Purification and Characterization of a Receptor for Human Parathyroid Hormone and Parathyroid Hormone-Related Peptide

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Summary

The human parathyroid hormone receptor (hPTH1R), containing a nine-amer acid sequence of rhodopsin at its C-terminus, was transiently expressed in COS-7 cells and solubilized with 0.25% n-dodecyl maltoside. Approximately 18 µg of hPTH1R were purified to homogeneity per mg of crude membranes by single-step affinity chromatography using 1D4, a monoclonal antibody to a rhodopsin epitope. The N-terminus of the hPTH1R is Y23, consistent with removal of the 22 amino-acid signal peptide. Comparisons of hPTH1R by quantitative immunoblotting and Scatchard analysis revealed that 75% of the receptors in membrane preparations were functional; there was little, if any, loss of functional receptors during purification. The binding affinity of the purified hPTH1R was slightly lower than membrane-embedded hPTH1R (Kd=16.5+/−1.3 vs 11.9+/−1.9 nM) and the purified receptors bound rat [Nle$^{8,21}$,Y$^{34}$] PTH (1-34)-NH$_2$ [PTH (1-34)], and rat [I$^5$,W$^{23}$,Y$^{36}$] PTHrP (5-36)-NH$_2$ with indistinguishable affinity. Maximal displacement of $^{125}$I-PTH (1-34) binding by rat [α-amino isobutylic acid (Aib)$^{1,3}$,Nle$^8$,Q$^{10}$,Har$^{11}$,A$^{12}$,W$^{14}$,R$^{19}$,Y$^{21}$]-PTH(1-21)-NH$_2$ and rat [Aib$^{1,3}$,Q$^{10}$,Har$^{11}$,A$^{12}$,W$^{14}$] PTH (1-14)-NH$_2$ of 80% and 10%, respectively, indicate that both N-terminal and juxtamembrane ligand binding determinants are functional in the purified hPTH1R. Finally, PTH stimulated $[^{35}]$S GTP$\gamma$S incorporation into Go$\delta$s in a time- and dose-dependent manner, when recombinant hPTH1R, Go$\delta$s- and βγ-subunits were reconstituted in phospholipid vesicles. The methods described will enable structural studies of the hPTH1R and they provide an efficient and general technique to purify proteins, particularly those of the class II G protein-coupled receptor family.
Introduction

The parathyroid hormone (PTH)/PTH-related protein receptor (PTH1R) is a member of the class II G protein-coupled receptor (GCPR) family (1). It binds PTH, the major regulator of blood calcium and PTHrP, an autocrine/paracrine factor (1), and an important developmental regulator, particularly of the skeleton (2,3). The molecular mechanisms that underlie PTH1R function are of high interest because of their physiological and pathophysiological importance, and because the PTH1R is a target for drugs to treat osteoporosis. Hyperparathyroidism, a disease due to overproduction of PTH, leads to hypercalcemia and bone loss, but PTH, when administered to both animals and humans at specified doses and schedules, increases bone mass (4-6) and reduces the risk of bone fracture (6). Therefore, PTH is a promising therapy for reversing, not merely halting, bone loss. Secretion of PTHrP by cancers is the most common paraneoplastic syndrome, frequently leading to morbidity and mortality due to hypercalcemia (7-9).

The PTH1R, like all GPCRs, has seven hydrophobic segments that probably form membrane-spanning α-helices. The class II subfamily of GCPRs bind peptide ligands of intermediate length, such as secretin, glucagon, calcitonin, corticotropin-releasing hormone, vasoactive intestinal polypeptide and pituitary adenylyl cyclase-activating polypeptide (PACAP) (10). Ligand binding domains of many of these receptors reside in the both the N-terminal ectodomain and the juxtamembrane region [(11-17), for review see (18)]. Activation of the PTH1R stimulates multiple effectors, including adenylyl cyclase, phospholipase C and phospholipase D (19-21).

