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# Preparation and Characterization of [$N^{\alpha}$-(4-Azido-2-nitrophenyl)Ala[1],Tyr[36]]-Parathyroid Hormone Related Peptide (1–36)Amide: A High-Affinity, Partial Agonist Having High Cross-Linking Efficiency with Its Receptor on ROS 17/2.8 Cells[†]

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ABSTRACT: The synthesis, purification, and structural analysis of the major compounds resulting from photoderivatization of [Tyr[36]]-parathyroid hormone related peptide (1–36)amide [[Tyr[36]]PTHrP(1–36)amide] are described. The reaction of the synthetic peptide with 4-fluoro-3-nitrophenyl azide under nonaqueous conditions yields three major products (peaks D-1, D-2, and G), which were purified to homogeneity by reverse-phase high-performance liquid chromatography. Subsequent amino acid analysis showed that the peptides of peaks D-1 and G each lack one lysine residue, while the peptide in peak D-2 lacks one alanine residue, suggesting that these residues are chemically modified by photoderivatization. Sequence analysis of the photoderivatized peptides revealed that compounds D-1 and G were derivatized on Lys[13] and Lys[11], respectively. Compound D-2 was N-blocked, indicating that this compound is derivatized on the $\alpha$-amino function of Ala[1]. Both Lys residues of D-2 were quantitatively recovered upon sequencing after digestion with endoproteinase Glu-C. Compounds D-2 and G had apparent $K_d$s of $1 \times 10^{-9}$ M and $0.6 \times 10^{-9}$ M, respectively, for their receptors on ROS 17/2.8 cells, which are identical with or similar to that of the underivatized [Tyr[36]]PTHrP(1–36)amide. Compound G had the same adenylate cyclase stimulating potency as the underivatized, synthetic [Tyr[36]]PTHrP(1–36)amide, whereas compound D-2 was only a partial agonist, having about 25% of the maximal cAMP production. Compound D-1, which is modified on Lys[13], retained only 2–4% of its receptor binding affinity and biological activity relative to that of its parent compound. After radioiodination and incubation with ROS 17/2.8 cells in the dark, D-2 and G bound equivalently to the receptor protein (specific binding 18.0 ± 3.6% and 16.2 ± 1.3%, respectively), whereas [Nle[8],$N^{\epsilon}$-(4-azido-2-nitrophenyl)Lys[13],Nle[18],Tyr[34]]bPTH(1–34)amide (NAP-NlePTH) and D-1 bound less well (specific binding 5.6 ± 2.9% and 3.7 ± 2.4%, respectively), as was predicted from their dissociation constants. After photolysis and analysis by SDS–PAGE and autoradiography, labeling of the 80 000-dalton receptor protein was approximately 5–10 times more efficient with D-2 than with G. This improved cross-linking efficiency of Ala[1]-photoderivatized [Tyr[36]]PTHrP(1–36)amide presumably reflects a steric advantage for this derivative, such as a closer proximity of the azide function at that position to the receptor protein, and suggests that this compound potentially is useful for receptor identification and isolation.

The parathyroid hormone related peptide (PTHrP) [synonyms: human adenylate cyclase stimulating peptide (hACSP), humoral hypercalcemia of malignancy factor (HHMF), parathyroid hormonelike peptide (PTHLP)], a peptide whose secretion is thought to cause the syndrome of humoral hypercalcemia of malignancy (HHM), has recently been isolated and cloned (Suva et al., 1987; Mangin et al., 1988). It is now widely believed that similarities in the clinical and laboratory findings in the syndrome of HHM and in primary hyper-

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parathyroidism are due to the actions of amino-terminal domains in the PTH and PTHrP molecules, which are structurally and functionally highly homologous [for review, see Orloff et al., (1989a)]. PTHrP with a primary sequence longer than the amino-terminal 74 amino acids appears to be important for the fetus-directed placental transfer of calcium and may serve further physiological functions during neonatal development (Abbas et al., 1989; Budayr et al., 1989). Thus far, only synthetic amino-terminal fragments have been well characterized: amino-terminal peptides of both PTH and PTHrP bind to the same receptor protein, activate the same postreceptor events in osteoblastic cell clones and renal plasma membranes, and cause similar degrees of hypercalcemia in vivo (Horiuchi et al., 1987; Kemp et al., 1987; Stewart et al., 1988; Jüppner et al., 1988; Nissenson et al., 1988; Shigeno et al., 1988a; Orloff et al., 1989b).

