Probing the Bimolecular Interactions of Parathyroid Hormone and the Human Parathyroid Hormone/Parathyroid Hormone-Related Protein Receptor. 2. Cloning, Characterization, and Photoaffinity Labeling of the Recombinant Human Receptor[†,‡]

Amy E. Adams,[§] Mark Pines,[∥] Chizu Nakamoto,[∥] Vered Behar,[∥] Qui Ming Yang,[∥] Roberto Bessalle,[∥] Michael Chorev,[∥] Michael Rosenblatt,[∥] Michael A. Levine,[⊥] and Larry J. Suva*[,∥]

Division of Bone and Mineral Metabolism, Harvard-Thorndike and Charles A. Dana Research Laboratories, Department of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts 02215, Harvard−Massachusetts Institute of Technology Division of Health Sciences and Technology, Harvard Medical School, Boston, Massachusetts 02215, and Division of Endocrinology and Metabolism, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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ABSTRACT: Parathyroid hormone (PTH) acts to regulate calcium homeostasis by interacting with a G-protein-coupled receptor that also binds PTH-related protein (PTHrP). In this report we describe the cloning, characterization, and biological activity of the cloned human (h) PTH/PTHrP receptor (Rc) and cross-linking of a benzophenone-substituted PTH analog, [Nle^{8,18},Lys^{13}(ϵ-*pBz*$_2$),L-2-Nal^{23},Tyr^{34}]bPTH(1−34)-NH_2 (K13), to cells endogenously expressing the Rc and cells transiently or stably transfected with the human Rc. A full-length cDNA clone was isolated and fully sequenced from a human kidney cDNA library. Northern blot analysis of normal human tissues revealed a limited tissue distribution: a single transcript of ~2.3 kb was detected in kidney, lung, placenta, and liver. In human embryonic kidney cells (HEK-293, clone C-21) stably transfected with hPTH/PTHrP Rc, a single 85−90 kDa Rc−hormone complex was formed after photolysis in the presence of K13. This covalent cross-linking reaction was specifically inhibited by excess quantities of biologically active 1−34 analogs of bovine (b) PTH or hPTHrP but not by C-terminal and midregion PTH peptides. Photoincorporation of ^{125}I-labeled K13 into the Rc occurred with high efficiency (60−70%), approximately an order of magnitude greater than that achieved with conventional aryl azide cross-linking reagents. These results support the feasibility of our approach for specifically cross-linking a tagged PTH analog to the Rc, as a first step in the effort to identify directly the amino acid residues that constitute the Rc binding site.

Parathyroid hormone (PTH)[1] regulates calcium levels in blood through actions on bone and kidney via activation of a G-protein-coupled seven-transmembrane-segment-containing receptor (Jüppner et al., 1991; Abou-Samra et al., 1992; Adams et al., 1993; Schipani et al., 1993) that it shares with PTH-related protein (PTHrP) (Suva et al., 1987). Structure−function studies of PTH and PTHrP with their common receptor allow examination of the structural elements in these hormones which are involved at the molecular level in the expression of biological activity.

Characterization of the structural features of PTH required for bioactivity has been studied extensively over the past two decades (Rosenblatt 1986; Chorev & Rosenblatt, 1994); however, information regarding the structure of the receptor and the nature of its interaction with hormone have been limited because the cloned Rc was not available until recently (Jüppner et al., 1991; Abou-Samra et al., 1992; Schipani et al., 1993; Adams et al., 1993; Pausova et al., 1994; McCuaig et al., 1994). Studies of the effects produced by mutation or deletion of domains within the Rc provide insight into important structural features of the Rc; however, conformational and long-range intramolecular effects resulting from mutations or deletions in the Rc cannot be excluded as the mechanism for the observed change in Rc properties. Furthermore, neither hormone nor Rc structure−function-oriented investigations alone can predict or identify the specific amino acid-to-amino acid contacts between hormone and receptor at their interface during binding and activation.

In addition to the availability of high-efficiency cross-linking (Nakamoto et al., 1995), the effort to identify hormone−Rc amino acid "contact points" requires a large amount of human Rc in order to perform microsequence analysis of the covalently attached Rc−hormone conjugate. Recently, we have succeeded in stably expressing hPTH/PTHrP Rc at a level greater than that observed in any native

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* To whom correspondence should be addressed.

[§] Harvard−MIT Division of Health Sciences and Technology.

[∥] Harvard-Thorndike and Charles A. Dana Research Laboratories.

[⊥] Johns Hopkins University School of Medicine.