Understanding hormone-PTH1R and PTH1R-G protein interactions have relied largely on determination of functional consequences resulting from mutations in either the hormone or the receptor (13,22-36), analysis of receptor fragments after cross-linking to radioiodinated, p-benzoyl-L-phenylalanine-modified ligands (13,37-40),
crystallographic resolution of PTH (41), and NMR of PTH (42-58), PTHrP (59-68), and short segments of the PTH1R [(69,70), for review see (18,71,72)]. These data have been interpreted largely by molecular modeling, based initially on two-dimensional (73), and more recently on three-dimensional resolution (74), of bovine rhodopsin, and on calculations of hydrophobicity to assign membrane-embedded segments. The transmembrane domains of receptors in the class II GPCR family, including the PTH1R, however, have been predicted to differ in their arrangement from those of rhodopsin (75); these likely differences especially highlight the need for structural data. Obtaining detailed biochemical and biophysical information concerning the class II GCRPs has been hindered both by low endogenous expression and by difficulties in purifying biologically active receptors. Several laboratories (76-78) have solubilized functional PTH1Rs, but only very limited purification has been achieved (78). Thus far only the receptor for pituitary adenylyl-cyclase activating polypeptide receptor has been purified in a functionally active state (79). This has allowed recent conformational studies of PACAP (1-21) bound to its receptor by NMR spectroscopy (80), but has yet to provide information concerning structural features of the receptor.

Here, we report the solubilization and purification of the human (h)PTH1R from COS-7 cells in high yield and to apparent homogeneity by single-step affinity chromatography. Purified hPTH1Rs retain high-affinity binding and PTH treatment of the purified receptors promotes incorporation of GTPγS into Gs dose-dependently, when they are reconstituted in phospholipid vesicles with recombinant Gαs- and βγ-subunits.
Experimental Procedures

Peptides and Reagents. Rat [Nle$^{8,21}, Y^{34}$] PTH (1-34)-NH$_2$ [PTH (1-34)], rat [I$^5, W^{23}, Y^{36}$] PTHrP (5-36)-NH$_2$ [PTHrP (5-36)], rat [α-amino isobutylic acid (Aib)$^{1,3}$, Nle$^8$,Q$^{10}$,Har$^{11}$,A$^{12}$,W$^{14}$,R$^{19}$,Y$^{21}$] PTH (1-21)-NH$_2$ [(Aib)-PTH (1-21)], rat [Aib$^{1,3}$,Q$^{10}$,Har$^{11}$,A$^{12}$,W$^{14}$] PTH (1-14)-NH$_2$ [(Aib)-PTH (1-14)] (81), a nine amino-acid peptide of carboxyl-terminus of rhodopsin (TETSQVAPA), and three sequences from the rat PTH1R, YPESKENKDVTGSRRRGRPC, FCNGEVQAEIRKSWSRWTLAL and SGLDEEASGSARPPLQLQEGWETVM were prepared on an Applied Biosystems model 431A peptide synthesizer using F-moc (N-(9-fluorenyl)methoxy-carbonyl protecting group chemistry and trifluoracetic acid-mediated cleavage/protection (MGH Biopolymer Synthesis Facility, Boston, MA). Peptides were purified by HPLC and lyophilized. The purity, identity and stock concentration of each compound was determined by analytical HPLC, matrix-assisted laser desorption/ionization mass spectroscopy and amino acid analysis. Peptides were reconstituted in 10 mM acetic acid and stored at –80°C.