Our interest in purifying the common "PTH/PTHrP" receptor led us to explore whether photoderivatized PTHrP might serve as a more effective ligand than analogues of PTH (Jüppner et al., 1988; Shigeno et al., 1988b,c) or alternative bifunctional cross-linkers (Karpf et al., 1988). In this report, we show that the reaction of PTHrP with 4-fluoro-3-nitrophenyl azide (FNPA) yields multiple reaction products that represent additions of the photoreactive group to the $\alpha$-amino group of the amino-terminal alanine or to the $\epsilon$-amino group of either of the two lysine residues. We have purified several of these products to homogeneity and characterized some of their chemical properties. Subsequent testing of their biological properties revealed marked differences that are related to modifications of specific amino groups; specifically, photoderivatization of the $\alpha$-amino function of the amino-terminal alanine yields an analogue that can be cross-linked to its receptor with high efficiency and thus may be more effective in purifying solubilized receptors through their covalent attachment to this ligand. Our results also provide insights into biological properties of the PTHrP/PTH family of peptides and may potentially be useful in the design of future receptor antagonists.

## Materials and Methods

*Materials.* A tyrosine-containing analogue of PTHrP, [Tyr$^{36}$]PTHrP(1–36)amide, was provided by Dr. A. F. Stewart, Yale University School of Medicine, New Haven, CT (Stewart et al., 1988). [Nle$^{8,18}$,Tyr$^{34}$]bPTH(1–34)amide was from Bachem Fine Chemicals (Torrance, CA). 3-Isobutyl-1-methylxanthine (IMBX) was from Sigma (St. Louis, MO). FNPA, triethylamine (TEA), trifluoroacetic acid (TFA), and heptafluorobutyric acid (HFBA) were from Pierce (Rockwood, IL), and dimethyl sulfoxide (DMSO) was from Aldrich (Milwaukee, WI). Endoproteinase Glu-C was purchased from Boehringer, Mannheim (Mannheim, West Germany). Premixed low molecular weight standards for SDS–PAGE were from Pharmacia (Uppsala, Sweden). The antiserum against succinylated cAMP was provided by Dr. Kevin Catt, NIH, Bethesda, MD. Sodium [$^{125}$I]iodide (2200 Ci/mmol) was obtained from E. I. Du Pont de Nemours and Co., Inc. (Boston, MA). Model 510 HPLC pumps, model 680 automatic gradient former, model 440 absorbance detector, and $\mu$Bondapak C-18 columns (3.9 mm × 30 cm) were from Waters (Bedford, MA). All other reagents employed in this study were of the highest grade available.

Protein sequencing was performed with solvents and reagents from Applied Biosystems (Foster City, CA) on an Applied Biosystems Model 477A protein/peptide sequencer. The phenylthiohydantoin (PTH) amino acid derivatives were analyzed on-line with an Applied Biosystems Model 120A PTH analyzer. Hydrolysates for amino acid analysis were examined on a Beckman System 6300 (Palo Alto, CA) high-performance amino acid analyzer.

*Cell Culture.* ROS 17/2.8 cells, a clonal, osteoblastic cell line derived from a rat osteosarcoma, were obtained from Dr. G. Rodan (Merck Sharp and Dohme Laboratories, West Point, PA). Cells were maintained in 6- or 24-multiwell plastic culture dishes as monolayer cultures with HAM F-12 medium supplemented with 5% (v/v) heat-inactivated fetal calf serum and 2 g/L $NaHCO_3$ at 37 °C in a humidified 95% air/5% $CO_2$ atmosphere. For three days prior to each experiment, the medium was changed daily.

*Photoderivatization and Radioiodination of [Tyr$^{36}$]-PTHrP(1–36)amide and [Nle$^{8,18}$,Tyr$^{34}$]bPTH(1–34]amide.* All procedures were performed in the dark under a Kodak safelight, and all reaction products were protected from light. FNPA-derivatized [Tyr$^{36}$]PTHrP(1–36)amide was prepared as described (Jüppner et al., 1988). In brief: [Tyr$^{36}$]-PTHrP(1–36)amide (final concentration 620 $\mu$g/mL), FNPA, and TEA were dissolved in DMSO, at a molar ratio of 1:90:16 and incubated for 24 h at 40 °C. The reaction was stopped by freezing at −70 °C. After lyophilization, the reaction mixture was dissolved in 20% acetonitrile, containing 0.1% trifluoroacetic acid (TFA), and then subjected to reverse-phase $C_{18}$ HPLC, using a linear gradient of acetonitrile (30–45%) in 0.1% TFA at a constant flow rate of 2 mL/min over 30 min. Fractions (0.3 min) were collected in borosilicate glass tubes. Those fractions containing the major reaction products were pooled, lyophilized, and rechromatographed on a $C_{18}$ column (acetonitrile gradient of 30–45% in 0.1% TFA over 30 min for peptide peak D-1 and 35–50% over 30 min for peptide peak G). Compound D-2 was repurified by using an acetonitrile gradient of 40–60% with 0.1% HFBA as the counterion. UV absorbance was monitored at 229 nm. The various, photoderivatized forms of [Tyr$^{36}$]PTHrP(1–36)amide were radioiodinated and individually purified by HPLC as recently described (Shigeno et al., 1988b). $^{125}$I-Labeled [Tyr$^{36}$]PTHrP-(1–36)amide and radioiodinated compounds D-1, D-2, and G each resolved into two peaks upon reverse-phase chromatography; the first radioactivity peak was used for experiments. Radioactive peptides were stored at −20 °C for up to 1 week without loss of activity. [Nle$^{8}$,$N^{\epsilon}$-4-azido-2-nitrophenyl)-Lys$^{13}$,Nle$^{18}$,Tyr$^{34}$]bPTH(1–34)amide (NAP-NlePTH, compound 6) was prepared and radioiodinated as described (Shigeno et al., 1988b, 1989).