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[1] Abbreviations used are in accordance with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (1975) *J. Biol. Chem. 250,* 3215−3216 and (1985) *J. Biol. Chem. 260,* 14−42, and IUPAC−CNOC (1979) *Eur. J. Biochem. 86,* 9−25. In addition: BP, benzophenone; C-21, HEK-293 cells stably transfected with the human parathyroid hormone/parathyroid hormone-related protein receptor; COS-7, receptor-negative monkey kidney cell line; HEK-293, parathyroid hormone/parathyroid hormone-related protein receptor-negative human embryonic kidney cell line; IBMX, 3-isobutyl-1-methylxanthine; K13, [Nle^{8,18},Lys^{13}(ϵ-*pBz*$_2$),L-2-Nal^{23},Tyr^{34}]bPTH-(1−34)NH_2; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; Rc, receptor; ROS17/2.8, rat osteogenic sarcoma cell line; Saos-2/B10, human osteogenic sarcoma cell line.

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hPTH/PTHrP Rc-expressing cell line. We have both transiently and stably expressed the cloned hPTH/PTHrP Rc in monkey kidney (COS-7) (Adams et al., 1993) and in human embryonic kidney (HEK-293) cells (Pines et al., 1994).

In this report, we describe the cloning, biological properties, and photoaffinity labeling of the transfected hPTH/PTHrP Rc. Biological properties of both the stably and transiently transfected recombinant hPTH/PTHrP Rc, as assessed by ^{125}I-PTH binding, PTH-stimulated cAMP accumulation, and adenylyl cyclase activity, are virtually identical to those of the native Rc. Specific high-affinity cross-linking to the hPTH/PTHrP Rc using a benzophenone-(BP-) substituted PTH analog, [Nle^{8,18},Lys^{13}(ϵ-*pBz*$_2$),L-2-Nal^{23},Tyr^{34}]bPTH(1−34)NH$_2$ (K13), was achieved with high efficiency (60−70%), approximately an order of magnitude greater than cross-linking achieved with conventional aryl azide cross-linking reagents (Kaufmann et al., 1994; Shigeno et al., 1987; Goldring et al., 1984). The degree of photoaffinity labeling obtained with transfected receptor was much higher than that observed with native (human or rat) Rc, suggesting a dependence on Rc number for signal strength, as has been reported for other parameters of hormone activity, such as ^{125}I-bPTH binding, cAMP accumulation, and increased intracellular calcium ([Ca^{2+}]$_i$) transients (Pines et al., 1994, 1995). In addition, this photoaffinity labeling approach detects multiple cross-linked hormone−Rc conjugates with different molecular weights for native and transiently and stably transfected Rc. Such differences in the apparent molecular weights of cross-linked PTH/PTHrP Rc have been reported previously using aryl azide cross-linking agents (Kaufmann et al., 1994; Shigeno et al., 1987; Goldring et al., 1984; Karpf et al., 1987) and suggest that the size differences may be due, at least in part, to altered Rc posttranslational processing such as glycosylation, Rc degradation, or high-level Rc overexpression achieved by transient transfection. These results indicate that a photoaffinity-based cross-linking approach holds promise for mapping directly the bimolecular interface of PTH or PTHrP and their common Rc.

EXPERIMENTAL PROCEDURES

General Peptide Synthesis and Purification. Standard [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$[bPTH-(1−34)] and [Nle^{8,18}, Lys^{13}(ϵ-*p*Bz$_2$),L-2-Nal^{23},Tyr^{34}]bPTH(1−34)NH$_2$ (K13) were synthesized as described (Nakamoto et al., 1995), using an Applied Biosystems 430A automated peptide synthesizer, and peptide concentrations were determined by amino acid analysis. Biological characterization (^{125}I-bPTH-(1−34) binding and PTH-stimulated adenylyl cyclase activity) of the BP-containing analog K13, in Saos-2/B10 cells, was performed as described in the preceding paper (Nakamoto et al., 1995). [Tyr^{52}]hPTH(52−84)NH$_2$ [hPTH(52−84)], hPTH-(27−52)NH$_2$ [hPTH(27−52)], and hPTHrP(1−34)NH$_2$ [hPTHrP(1−34)] were purchased from Bachem (Torrence, CA).

Radioiodination. Iodination of PTH analogs was carried out on the Tyr^{34} residue employing IodoGen (Chorev et al., 1990). K13 (35 μg, 7.7 nmol) was dissolved in 10 mM acetic acid (40 μL) and diluted with 100 mM sodium phosphate buffer, pH 7.4 (60 μL). The solution was then added to a borosilicate tube (12 × 75 mm) coated with IodoGen (10 mg, 0.23 mmol), followed by the addition of Na^{125}I (2.0 mCi, 20 μL), specific activity 2200 Ci/mmol (Amersham Corp., Arlington Heights, IL). The reaction was carried out for 10 min at room temperature with occasional hand mixing. The reaction was quenched by the addition of 0.1% TFA in H$_2$O (300 μL). The mixture was loaded onto an analytical HPLC system equipped with a Vydac C-18 protein column (5 μm, 0.21 × 15 cm) and a solvent system of (A) 0.1% TFA in H$_2$O and (B) 0.1% TFA in acetonitrile. Separation was carried out using a linear gradient of 32−40% B in A over 30 min, at a flow rate of 1 mL/min, monitored by UV (220 nm) and in-line radioactive detectors. The radiolabeled peptide peak (t_r = 13 min with k' = 12 min and t_r = 15.3 min with k' = 14.3 min for K13 and ^{125}I-labeled K13, respectively) was collected (0.5-mL fractions) into borosilicate tubes containing 10% fetal bovine serum (FBS), heat-inactivated for 4 min at 100 °C, in 100 mM Tris buffer, pH 7.4 (500 μL). Fractions containing radiolabeled peptide were pooled and stored in borosilicate tubes at −80 °C.