FuGene 6 and $n$-dodecyl maltoside (DM) were purchased from Roche Molecular Biochemicals (Indianapolis, IN), β-D glucopyranosides ($n$-hexyl, septyl, octyl, nonyl, decyl and dodecyl) were purchased from Calbiochem (La Jolla, CA), CHAPS, CHAPSO, digitonin, and sodium cholate were purchased from Sigma (St. Louis, MO), and Na$^{125}$I (2125 Ci/mmol), [35S] GTPγS (1250 Ci/mmol) and [3H] 1,2-dipalmitoyl-sn-glycero-phosphocholine (DPPC, 76 Ci/mmole) were purchased from DuPont-NEN (Boston, MA). 1-palmitoyl-2-oleoyl-sn-glycero-3 phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), and 1,2-dimyristoyl-sn-glycero-phosphate (DMPA) were obtained as chloroform solutions (Avanti Polar Lipids Inc., Alabaster, AL). Other reagents of the highest purity available were obtained from Fisher Scientific International Inc. (Pittsburgh, PA).
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Baculoviral vectors encoding recombinant G proteins, \(\alpha\)-, hexahistidine-tagged \(\alpha\)-, \(\beta\)-, \(\gamma\)- and hexahistidine-tagged \(\gamma\)-subunits, were generous gifts from Dr Henry R. Bourne (University of California, San Francisco, CA).

\textbf{Antibodies.} The monoclonal antibody, 1D4, which recognizes the rhodopsin epitope, TETSQVAPA (82) was purchased from the National Cell Culture Center (Minneapolis, MN). G48, a polyclonal antiserum raised in a sheep, contains antibodies that recognize epitopes within the sequences of the rat PTH\textsubscript{1}R, YPESKENKDVTGSR\textsubscript{RR}GR\textsubscript{PC}, FCN\textsubscript{GEVQAEIRK}SR\textsubscript{WTLAL} and SGL\textsubscript{DEEASGSAR}PPLLQ\textsubscript{E}GW\textsubscript{ETVM}. The first sequence is located in the N-terminal ectodomain and the last two sequences are located in the receptor’s intracellular C-terminal tail. For some experiments recognition of G48 was restricted to epitopes in the C-terminal tail by immuno-depletion using YPESKENKDVTGSR\textsubscript{RR}GR\textsubscript{PC} coupled to CNBr-activated Sepharose 4B, following a protocol provided by the manufacturer.

\textbf{Cell Culture.} COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Cellgro Mediatech Inc., Herndon, VA), supplemented with 10\% of fetal bovine serum (FBS) (HyClone, Logan, UT) at 37\(^\circ\)C in a humidified atmosphere containing 95\% air and 5\% CO\(_2\). Sf9 cells (1.5x10\(^6\) cell/ml) (Invitrogen Life Technologies, Carlsbad, CA) were cultured in 500 or 900 ml of TNM-FH Insect Medium (Pharmingen, San Diego, CA) in 1L or 2L spinner flasks (Kontes, Vineland, NJ) in a humidified atmosphere containing 100\% air at 27\(^\circ\)C.

\textbf{Construction of the hPTH\textsubscript{1}R plasmid.} Mutations were introduced into the hPTH\textsubscript{1}R by site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA). Five amino acids in exon 2 of the hPTH\textsubscript{1}R were replaced with the corresponding amino acids from the rat PTH\textsubscript{1}R (E92K, D94N, E96D, A97V, Y103R) to allow efficient detection by G48 of hPTH\textsubscript{1}Rs expressed on the cell surface. The 27-mer
encoding the epitope (TETSQVAPA) recognized by 1D4, was inserted in-frame immediately upstream of the stop codon at the 3’-end of the hPTH1R sequence. The modified hPTH1R cDNA was subcloned at BamH1 and XbaI sites in a pcDNA3 expression vector (Invitrogen, Carlsbad, CA). The accuracy of the modified receptor construct was confirmed by sequencing the entire receptor (Tuft University, Department of Physiology, Sequencing Core Facility, Boston, MA).

**Transient transfection of COS-7 cell.** All plasmid DNAs were prepared using the EndoFree plasmid Maxi kit (Qiagen Inc., Valencia, CA) and stored at –20˚C in sterile buffer containing 10mM Tris-HCl and 1mM EDTA (pH 7.5). Transfection of COS-7 cells with purified plasmid containing the modified hPTH1R cDNA was performed using FuGene 6 transfection reagents. In brief, 10 µg of DNA in 1 ml of DMEM were mixed with 30 µl of FuGene 6 for 20 min at 21˚C and the mixture was added to 60-75% confluent COS-7 cells in a 15-cm dish. Cells were harvested after 72 h for preparation of membranes. For studies of hPTH1R on intact cells, COS-7 cells were grown in 24-well plates and transfected with 250 ng of cDNA and 0.75 µl of FuGene 6 per well. Cells were studied 72 h after transfection.