*Amino Acid Analysis and Peptide Sequencing.* Hydrolysis of [Tyr$^{36}$]PTHrP(1–36)amide and its derivatives was performed in constantly boiling 6 M HCl containing 2-mercaptoethanol (0.05% v/v) for 24 h at 110 °C in vacuo. The resulting hydrolysates were analyzed with a Beckman System 6300 high-performance amino acid analyzer. The data were calculated on the basis of a peptide mass of 4295 daltons. For peptide sequencing, precycled polybrene-coated filters were loaded in the dark and protected from light throughout the procedure. Endoproteinase Glu-C treatment of compound D-2 was performed in 50 mM ammonium acetate buffer, pH 4.0 (final volume 0.04 mL), for 24 h at room temperature at a peptide to enzyme ratio of 10:1 (w/w). The products were then sequenced.

*Radioreceptor and Adenylate Cyclase Assays.* [Tyr$^{36}$]-PTHrP(1–36)amide was radioiodinated and purified by HPLC according to previously described methods and served as tracer for the radioreceptor assay, which was performed as described in 24-multiwell plates (Jüppner et al., 1988; Shigeno et al., 1988b; Yamamoto et al., 1988). Intracellular cAMP was

Table I: Amino Acid Composition of [Tyr[36]]PTHrP(1–36)amide and Photoderivatized PTHrP Compounds[a]

| amino acid | residues expected | [Tyr[36]]PTHrP-(1–36)amide ($n$ = 7) | D-1 ($n$ = 3) | D-2 ($n$ = 3) | G ($n$ = 3) |
|---|---|---|---|---|---|
| aspartic acid | 2 | 2.16 ± 0.12 | 2.12 ± 0.07 | 2.17 ± 0.08 | 2.20 ± 0.07 |
| threonine | 1 | 1.05 ± 0.08 | 1.06 ± 0.12 | 1.05 ± 0.09 | 1.08 ± 0.08 |
| serine | 2 | 1.95 ± 0.22 | 1.91 ± 0.07 | 2.02 ± 0.13 | 1.97 ± 0.20 |
| glutamic acid | 5 | 5.08 ± 0.27 | 5.05 ± 0.68 | 5.14 ± 0.14 | 5.19 ± 0.32 |
| proline | 0 | 0 | 0 | 0 | 0 |
| glycine | 1 | 1.23 ± 0.13 | 1.38 ± 0.40 | 1.38 ± 0.25 | 1.43 ± 0.30 |
| alanine | 3 | 2.93 ± 0.07 | 2.94 ± 0.10 | *2.33 ± 0.18* | 3.01 ± 0.21 |
| half-cystine | 0 | 0 | 0 | 0 | 0 |
| valine | 1 | 1.39 ± 0.45 | 1.49 ± 0.24 | 1.16 ± 0.02 | 1.46 ± 0.13 |
| methionine | 0 | 0 | 0 | 0 | 0 |
| isoleucine | 3 | 2.72 ± 0.13 | 2.70 ± 0.23 | 2.88 ± 0.06 | 2.69 ± 0.17 |
| leucine | 5 | 4.92 ± 0.20 | 4.95 ± 0.46 | 5.15 ± 0.21 | 4.91 ± 0.37 |
| tyrosine | 1 | 0.99 ± 0.06 | 0.92 ± 0.19 | 0.92 ± 0.17 | 0.95 ± 0.17 |
| phenylalanine | 2 | 1.97 ± 0.04 | 2.02 ± 0.05 | 2.08 ± 0.08 | 2.02 ± 0.08 |
| histidine | 5 | 4.78 ± 0.26 | 4.71 ± 0.80 | 4.52 ± 1.43 | 4.50 ± 0.49 |
| lysine | 2 | 2.04 ± 0.18 | *1.36 ± 0.11* | 1.99 ± 0.14 | *1.28 ± 0.02* |
| arginine | 3 | 3.13 ± 0.32 | 2.72 ± 0.65 | 3.00 ± 0.42 | 2.90 ± 0.34 |

[a] The amino acid compositions of HPLC-purified, photoderivatized PTHrP compounds D-1, D-2, and G and underivatized [Tyr[36]]PTHrP(1–36)amide are compared. Data are expressed as mean ± SD of results from analysis of three independent derivatizations for each photoderivatized product of PTHrP and seven analyses of either peak A or unmodified [Tyr[36]]PTHrP(1–36)amide.

measured as described (Abou-Samra et al., 1987).

