp substitution, reduced affinity at least 10-fold relative to the wild-type hormone. Positions 1, 2, and 3 appear not to be as important for receptor binding as position 4 since most of the mutations at these sites did not greatly reduce affinity. Four of the mutants, with changes at positions 1 and 3 displayed substantial cAMP-stimulating activity. The Ala₁, Val₁, Thr₁, and Gly₂ mutants all showed at least 25% of the activity of the
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The wild-type sequence is shown at the top of each column. The sequence of each mutant studied is indicated. Dashes indicate the wild-type codon; asterisks indicate synonymous codon changes. Mutations resulting in 25% or greater of the cAMP-stimulating activity of the wild-type hormone are boxed.

| Ser 1 Val 1 Ser 2 Val 2 Ser 3 Val 3 Ser 4 Val 4 | Glu 1 | Glu 2 | Glu 3 | Glu 4 |
|-----------------------------------------------|--------|--------|--------|--------|
| Ala                                           | Val    | Gin    | Val    | Gin    |
| Ala                                           | Leu    | Cys    | Ala    | Glu    |
| Val                                           | *      | *      | Ala    | Glu    |
| Tyr                                           | Tyr    | Gly    | *      | *      |
| Tyr                                           | *      | Cys    | *      | Arg    |
| Pro                                           | *      | Phe    | Cys    | *      |
| Pro                                           | *      | *      | Tyr    | *      |
| Asp                                           | Leu    | Gly    | *      | *      |
| Leu                                           | Ile    | Asn    | *      | *      |
| Ala                                           | *      | Lys    | Val    | Glu    |
| Ser                                           | Leu    | Val    | Arg    | Glu    |
| Arg                                           | *      | Ala    | *      | Asn    |
| Glu                                           | Thr    | Cys    | Val    | Gly    |
| Gly                                           | Asp    | Glu    | Val    | Gly    |
| Gly                                           | Ile    | Asp    | Glu    | *      |
| Ile                                           | *      | *      | Glu    | Val    |
| Asn                                           | Ile    | *      | Asp    | Arg    |
| Ala                                           | *      | Gly    | *      | *      |
| Gly                                           | *      | Gly    | His    | *      |
| Gly                                           | *      | Lys    | *      | Val    |
| Asp                                           | *      | *      | *      | *      |
| Ala                                           | *      | Gly    | *      | *      |
| Gly                                           | *      | Gly    | His    | *      |
| Lys                                           | *      | Lys    | *      | Val    |
| Val                                           | *      | *      | Asp    | Gly    |
| Asp                                           | *      | *      | *      | *      |

TABLE I

Mutations in codons 1–4 of human PTH

Wild-type sequence is shown at the top of each column. The sequence of each mutant studied is indicated. Dashes indicate the wild-type codon; asterisks indicate synonymous codon changes. Mutations resulting in 25% or greater of the cAMP-stimulating activity of the wild-type hormone are boxed.

Mutations in codons 1–4 of human PTH

Phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine was included in the assay buffer.

[Arg<sup>2</sup>,Tyr<sup>4</sup>]hPTH(1–34)NH<sub>2</sub> was tested as a PTH antagonist in an in vitro ROS cell assay, and, as shown in Fig. 3, it was nearly as effective as [Tyr<sup>4</sup>]hPTH(3–34)NH<sub>2</sub> (previously recognized as a potent inhibitor of PTH in vitro by Goltzmann et al., 1975) in inhibiting stimulation of cAMP production by [Tyr<sup>4</sup>]hPTH(1–34)NH<sub>2</sub>. Either the large size or the positive charge of the arginine side chain could be responsible for the inhibitory effect on receptor activation. To investigate these possibilities further, we synthesized two analogs containing lysine and citrulline (Cit) at position 2. The side chain of citrulline is approximately the same size and shape as that of arginine but carries an oxygen atom in place of a guanadino nitrogen. Thus, this side chain, although polar, is uncharged. The side chain of lysine is smaller than that of arginine but it is positively charged. Both of these analogs bind to the ROS cell receptor although with lower affinity than [Arg<sup>2</sup>,Tyr<sup>4</sup>]hPTH(1–34)NH<sub>2</sub> (Table II). Like the arginine analog, however, both the lysine and citrulline analogs only weakly stimulated cAMP production showing less than 10% of the maximal cAMP response at a dose of 10<sup>−6</sup> M (Fig. 4).

The interaction of [Arg<sup>2</sup>,Tyr<sup>4</sup>]hPTH(1–34)NH<sub>2</sub> with the opossum kidney cell receptor differed dramatically from its interaction with the ROS cell receptor. In OK cells, at a dose of 1 μM, the Arg<sup>2</sup> peptide stimulated cAMP production as well as the native hormone. In contrast, this peptide exhibited less
Our data indicate that each of the first 4 residues of PTH are important for both receptor binding and activation. One among 53 different mutants surveyed did not show reduced activity. Interestingly, this mutation of Ser to Ala at position 1 corresponds to the only evolutionary divergence seen among the first 4 residues of the eight known PTH or PTHrP peptide sequences. Position 1 is alanine in five of the proteins and serine in the other three while positions 2, 3, and 4 are invariably valine, serine, and glutamic acid, respectively. The high degree of mutational intolerance observed for residues 1-4 in vivo appears to parallel their strong phylogenetic conservation.