Cloning of the PTH/PTHrP Receptor. A human kidney λgt10 cDNA library (Clontech, Palo Alto, CA) was screened in duplicate, as described previously (Suva et al., 1987), using a ^{32}P-labeled 1.4-kb polymerase chain reaction- (PCR-) derived hPTH/PTHrP Rc fragment (Adams et al., 1993). Approximately 30 strongly hybridizing phage were identified and plaque-purified twice, as described (Sambrook et al., 1989). Insert DNA was excised with *Eco*RI (Pharmacia, Nutley, NJ) and visualized on an ethidium bromide-stained 1.0% agarose gel. The two largest independent cDNA clones were subcloned into pUC18+ (Pharmacia) and completely sequenced using Sequenase (USB, Cleveland, OH). The full-length cDNA was then inserted in both orientations into mammalian expression vectors pRSV (Promega, Madison, WI) and pcDNA1/Neo (Stratagene, La Jolla, CA) for transfection. The sequence described in this paper has been submitted to GenBank (Accession Number U17418).

Cell Culture and Transfection. All tissue culture media, supplements, and sera were purchased from Gibco−BRL (Gaithersburg, MD) and tissue culture plastics were from Corning (Corning, NY). COS-7 cells were kindly provided by Dr. Steven Goldring (Massachusetts General Hospital) and maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% FBS. Transient transfections were performed with 1-5 μg of hPTH/PTHrP Rc in pRSV, using calcium phosphate or DEAE-dextran (Sigma, St. Louis, MO), as described previously (Suva et al., 1991). Saos-2/B10 cells and ROS17/2.8 cells were a generous gift of Dr. Sevgi Rodan (Merck Research Laboratories). Saos-2/B10 cells (Rodan et al., 1989) were maintained in RPMI 1640 medium supplemented with 10% FBS and 10 mM glutamine and ROS17/2.8 cells were maintained in Hams F-10 supplemented with 5% FBS. Human embryonic (HEK-293) kidney cells were the generous gift of Dr. Vikas Sukhatme (Beth Israel Hospital) and were maintained in D-MEM supplemented with 10% FBS. HEK-293 cells stably transfected with full-length hPTH/PTHrP Rc cDNA in pcDNA/Neo were maintained in D-MEM supplemented with 10% FBS with 500 μg/mL G418 (Sigma) as described (Pines et al., 1994).

hPTH/PTHrP Receptor Binding, cAMP Production, and Adenylyl Cyclase Activity. Measurement of specific hPTH/

PTHrP Rc binding was performed as described previously (Pines et al., 1994), using monolayers of transiently and stably transfected cells. Cells were washed once with binding buffer (5% FBS-supplemented appropriate growth medium) before incubation with ^{125}I-labeled [Nle^{8,18},Tyr^{34}]-bPTH-(1−34)NH_2 [^{125}I-bPTH-(1−34)] (200 000 cpm/mL), with or without nonradioactive bPTH-(1−34), in binding buffer for 2 h at RT. Cells were washed twice with PBS, before being solubilized in 1 mL of 0.1 M NaOH. Aliquots were taken for determination of bound radioactivity by scintillation counting (Packard 2200 CA Tri-carb liquid scintillation counter). Specific binding was expressed as cpm bound/100 000 cells, where cell number was measured in a Coulter counter (Coulter Electronics) (Nakamoto et al., 1995). For cyclic AMP (cAMP) determination, cells in 24-well tissue culture plates were incubated with various PTH peptides for 20 min in growth medium in the presence of 1 mM IBMX. The incubation was terminated by the addition of perchloric acid (final concentration of 30%) and the samples were neutralized with potassium bicarbonate and acetylated, and the total cAMP (medium + cells) was determined by radioimmunoassay (RIA) as described previously (Pines et al., 1994). Adenylyl cyclase activity was measured as described in the preceding paper (Nakamoto et al., 1995), except that transiently transfected COS-7 cells were used. Curves were fitted by CA Cricket Graph III, version 1.0 (Computer Associates).