*Photoaffinity Labeling of ROS 17/2.8 Cells and SDS–PAGE Analysis.* Confluent ROS 17/2.8 cells grown in 6-multiwell plates were rinsed once with 2.5 mL of cold binding buffer [50 mM Tris-HCl (pH 7.7), 5 mM KCl, 100 mM NaCl, 2 mM $CaCl_2$, 5% (v/v) heat-inactivated horse serum, and 0.5% (v/v) heat-inactivated fetal calf serum]. The cell monolayer was then incubated in the dark (4 h, 15 °C) with $^{125}$I-labeled photoderivatized analogues (0.9 nM) in the absence or presence of competing ligands (range, $3 \times 10^{-9}$ to $1 \times 10^{-6}$ M); final incubation volume was 1 mL. After the incubation mixture was discarded, the cells were rinsed once with 2.5 mL of binding buffer. Subsequently, 1.0 mL of binding buffer was added to each well. The plate was then placed on ice, exposed to an UV light source (cell to light distance approximately 10 cm) for 20 min as previously described (Jüppner et al., 1988; Shigeno et al., 1988b). After photolysis, the cells were rinsed twice with 2.5 mL of phosphate-buffered saline and then solubilized with 0.5 mL of SDS–PAGE sample buffer [4% (w/v) sodium dodecyl sulfate (SDS), 80 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, and 100 mM dithiothreitol (DTT)]. After the samples were boiled they were subjected to SDS–PAGE analysis (5–15% acrylamide) according to the method of Laemmli (1970) with subsequent autoradiography at −80 °C, using Cronex Lightning Plus intensifying screens and Kodak X-Omat AR films. Quantification of the efficiency of receptor cross-linking was determined by direct counting of gel slices.

## Results

The reaction mixture of synthetic [Tyr[36]]PTHrP(1–36)-amide and FNPA (24 h at 40 °C), was resolved in four major peaks by HPLC (peaks A, D-1, D-2, and G) (Figure 1). Peak A coeluted with the parent compound with a retention time of 8.7 min, whereas peaks D-1, D-2, and G were eluted at 15.2, 16.5, and 23.5 min, respectively. The HPLC fractions containing each of these major photoderivatized peptide peaks were pooled. After rechromatography of each pool, the peptides then were subjected to amino acid analysis and peptide sequencing. Minor compounds were not investigated since the amounts generated were insufficient for further analysis. Two absorbance peaks (both exceeding the sensitivity of the UV detector) with retention times of 11.3–12.4 min were constantly observed volatile byproducts of the reaction, which disappeared upon repetitive lyophilization (data not shown).

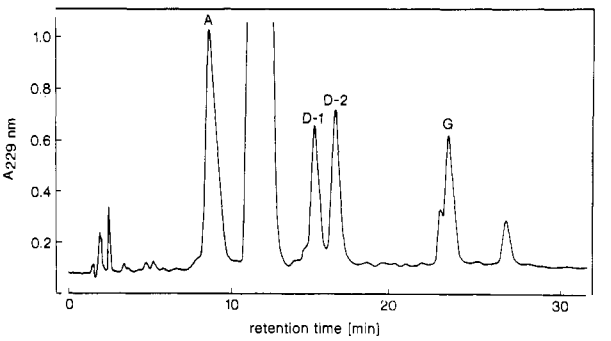


FIGURE 1: HPLC profile of photoderivatized [Tyr[36]]PTHrP(1–36)amide. A 640-μg quantity of [Tyr[36]]PTHrP(1–36)amide was dissolved in 1000 μL of DMSO and reacted with FNPA and TEA as described under Materials and Methods for 24 h at 40 °C. After lyophilization, the reaction mixture was dissolved in 20% acetonitrile containing 0.1% TFA, and the products were resolved by HPLC on a μBondapak C-18 column with a linear gradient of 30–45% acetonitrile in 0.1% TFA over 30 min (flow rate 2 mL/min). The resulting peptide peaks were subsequently repurified by using a gradient of 30–45% acetonitrile (0.1% TFA) for compound D-1 and 35–50% acetonitrile (0.1% TFA) for peptide peak G. Compound D-2 was repurified by using a gradient of 40–60% acetonitrile (0.1% HFBA). Absorbance was monitored at 229 nm; the data are representative of six independent experiments.