All of the mutants with multiple substitutions were inactive in the ROS cell assay for cAMP stimulation and thus were at least 50-fold less potent than hPTH(1-34). Most of the single mutants which retained detectable activity had changes at positions 1 or 3. The Ala1, Val1, Thr3, and Gly3 mutants each showed at least 25% of the cAMP-stimulating activity of the wild-type hormone. Some of the position 1 and 3 mutants, however, including Tyr1, Asp3, Pro3, Ile3, and Asn3, were inactive; these results suggest that the normal serine residues at these two positions play some role in receptor interaction. The effects of several modifications at position 1 and 3 on PTH function have been reported previously. The 2-34 fragment of bovine PTH (bPTH), as well as the Tyr1, Ser3, and Val3 derivatives of bPTH(1-34), had from 5 to 60% of the cAMP-stimulating potencies of bPTH(1-34) (Rosenblatt, 1981). More recently, Cohen et al., (1991) studied the effects on hormone action of a series of substitutions at positions 3 and 6. Their position 3 mutants showed reductions in bioactivity which appeared to be proportional to the volume of the substituted side chain. Our data, which includes four position 3 mutants (hPTH(1-34)) different from the six they described, is in accord with this concept, since glycine and threonine at position 3 of hPTH(1-34) resulted in only slight decreases in activity while Ile and Asn caused more severe reductions.

In the screening assay, none of the position 2 mutants displayed detectable cAMP-stimulating activity in ROS cells. This result suggests that valine 2 of PTH is a critical determinant of receptor activation. The activities of the synthetic peptides [Arg2, Tyr3']hPTH(1-34)NH2 and [Ala2, Tyr3']hPTH(1-34)NH2 support this concept. These peptides were severely defective in activating the ROS cell receptor. The reduction in cAMP-stimulating activity observed for these analogs was not in proportion to their reduction in binding affinity. The affinity of the Ala2 peptide was only 30-fold reduced relative to that of the parent peptide [Tyr2]hPTH(1-34)NH2 yet it's CAMP-stimulating activity was decreased by over a factor of 100. The binding affinity of [Arg2, Tyr3']-hPTH(1-34)NH2 was reduced only 5-fold, but, at a dose at which nearly complete receptor occupancy occurs (10-8 M), this analog induced only 10% of the maximum cAMP response. The synthetic Arg2 peptide resembled hPTH(3-34) in its ability to antagonize the action of hPTH(1-34) in an in vitro ROS cell assay.

The magnitude of the decrease in cAMP-stimulating activity resulting from the Arg2 substitution, and from the deletion of residues 1 and 2, demonstrates the important role of position 2 of PTH in hormone action. This site does not appear to be as important for binding as it is for activation, since both the 3-34 and Arg2 peptides have high binding affinities. Position 2 of PTH does not appear to have a critical role in maintaining secondary structure, since Zull and co-workers (1990) found that the circular dichroism spectra of PTH(1-34) and PTH(3-34) were identical. The conserved valine residue at position 2, therefore, appears to be critically involved in triggering signal transduction. Previous studies have indicated that other sites in parathyroid hormone may also be involved in this process. For example, PTHrP(7-34)

![Diagram](image-url)
Mutagenesis of Human PTH(1-84)

Table II
Receptor binding and activation properties of [Tyr-34]hPTH(1-34)NH2 analogs in ROS 17/2.8 and OK cells
The substitutions of the different peptides tested are indicated at the left. Half-maximal dissociation (Kd) and activation values (kcat) were determined as described under “Experimental Procedures” and represent the average (±S.E.) of three separate experiments. Asterisks indicate that at a dose of 10 μM maximum cAMP stimulation was not observed.

| Peptide             | ROS 17/2.8 cells | Opposum kidney cells |
|---------------------|------------------|----------------------|
|                     | Kd              | kcat                |                     |
|                     | (nM)            | (nM)                |
| [Tyr34]hPTH(1-34)NH2| 4 ± 1           | 1 ± 0.2              | 0.8 ± 0.1           |
| Val2→Ala            | 117 ± 53        | 200 ± 39             | 17 ± 4.6            |
| Val2→Arg            | 31 ± 10         | *                    | 64 ± 9.5            |
| Glu4→Ala            | 653 ± 270       | 468 ± 16             | ND*                 |
| Val2→Cit            | 144 ± 34        | *                    | ND*                 |
| Val2→Lys            | 735 ± 91        | *                    | ND*                 |
| [Tyr34]hPTH(3-34)NH2| 89 ± 1          | *                    | ND*                 |

*ND, not determined.