RNA Isolation and Analysis. Total cellular RNA was isolated by guanidinium isothiocyanate (GT) and phenol extraction of samples homogenized in GT buffer as previously described (Suva et al., 1991; Chomczynski & Sacchi, 1987). Prepared RNA was washed with 70% ethanol, resuspended in water, and precipitated with ethanol. Total RNA (20 μg) was separated by electrophoresis through a 1% agarose gel containing 0.66 M formaldehyde and transferred to nylon. Filters were prehybridized in a buffer containing 50% formamide, 5× SSC (1× SSC = 0.15 M NaCl/0.015 M trisodium citrate), 50 mM sodium phosphate, pH 7.0, 5× Denhardt's solution, 1% skim milk powder, 100 μg/mL sonicated salmon sperm DNA, and 0.1% SDS, at 42 °C and then hybridized in the same buffer containing ^{32}P-labeled cDNA probes at 10^6 cpm/mL for 18 h and washed twice at 50 °C in 0.1× SSC/0.1% SDS.

Hormone−PTH/PTHrP Receptor Cross-Linking. Saos-2/B10 cells, ROS17/2.8, transiently transfected COS-7, stably transfected HEK-293 (clone C-21), and parental HEK-293 cells were subcultured (2×10^5 cells/well) into 6-well tissue culture dishes and grown to near confluence. Cells were rinsed twice with PBS and 0.9 mL of binding buffer was added to each well. Competition was performed by pretreatment with either 10^{-5} M bPTH(1−34), hPTH(27−52), hPTHrP(1−34), or hPTH(52−84) or 0.1 mL of binding buffer (control) where indicated for 10 min at room temperature. All wells were then incubated 1 h at room temperature in the presence of $(1-5) \times 10^6$ cpm of ^{125}I-labeled K13 (~0.6−3 nM). Cells were irradiated for 30 min on ice in the tissue culture dishes, without lids, at a distance of 15 cm from 6 × 15-W 365-nm UV lamps in a Stratalinker 2400 (Stratagene). Cells were rinsed with complete medium and harvested with 500 μl of Laemmli sample buffer (Laemmli, 1970) containing 10% β-mercaptoethanol.

SDS−PAGE and Autoradiography. Photolabeled cells (50−100 μg of protein) in Laemmli sample buffer were electrophoresed in 7.5% polyacrylamide slab gels as described (Laemmli, 1970). Gels were dried with a Bio-Rad slab gel dryer and exposed at −80 °C to Kodak XAR-5 film with two intensifying screens for various times (6−48 h). The molecular weights of the radiolabeled peptides were determined from the following Bio-Rad molecular weight standards: broad range 6.5−205 kDa; Kaleidoscope prestained 6.9−202 kDa.

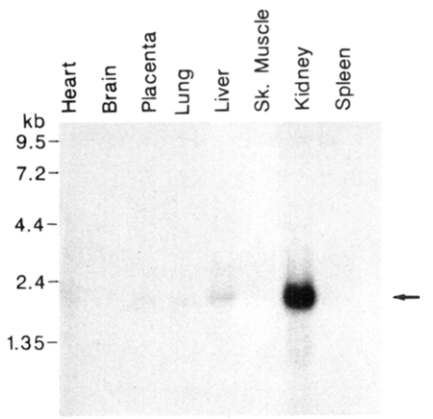

FIGURE 1: Human tissue distribution of the PTH/PTHrP receptor. Poly(A)$^+$ RNA (2 μg/lane) from normal human tissues was subjected to Northern blot analysis using a 2.4-kb human PTH/PTHrP receptor cDNA probe. Arrow shows the position of the single 2.3-kb mRNA transcript. Size markers of 9.5, 7.2, 4.4, 2.4, and 1.35 kb are also shown.

To determine photoincorporation of ^{125}I-labeled K13 into receptor protein, autoradiographs were aligned with the dried gels, radiolabeled peptides were excised, and radioactivity was measured. Alternatively, photoincorporation was determined by computer-enhanced scanning densitometry of the appropriately exposed autoradiographic films (NIH Image 1.54). Cross-linking efficiency refers to the percentage of total ^{125}I-K13 which formed a covalent adduct. This was calculated as ([{cpm in major band(s)}/{cpm in major band + cpm in all other bands}] × 100) in order to determine the amount of total radioactivity cross-linked that is incorporated into the Rc.

RESULTS

The hPTH/PTHrP Rc was cloned by screening 320 000 plaques from a normal human kidney cDNA library (Clontech) using a PCR-derived partial human cDNA as a probe. Two independent, full-length clones were isolated and completely sequenced, including clone pPTHRc4.2. Comparison of the pPTHRc4.2 sequence with GenBank sequence repositories revealed that this clone was identical to the previously published hPTH/PTHrP Rc sequence (Schipani et al., 1993; Schneider et al., 1993). Northern blot analysis using ^{32}P-labeled pPTHRc4.2 cDNA, revealed a single 2.3-kb mRNA transcript in kidney, with low amounts of an identical size transcript detectable in liver, lung, and placenta (Figure 1).