Acid hydrolysis of peak A confirmed the predicted amino acid composition of the parent peptide. Compared to the parent peptide, each of the three other major peptide peaks differed from the predicted composition. Peaks D-1 and G lost more than 35% of their expected lysine residues, and peak D-2 lost more than 20% of its expected alanine residues (Table I). All three major derivatized compounds and the parent compound [Tyr[36]]PTHrP(1–36)amide were subjected to sequence analysis (Figure 2). The average yield based on the initial yield for phenylthiohydantoin derivatives of Ala (cycle 1) and Val (cycle 2) was 77.2 ± 12.3% ($n$ = 4) for compounds A, D-1, and G, with an average repetitive yield of 92.6 ± 3.94% based upon Ser (cycles 3 and 14), Glu (cycles 4, 30, and 35), Gln (cycles 6 and 16), and Asp (cycles 10 and 17). The percent recovery of phenylthiohydantoin derivatives of amino acids per cycle based on the amount of peptide subjected to sequence analysis was similar for peptide peaks A, D-1 and G, except for the absence of lysine at cycle 13 for compound D-1 and at cycle 11 for compound G (Figure 2). Compounds D-1 and G thus were [$N^{\epsilon}$-(4-azido-2-nitrophenyl)-Lys[13],Tyr[36]]PTHrP(1–36)amide and [$N^{\epsilon}$-(4-azido-2-nitro-

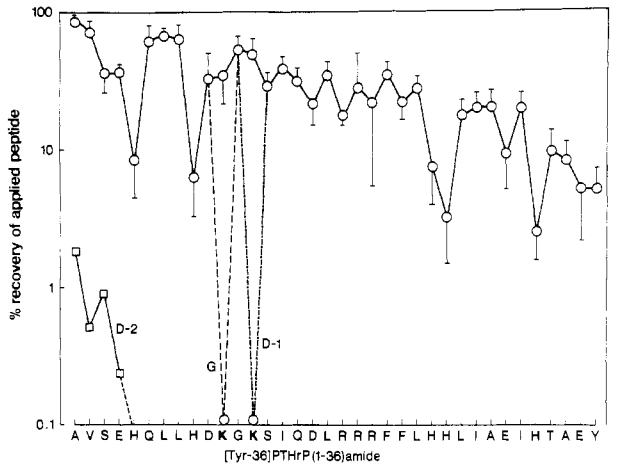


FIGURE 2: Sequence analysis of nitrophenyl azide derivatized [Tyr$^{36}$]PTHrP(1–36)amide. Peptide peaks A (874 pmol), D-1 (325 pmol), D-2 (881 pmol), and G (650 pmol) were purified by HPLC and sequenced. The recovery of PTH amino acids per cycle was normalized to adjust for differences in the initial amount of peptide. The data for peptide peaks, A, D-1, and G (○) are summarized as mean ± SD (exceptions: for both lysine residues the mean was calculated from either peaks A and D-1 or peaks A and G). The data are representative of sequence analysis of the products of two independent derivatizations. Data from Edman degradation of D-2 (□) are also representative of two sequence analyses of independent derivatizations.

phenyl)Lys$^{11}$,Tyr$^{36}$]PTHrP(1–36)amide, respectively.

Compound D-2 gave an average initial amino acid yield of 8.5 ± 3.8% ($n$ = 3), indicating that the bulk of this compound is N-blocked due to photoderivatization of the $\alpha$-amino function of Ala$^1$. To eliminate contaminating components that are not N-blocked, compound D-2 was repurified by using an acetonitrile gradient with 0.1% HFBA instead of 0.1% TFA as the counterion. Sequencing of the repurified peptide gave an initial yield of only 1–2% (data of two independent derivatizations and two consecutive sequencing experiments), indicating that over 98% of compound D-2 was blocked on its amino terminus. This finding, that D-2 was modified at the $\alpha$-amino group of Ala$^1$, was consistent with the amino acid analysis, which showed low recovery of this residue. An aliquot of TFA-purified compound D-2 was digested with endoproteinase Glu-C. The mixture was then submitted to sequence analysis. On the basis of specificity of the enzyme, proteolysis of compound D-2 was expected to generate three peptides, 1–4, 5–30, and 31–35, and a free tyrosinamide from cleavage after the Glu at position 35. The recovery of Ala$^1$ was 6.9%, which is similar to the initial yield obtained when sequencing the TFA-purified intact D-2 compound. On the basis of the recovery of Leu$^7$ and Ile$^{31}$, the initial yields for fragments 5–30 and 31–35 were 72.1% and 69.6%, respectively. Both lysine residues contained in the 5–30 fragment were recovered quantitatively at cycle 7 and 9, thus providing further evidence that compound D-2 was exclusively derivatized on the $\alpha$-amino group of Ala$^1$ (data not shown). These cumulative data strongly indicate that peak D-2 is [$N^{\alpha}$-(4-azido-2-nitrophenyl)Ala$^1$,Tyr$^{36}$]PTHrP(1–36)amide.