Fig. 3. PTH antagonist activity of the Arg2 analog. ROS 17/2.8 cells were preincubated either without or with PTH antagonists (shaded, [Arg2,Tyr34]hPTH(1-34)NH2; hatched, [Tyr34]hPTH(3-34)NH2) at a dose of 1 × 10-6 M for 15 min at 21 °C. Subsequently, [Tyr34]hPTH(1-34)NH2 (5 × 10-6 M) was added to all wells and after an additional 25 min at 37 °C, intracellular cAMP levels were determined as described under “Experimental Procedures.” Values are the mean (± S.E.) of triplicate wells.

Fig. 4. Activity of PTH analogs with substitutions of valine 2. ROS 17/2.8 cells were stimulated (25 min/37°C) with [Tyr34]hPTH(1-34)NH2 derivatives carrying the indicated substitutions at position 2 (valine is the native residue at this position). The dose of [Tyr34]hPTH(1-34)NH2 was 1 × 10-6 M; others were at 1 × 10-5 M. Values are the mean (± S.E.) of triplicate wells.

exhibits weak partial agonism (McKee et al., 1988); this result indicates that some activation determinants lie carboxyl-terminal to residue 6 in PTHrP. Nutt and coworkers (1990) demonstrated that the substitution of lysine 11 with leucine abolishes the residual agonism of PTHrP(7-34); this site may, therefore, represent or influence a secondary activation determinant.

In an attempt to define the structural basis for the effect of the arginine 2 substitution on receptor activation, we analyzed two analogs having position 2 substitutions of ci-
trulline or lysine. Like the arginine-substituted peptide, the lysine 2 and citrulline 2 analogs displayed only weak agonism in ROS cells, yet each showed only modestly reduced binding affinities. Since the citrulline analog is uncharged, this result indicates that it is not the positive charge of the arginine side chain which is solely responsible for the marked decline in cAMP activation. One interpretation of these results is that at position 2 amino acid side chains that are polar or occupy a large volume cannot activate the ROS cell receptor. The ability of the Ala*** peptide to fully stimulate the ROS cell PTH receptor at high doses is consistent with this idea.

In opossum kidney cells, [Arg**,Tyr**]**hPTH(1–34)NH₂ displayed full agonism, whereas in bone-derived ROS cells it was only a weak agonist (Table II and Figs. 2 and 5). In contrast, PTH(3–34) is a very weak agonist in both cell lines (Cole et al., 1990). The Arg** compound, therefore, can detect structural differences between the OK and ROS cell receptors. The increased agonist activity of the Arg** peptide in OK cells relative to ROS cells is not due simply to a higher binding affinity in the OK cells; Arg** has a lower affinity for the OK cell receptor than it does for the ROS cell receptor. In contrast, hPTH 1–34 and the Ala*** analog demonstrate higher affinities for the OK cell receptor than for the ROS cell receptor. Other studies have also shown higher affinities of PTH in kidney-based systems relative to bone-based systems (Cohen et al., 1991; Caulfield et al., 1990). In most of these comparisons, as well as in our study, however, the two tissue types were derived from different species. Thus, it is uncertain whether the differences in binding affinities and agonist specificity observed between the ROS and OK cells are due to the different species origin of the cell lines or to PTH receptor heterogeneity such that the two cell types express a distinct PTH receptor subtype. The position-2 PTH analogs described here should prove useful for probing the activation domains of the receptors from different cell types or species.

None of the hPTH(1–84) mutants with substitutions from Glu to showed detectable binding affinity or cyclase activation, with the exception of the conservative change to aspartic acid. Furthermore, the synthetic peptide, [Ala**,Tyr***]**hPTH(1–34)NH₂, was reduced 100-fold in binding affinity. This pattern of binding and activation emphasizes the importance of the carboxylic acid at position 4. A simple interpretation of this result is that the carboxyl of Glu** is involved in a direct receptor contact which is critical for the establishment, or the maintenance, of the receptor-bound state. Alternatively, Glu** may play a conformational role and mutations at this site indirectly impair receptor binding by disrupting the hormone’s ordered structure. Recently, Barden and Kemp (1989) proposed a structural model of hPTHrP(1–36) based on their NMR analysis. In this model, the carboxyl of Glu** is proposed to form a salt bridge with the imidazole side chain of His**.

Most amino acid replacements at position 4 would be expected to disrupt such a salt bridge, and destabilize the peptide’s conformation. Our observations of Asp** bioactivity are consistent with either of these models. Biophysical analysis of position 4 analogs are needed to clarify this issue, as competitive binding assays cannot distinguish altered receptor interactions from conformational defects.

Our experiments demonstrate that site-directed mutagenesis coupled with COS cell expression systems can be used to probe a peptide hormone for sites involved in receptor binding and activation. Our main findings are that the valine residue at position 2 is an important determinant of receptor activation and that the glutamic acid residue at position 4 is important for receptor binding. By using a series of olinogluconolide primers spanning residues 1–34, we are now applying this screening strategy to identify critical functional sites throughout the biologically active region of the hormone. Use of assays that measure PTH stimulation of second messengers other than cAMP may permit the discovery of analogs that favor one intracellular signal over another. Systematic comparisons of the interactions of analogs with PTH receptors from diverse tissues and species should facilitate precise analysis of hormone-receptor interactions.

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