Full-length hPTH/PTHrP Rc cDNA was inserted into the mammalian expression vectors pRSV and pcDNAINeo yielding pRc/RSV for transient transfection and pRc/Neo for stable transfection. Functional expression of the transiently transfected recombinant hPTH/PTHrP Rc was assessed by Northern blot analysis, competitive binding of ^{125}I bPTH-(1−34), and PTH-stimulated adenylyl cyclase activity (Figure 2). Northern blot analysis of 20 μg of total RNA obtained

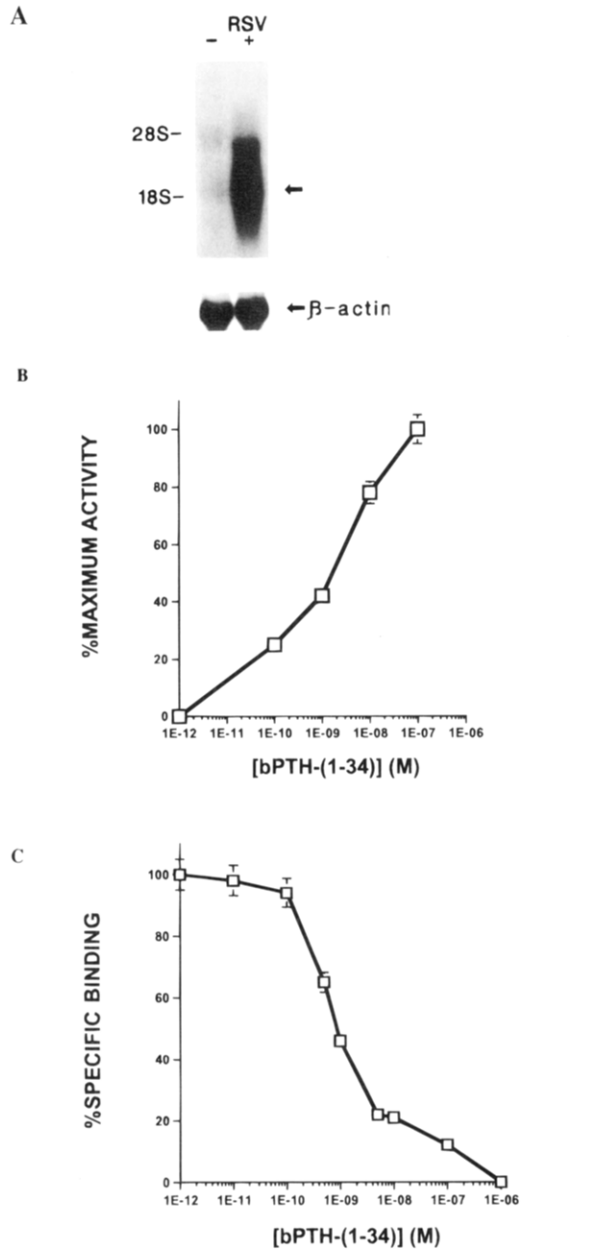

FIGURE 2: Characterization of transiently transfected COS-7 cells. Full-length hPTH Rc was transfected into COS-7 cells. (A) Northern blot of 20 μg of total RNA from COS-7 cells transfected with (+) or without (−) 2.5 μg of pRSV-receptor construct. (B) PTH-stimulated adenylyl cyclase activity of cells from the same transfection. (C) Competition for ^{125}I-bPTH-(1−34) binding by bPTH(1−34) in cells from the same transfection. Similar results were obtained in two additional experiments.

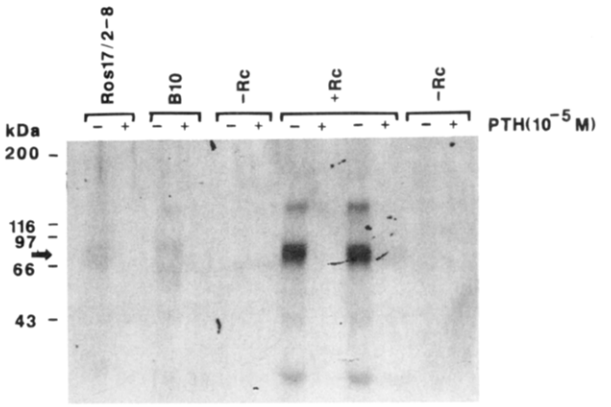

FIGURE 3: Autoradiograph of photolabeled COS-7, Saos-2 B10, and ROS17/2.8 cells. COS-7 cells transiently transfected with hPTH/PTHrP Rc (+Rc) or mock transfected (−Rc) Saos-2 B10 and ROS17/2.8 cells were photolabeled with ^{125}I K13 PTH, in the presence (+) or absence (−) of 10^{-5} M bPTH(1−34). Arrow indicates the position of the major photolabeled species (70−90 kDa) in all cell types. Size markers (kilodaltons) are also shown. Autoradiograph is typical of three additional experiments.

from mock-transfected and transiently transfected COS-7 cells revealed very high-level expression mRNA in cells transfected with hPTH/PTHrP Rc (Figure 2A). ^{125}I-bPTH-(1−34) binding ($IC_{50} \sim 1$ nM) and PTH-stimulated adenylyl cyclase activities ($EC_{50} \sim 2$ nM) were comparable to that of Saos-2/B10 cells (Figure 2B,C) (Nakamoto et al., 1995).