The apparent affinity of the photoderivatized PTHrP compounds for their receptors on ROS 17/2.8 cells was assessed by using varying doses of each peptide peak in the dark in a radioreceptor assay with radioiodinated [Tyr$^{36}$]PTHrP(1–36)amide (Figure 3a). All compounds completely competed with the radioactive ligand. The apparent $ID_{50}$s of both [Tyr$^{36}$]PTHrP(1–36)amide and compound G were 0.6 × 10$^{-9}$ M, and that of compound D-2 was 1.0 × 10$^{-9}$ M. Compound D-1 had an apparent affinity of 0.3 × 10$^{-7}$ M, which is about

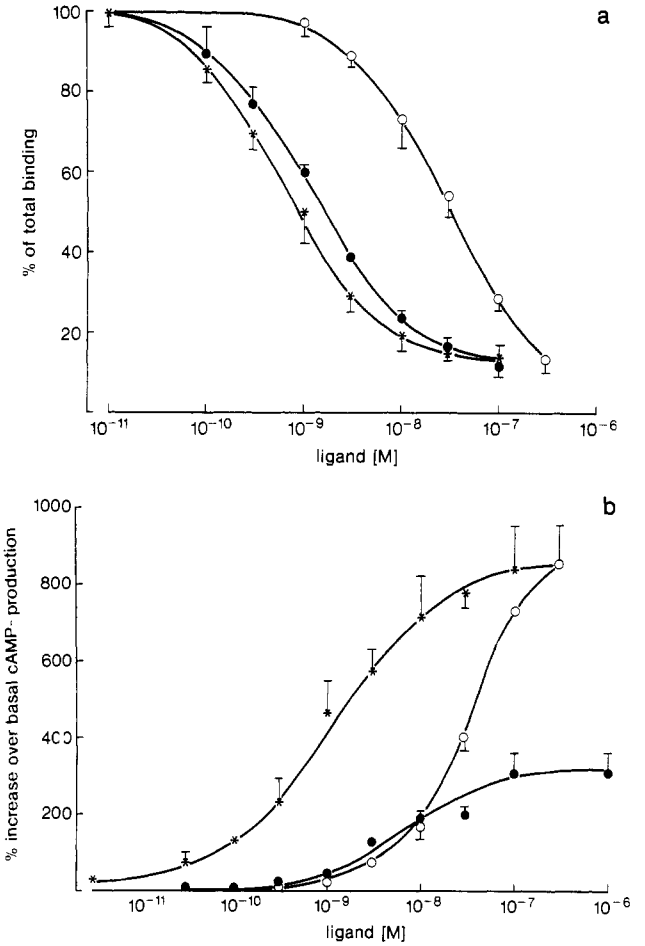


FIGURE 3: Biological activity of [Tyr$^{36}$]PTHrP(1–36)amide analogues. NlePTH and [Tyr$^{36}$]PTHrP(1–36)amide and its photoderivatized analogues were tested in the radioreceptor assay (a) and the cAMP bioassay (b) with ROS 17/2.8 cells. (a) Inhibition of binding of radioiodinated [Tyr$^{36}$]PTHrP(1–36)amide by [Tyr$^{36}$]PTHrP(1–36)amide, NlePTH, and compounds A and G (*), D-1 (○), and D-2 (●). The data represent the mean ± SD of four independent experiments performed in triplicate with material from three independent derivatizations. (b) Stimulation of intracellular cAMP accumulation by [Tyr$^{36}$]PTHrP(1–36)amide, NlePTH, and compounds A, G (*), D-1 (○), and D-2 (●). The data represent the mean ± SD of two independent experiments performed in duplicate with material from two independent derivatizations. Since [Tyr$^{36}$]PTHrP(1–36)amide, NlePTH, and compounds A and G were equipotent and were used at the same concentrations in both (a) and (b), results of the studies with these four compounds are represented by an identical symbol (*).

2% of the activity of the parent, underivatized peptide.

The potencies of [Tyr$^{36}$]PTHrP(1–36)amide and compound G to increase the accumulation of intracellular cAMP in ROS 17/2.8 cells were similar to their potencies in the radioreceptor assay. Half-maximal stimulation was achieved by both peptides at a dose of 1.0 × 10$^{-9}$ M. Compound D-1 was a full agonist, giving half-maximal stimulation at 0.4 × 10$^{-7}$ M, or only 3% of the activity of the underivatized peptide and compound G. Compound D-2 was only a partial agonist: maximal cAMP production increased about 30-fold over basal, reaching a plateau at approximately 1 × 10$^{-7}$ M, which corresponds to about 25% of the levels maximally stimulated by fully active compounds (Figure 3b).