A series of BP-containing PTH analogs have been synthesized and biological activity has been characterized in Saos-2 B10 cells (Nakamoto et al., 1995). We next examined the ability of one biologically active BP-containing PTH analog, K13 [see Experimental Procedures and Nakamoto et al. (1995)] to photoaffinity-label native hPTH/PTHrP Rc and transiently transfected hPTH/PTHrP Rc. Mock-transfected and transiently transfected COS-7 cells were incubated with radiolabeled K13, in the presence or absence of competing 10^{-5} M bPTH-(1−34), and cross-linked to the hPTH/PTHrP Rc (see Experimental Procedures). Equal amounts (50−100 μg) of cross-linked cell extracts were analyzed by SDS−7.5% PAGE and autoradiography. A specific photoaffinity-labeled product was observed only in Rc-transfected COS-7 cells (Figure 3). In transiently transfected cells, a predominant broad band was detected at molecular mass ~70−90 kDa, with minor bands at 130, 43, and 15 kDa, which were all competed by 10^{-5} M bPTH-(1−34). In addition, Figure 3 shows the cross-linking of K13 to native PTH/PTHrP Rcs expressed in ROS17/2.8 (rat) and Saos-2/B10 cells (human). In ROS17/2.8, a specific photoaffinity-labeled doublet at 90 and 70 kDa was observed (Figure 3). Furthermore, photoaffinity labeling of Saos-2/B10 cells revealed specific labeling of a major doublet at 90 and 60 kDa, with minor bands at 130 and 15 kDa, which were weaker in intensity but of similar size to those seen in transiently transfected COS-7 cells (Figure 3).

We have previously reported the production and partial characterization of HEK-293 cells stably expressing high levels of the hPTH/PTHrP Rc (Pines et al., 1994). Analysis of the stably transfected cells showed that the levels of Rc mRNA, PTH-stimulated cAMP accumulation, and ^{125}I-PTH-(1−34) binding in the stably transfected cells all correlate well with Rc number (Pines et al., 1994). Since the overexpression of the hPTH/PTHrP Rc is essential for the success of our photoaffinity-based approach and subsequent identification of the points of bimolecular interaction between hormone and Rc by microsequencing, we examined the biological activity of K13 and photoaffinity labeling of the hPTH/PTHrP Rc by K13 in HEK-293 clone C-21 (expressing ~400 000 Rc/cell; Pines et al., 1994) (Figures 4 and 5). As shown in Figure 4A, K13-stimulated cAMP accumulation in C-21 cells were virtually indistinguishable from the cAMP stimulation elicited by unsubstituted bPTH-(1−34). Competition by K13 for ^{125}I-PTH-(1−34) binding was similar to that of native PTH-(1−34) lacking the BP moiety in C-21 cells [$IC_{50} \sim 50$ nM for K13 and 90 nM for PTH-(1−34)] (Figure 4B). The analog was then used to photoaffinity-label the recombinant hPTH/PTHrP Rc expressed in C-21 cells (Figure 5). Specific high-affinity photoaffinity labeling

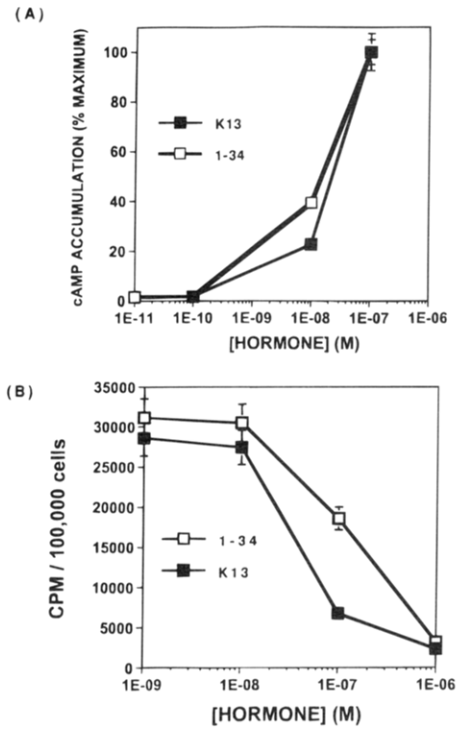

FIGURE 4: Biological activity of benzophenone-substituted bPTH-(1−34) analogs in stably transfected C-21 cells. (A) bPTH(1−34)- and benzophenone-substituted bPTH(1−34)-stimulated cAMP accumulation in C-21 cells. (B) Competition for ^{125}I-bPTH(1−34) binding by bPTH(1−34) and K13 bPTH(1−34) in C-21 cells. Similar results were obtained in two additional experiments.