Equimolar concentrations of each radiolabeled, photoderivatized PTHrP compound and NAP-NlePTH bound differently to receptors on ROS 17/2.8 cells: specific binding values of NAP-NlePTH, D-1, D-2, and G were 5.6 ± 2.9%, 3.7 ± 2.4%, 18.0 ± 3.6%, and 16.2 ± 1.3%, respectively (Table II). SDS–PAGE analysis and autoradiography revealed that all

Table II: Specific Binding of $^{125}$I-Labeled Photoderivatized NAP-NlePTH and PTHrP Compounds[a]

| radioiodinated, photoderivatized compound | % specific binding ($n$ = 3) |
|---|---|
| NAP-NlePTH | 5.6 ± 2.9 |
| D-1 | 3.7 ± 2.4 |
| D-2 | 18.0 ± 3.6 |
| G | 16.2 ± 1.3 |

[a] Confluent ROS 17/2.8 cells were incubated with equal concentrations of the HPLC-purified photoactive radioligands (0.9 nM) for 4 h at 15 °C, in the dark. Data are expressed as the mean ± SD of three independent experiments ($n$ = 3).

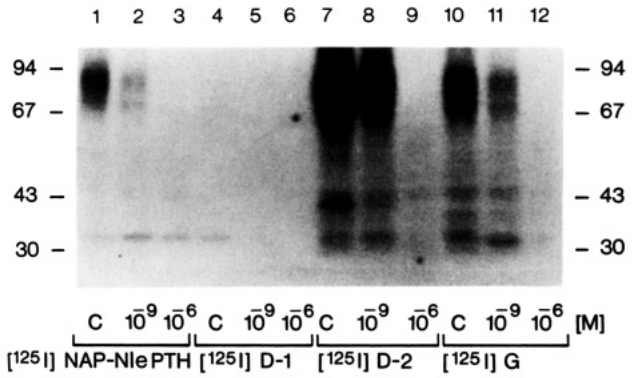


FIGURE 4: Photoaffinity labeling of PTH/PTHrP receptors on ROS 17/2.8 cells with radiolabeled [Nle$^{8,18}$,Tyr$^{34}$]bPTH(1–34)amide and [Tyr$^{36}$]PTHrP(1–36)amide. Confluent ROS 17/2.8 cells were incubated in the dark in the absence (C) or presence of competing [Tyr$^{36}$]PTHrP(1–36)amide ($10^{-9}$ and $10^{-6}$ M) and equal amounts of radioactivity (0.9 nM) of either $^{125}$I-labeled photoderivatized NlePTH (lanes 1–3) or $^{125}$I-labeled photoderivatized PTHrP compounds D-1 (lanes 4–6), D-2 (lanes 7–9), or G (lanes 10–12), respectively, for 4 h at 15 °C, and then photolyzed for 20 min. The cells were then solubilized with 0.5 mL of SDS–PAGE sample buffer and subjected to linear SDS–PAGE (5–15%) with subsequent autoradiography at −80 °C for 3 days. $M_r$ standards (×10$^3$) are indicated.

radiolabeled, photoderivatized peptides specifically cross-link upon photolysis to an 80 000-dalton receptor band that is almost certainly the same receptor protein (Jüppner et al., 1988)(Figure 4). However, the intensity of receptor protein cross-linked with D-2 did not accurately reflect the binding of this radiolabeled, photoderivatized peptide to the ROS 17/2.8 cell surface receptor, whereas the intensity of labeling with the other photoderivatized ligands was predicted by their binding in the dark (Table II). In particular, direct quantification of receptor labeling with D-2 (Figure 4, lane 7) showed that about 30% of the photoaffinity label which bound noncovalently to the receptor was covalently cross-linked upon photolysis, as contrasted to about 6% with G (Figure 4, lane 10). SDS–PAGE also showed that labeling of the 80 000-dalton receptor band is specific, being partially reduced by coincubation with $10^{-9}$ M unlabeled peptide and completely eliminated by $10^{-6}$ M unlabeled peptide.