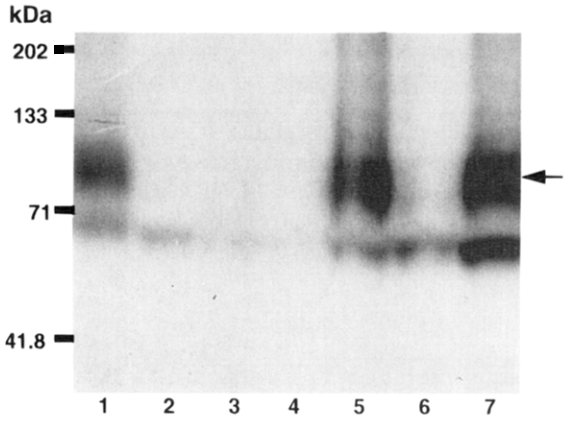

FIGURE 5: Competition of ^{125}I K13 bPTH(1−34) photolabeling of C-21 cells by various PTH peptides. C-21 cells were photoaffinity-labeled with ^{125}I K13 bPTH(1−34) alone (lane 1) or in the presence of 10^{-5} M PTH (lane 2). Parental HEK-293 cells were photoaffinity-labeled with ^{125}I K13 bPTH(1−34) alone (lane 3) or in the presence of 10^{-5} M PTH (lane 4). Photoaffinity-labeled C-21 cells preincubated with 10^{-5} M PTH or PTHrP peptides. Lane 5, hPTH-(27−52); lane 6, hPTHrP(1−34); lane 7, hPTH(52−84). Arrow indicates the position of the major photolabeled species (70−90 kDa). Size markers (kilodaltons) are also shown. Autoradiograph is typical of two additional experiments.

was observed in C-21 cells and not in parental HEK-293 cells (Figure 5, lines 1 and 3). SDS−PAGE analysis showed the cross-linked hormone−Rc complex with an apparent molecular weight of ~80−90 kDa. A minor, non-Rc-related band of ~70 kDa was observed in C-21 and parental HEK-293 cells (Figure 5). The broad 80−90-kDa photoaffinity-labeled complex was specifically competed by incubation with 10^{-5} M bPTH-(1−34) and 10^{-5} M hPTHrP(1−34) (Figure 5, lanes 2 and 6); however, no competition was observed by incubation with either 10^{-5} M hPTH(27−52) or 10^{-5} M hPTH(52−84) (Figure 5, lanes 5 and 7).

DISCUSSION

The goal of this work is to demonstrate and characterize an efficient and specific photoaffinity PTH ligand−receptor interaction that can form the basis for a subsequent effort to purify hormone−Rc conjugates and identify the amino acid "contact points" involved at the hormone−Rc interface. Toward this goal, we cloned the hPTH/PTHrP Rc and prepared and characterized stably transfected cell lines expressing the hRc.

In this report, we demonstrate the successful use of one photoreactive PTH agonist analog, K13 (Nakamoto et al., 1995), to specifically photoaffinity-label the cloned and expressed hPTH/PTHrP Rc. The photoaffinity labeling of cells expressing the hPTH/PTHrP Rc was achieved with an efficiency of 60−70%, much greater than that achieved with previously utilized aryl azide-based PTH/PTHrP cross-linking agents (Kaufmann et al., 1994; Shigeno et al., 1987; Goldring et al., 1984; Karpf et al., 1987).

Northern blot analysis of a variety of normal human tissues revealed a relatively limited tissue distribution for the hRc mRNA. High levels of a single 2.3-kb transcript was observed in kidney, with lower amounts of an identically sized transcript in liver, lung, and placenta (Figure 1). Little or no alternate mRNA species were detected using this full-length probe. However, the existence of other Rc mRNA species, possessing little or no sequence homology with this form of the hPTH/PTHrP Rc, cannot be excluded.

Using transiently transfected COS-7 cells, high levels of Rc mRNA expression were obtained (Figure 2A). Despite the high Rc mRNA levels achieved in these transiently transfected cells, PTH-stimulated adenylyl cyclase activity and ^{125}I-bPTH-(1−34) binding were not Rc-number dependent, as has been reported for other transient transfection systems (Bringhurst et al., 1993; Schneider et al. 1993). In transfected COS-7 cells, biologically active ^{125}I-K13 (Nakamoto et al., 1995) was used to specifically photoaffinity-label the transiently expressed hPTH/PTHrP Rc. Cross-linking revealed a broad band at 70−90 kDa; minor bands of ~130, 43, and 15 kDa were also seen in the transfected cells (Figure 3). Generation of these differently sized photoaffinity-labeled complexes was specifically inhibited by excess bPTH-(1−34), suggesting that each band contains some form or fragment of the hormone−Rc complex. The amount of cross-linked hormone−Rc complex/μg of protein in transiently transfected COS-7 cells is greater than that obtained with either ROS17/2.8 or Saos-2/B10 cells, which endogenously express ~20 000−30 000 Rc/cell (Figure 3; Pines et al., 1994), presumably reflecting the very high level of Rc expression achieved in transient transfection systems. Conservatively estimating a transfection efficiency of 30%, we calculate from our data on transiently transfected COS-7 cells that the cells express approximately 10^6 Rcs/cell, as has been reported previously (Schneider et al., 1994).