## Discussion

We previously had used chemically defined FNPA derivatives of NlePTH to characterize the physicochemical properties of PTH receptors on ROS 17/2.8 cells. These studies showed that radiolabeled [Nle$^8$,$N^\epsilon$-(4-azido-2-nitrophenyl)-Lys$^{13}$,Nle$^{18}$,Tyr$^{34}$]bPTH(1–34)amide had the highest receptor affinity of the multiple derivatized products and that the PTH receptor in ROS 17/2.8 cells is a $M_r$ = 80 000 glycoprotein with asparagine-linked oligosaccharides (Shigeno et al., 1988b,c). Karpf et al. identified renal PTH receptors with a similar $M_r$, using bPTH(1–34) as the radiolabeled ligand and $N$-hydroxysuccinimido 4-azidobenzoate (HSAB), a heterobifunctional cross-linking reagent (Karpf et al., 1988). However, our experience using HSAB and either $^{125}$I-labeled NlePTH or [Tyr$^{36}$]PTHrP(1–36)amide as radioligand showed much lower cross-linking efficiency when compared to results with radiolabeled [Nle$^8$,$N^\epsilon$-(4-azido-2-nitrophenyl)-Lys$^{13}$,Nle$^{18}$,Tyr$^{34}$]bPTH(1–34)amide (peak 6; Shigeno et al., 1988b, 1989) or FNPA-derivatized [Tyr$^{36}$]PTHrP(1–36)-amide (compound G) (unpublished data). Our findings (Jüppner et al., 1988) and those of others (Nissenson et al., 1988; Shigeno et al., 1988a; Orloff et al., 1989b) strongly indicate that amino-terminal fragments of both PTH and PTHrP bind to and regulate the same receptor with nearly identical properties, suggesting that the two ligands could be used interchangably to study their common receptor. However, unlike NlePTH, which contains three lysine residues, [Tyr$^{36}$]PTHrP(1–36)amide has only two lysines at positions 11 and 13. We thus undertook the evaluation of photoderivatized PTHrP analogous as alternative ligands with the expectation that fewer photoderivatized products would result from the reaction, thus increasing the yield of the desired product and facilitating its purification.

Amino acid and sequence analysis of FNPA-derivatized [Tyr$^{36}$]PTHrP(1–36)amide showed that several compounds were derivatized on a single amino acid: D-1 and G were derivatized on Lys$^{13}$ and Lys$^{11}$, respectively, and D-2 was derivatized on Ala$^1$. Sequence analysis of the products resulting from the digestion of D-2 with endoproteinase Glu-C yielded quantitative recovery of lysine, thus confirming that this product, also, was monoderivatized. The further characterization of these products revealed striking discrepancies in their biological properties.

Modification of Lys$^{13}$ lowered both the affinity of the peptide for the common PTH/PTHrP receptor and its potency to stimulate adenylate cyclase in ROS 17/2.8 cells. Although previous observations had shown that modification of Lys$^{13}$ in NlePTH also reduced receptor affinity and bioactivity, the effects of this change on the affinity of [Tyr$^{36}$]PTHrP(1–36)amide is more striking. Interestingly, low affinity and bioactivity has also been reported for the amino-terminal fragment of chicken PTH (Caulfield et al., 1988), where Lys$^{13}$, which is conserved in the PTH sequence of all mammalian species thus far determined, is replaced by Glu (Khosla et al., 1988). This implies that lysine at position 13, or perhaps the maintenance of a positive charge at that position, is of importance for receptor affinity. Modification of Lys$^{11}$, however, is well tolerated, resulting in no apparent loss in affinity or biological activity. Since position 11 is Leu in all known PTH sequences, the loss of the positive charge at this position within the primary structure of PTHrP might have been predicted to be a change of little biological consequence.

The most interesting data were, however, obtained with compound D-2, which is derivatized on Ala$^1$. Its binding to the receptor is essentially unimpaired, yet it is only a partial agonist with strikingly reduced biological activity. Earlier studies with PTH(1–34) revealed that amino-terminal modifications of bPTH(1–34)(deletion, extension, acetylation, or deamidation) yielded peptides nearly or completely devoid of activity in the adenylate cyclase bioassay (Rosenblatt, 1982), but PTH radioreceptor assays had not yet been developed. However, Goltzman et al. have shown that [deamino-Ala$^1$]-bPTH was an effective inhibitor of PTH-stimulated renal adenylate cyclase activity (Goltzman et al., 1975), implying that this compound retained high affinity for the PTH receptor. Furthermore, recent data have shown that when the

amino terminus of NlePTH was derivatized at $Ala^1$ with FNPA, its activity was only modestly impaired (compound 8; Shigeno et al., 1989). The high efficiency of cross-linking achieved by using compound D-2 could indicate that $Ala^1$ is in closer proximity to the receptor than is $Lys^{11}$ and/or that other steric characteristics favor the close interaction of the amino terminus with the receptor. High cross-linking efficiency of 30–50% has also been reported when angiotensin II was modified at its amino terminus by derivatization with phenyl azide (Guillemette et al., 1986). Our data predict that PTH/PTHrP analogues modified at their amino termini might serve dual functions as potent antagonists of hormone action and as high-efficiency cross-linkers for the purification of the receptor protein.

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