Photoaffinity labeling of cells endogenously expressing native rat or human PTH/PTHrP Rcs also produced multiple bands, the formation of which was inhibited by excess PTH. In ROS17/2.8 cells, a PTH-competable, photoaffinity-labeled doublet was observed at 70−90 kDa. In Saos-2/B10 cells, predominant bands at 60 and 90 kDa were observed, with

minor bands at 130, 43, and 15 kDa; all were competed by excess PTH-(1−34) (Figure 3). These additional products may represent multimeric forms of the PTH/PTHrP Rc, or Rc degradation products, or forms containing different degrees of glycosylation (Goldring et al., 1984; Karpf et al., 1987; Shigeno et al., 1988). Previous studies of cross-linking to PTH/PTHrP Rcs report inconsistent results using estimates of Rc content based on SDS gel analysis and/or Rc fragments with molecular sizes ranging from 14 to 135 kDa obtained from several species (Goldring et al., 1984; Karpf et al., 1987; Shigeno et al., 1987, 1988; Kauffman et al., 1994a). In particular, the recent cross-linking of *N*-hydroxysuccinimidyl 4-azidobenzoate-substituted chicken ^{125}I-PTHrP(1−36) to the transfected opossum PTH/PTHrP Rc in CHO cells revealed a major cross-linked band at 90 kDa, with a minor band at 14 kDa (Kaufmann et al., 1994b). This study, like many others, used a chemical cross-linking technique which is less selective and less efficient than the photoaffinity method and required autoradiography for 12−14 days to visualize cross-linked Rc, compared to 10−12 h for the approach we employed. The heightened efficiency of cross-linking achieved with benzophenone photoaffinity labeling may also explain the different hormone−Rc complexes revealed in our experiments.

There is a clear difference in the pattern of photoaffinity labeling of PTH/PTHrP Rcs from different species (i.e., rat vs human) and between endogenous and transfected human Rc (Figure 3). The difference between rat and human Rcs may well reflect different species-related posttranslational modifications, such as glycosylation, as has been reported for PTH/PTHrP Rcs across species (Karpf et al., 1987; Shigeno et al., 1988) and the evolutionarily related calcitonin Rc (Sexton et al., 1993). Presumably, the difference between endogenous and transfected human Rc relates to differences in Rc number (Bringhurst et al., 1993; Pines et al., 1994).

Since transient overexpression of the hPTH/PTHrP Rc into a monkey (COS-7) cell resulted in the photoaffinity labeling of multiple hormone−Rc fragments (Figure 3), we decided to examine the biological activity and cross-linking of K13 in a stably transfected human cell line expressing ~400 000 hPTH/PTHrP Rcs/cell (Pines et al., 1994). In these stably transfected cells (C-21), K13 stimulated cAMP accumulation and specifically competed for ^{125}I-bPTH-(1−34) binding with a potency similar to that of PTH-(1−34) (Figure 4). When K13 was used to photoaffinity-label the hPTH/PTHrP Rc, a single 80−90-kDa broad band labeled specifically, comparable to molecular weights reported previously for the PTH/PTHrP Rc (Figure 5) (Goldring et al., 1984; Karpf et al., 1987; Shigeno et al., 1987, 1988; Kauffman et al., 1994b). Taking into account a molecular weight of approximately 4 kDa for ^{125}I-labeled K13, the mass of the hPTH/PTHrP Rc stably transfected into HEK-293 cells is 76−86 kDa. In all photoaffinity cross-linking experiments described here, a specifically labeled hormone−Rc complex is observed with an apparent molecular weight of ~90 kDa (Figures 3 and 5), suggesting a size of ~86 kDa for both the rat and human Rcs. In addition, the photoaffinity-labeled hormone−Rc complex was specifically competed by N-terminal bPTH-(1−34) and hPTHrP(1−34), but not by midregion hPTH-(27−52) or C-terminal hPTH(52−84) fragments (Figure 5). The N-terminal specificity of photoaffinity labeling by K13 is in agreement with the N-terminal PTH and PTHrP peptide-specific, Rc-mediated effects on cAMP accumulation, ^{125}I-PTH binding, and $[Ca^{2+}]_i$ transients observed in C-21 cells (Pines et al., 1994, 1995). The high degree and relative lack of heterogeneity of the specific photoaffinity labeling achieved in stably transfected C-21 cells should facilitate future efforts to isolate cross-linked hormone−Rc conjugates for microsequence analysis.

The availability of biologically active BP-substituted PTH analogs, one of which photoaffinity labels the hPTH/PTHrP Rc with unusually high efficiency, provides an important new class of reagents for investigating PTH/PTHrP Rc function and evaluating the nature of the interaction of PTH, PTHrP, and analogs with their common Rc. Subsequent identification of the specific amino acid-to-amino acid contact points between hormone and Rc may facilitate the rational design of novel hPTH/PTHrP analogs and generate insights into the mechanism of hormone−Rc interaction.

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