**Preparation of the membrane fraction from COS-7 cells.** Confluent monolayers of COS-7 cells, grown in 15 cm dishes, were washed with PBS and harvested by scraping with a Teflon policeman in 5 ml of Buffer A (10 mM Tris-HCl and 4 mM EDTA, pH 7.4), containing proteinase inhibitors (10 µg/ml pepstatin, 2 µg/ml aprotinin, 10 µg/ml chymostatin and 10 µg/ml leupeptin) (Sigma, St. Louis, MO). Then, cell lysates were homogenized by 30 strokes with a Duall tissue grinder (Kontes, Vineland, NJ). The homogenates were centrifuged at 700 x g (Beckman J2-21 centrifuge, JLA rotor, Beckman Instruments, Berkeley, CA) for 10 min at 4˚C to remove nuclei and debris. Supernatants were centrifuged at 40,000 x g for 30 min at 4˚C, the supernatants were
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aspirated, and the membrane-enriched pellets were suspended in Buffer B (50 mM Tris-HCl, 0.15M NaCl, 2 mM CaCl₂, 5 mM KCl, 5mM MgCl₂, 4 mM EDTA, 20% glycerol, pH 7.5) with the same proteinase inhibitors at a protein concentration of 2 mg/ml, and stored at –80°C. Receptors in these crude membrane preparations were stable for at least one month under these conditions.

Solubilization and purification of hPTH1R. The membrane proteins were diluted to a concentration of 1 mg/ml with Buffer B, and then were solubilized by incubation with an equal volume of 0.5% DM in Buffer B (45 min, 4°C). A small amount of insoluble material was separated from the clear supernatant by ultracentrifugation at 100,000 x g for 30 min at 4°C (Beckman L8-55M, 70.1 TI rotor, Beckman Instrument, Berkeley, CA). The supernatant, diluted 2-fold with Buffer B, was added to 1 ml of Sepharose 4B to which 1D4 had been coupled using CNBr, and was placed on a rocker platform for 1.5 h at 4°C. The solution and resin then were transferred to Bio-Rad Econo column (0.7x5 cm, Bio-Rad, Hercules, CA), the resin was washed with 50 ml of Buffer B containing 0.05% DM, and the hPTH1R was eluted by competition with the 9 amino-acid rhodopsin peptide (100 µM, 2 ml of Buffer B containing 0.05% DM).

Quantification of the hPTH1R. Affinity-purified hPTH1R was concentrated approximately 25-fold on Microcon YM-50 filters (Millipore Co., Bedford, MA) by centrifugation at 12,000 x g for 40 min, and hydrolyzed for 24 h at 110°C in 6N HCl containing 0.05% β-mercaptoethanol. The hydrolysate was vacuum dried, re-dissolved in a citrate buffer, and amino acid analysis was performed on a Beckman System 6300 analyzer. Quantification was made after correction for the 9 amino-acid rhodopsin epitope. The same preparation was then used to develop a standard to rapidly assess receptor concentration and recovery by Western blotting. Purified hPTH1R (1.9 µg) was dissolved in 250 µl of buffer B containing 5 mg/ml of bovine serum albumin (BSA) and...
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serially diluted over a range of 1:25 (1.5 ng) to 1:3200 (11.8 pg) in the same buffer. Standards were established by “slot-blotting” 5 µl of each dilution onto nitrocellulose membranes (Bio-Dot SF apparatus, Bio-Rad, Hercules, CA) and stored for future use. Samples of solubilized crude membrane and purified hPTH1R at several dilutions in Buffer B containing 0.05% DM were then applied to the nitrocellulose membranes. The membranes were blocked by treatment with PBS containing 5% dry milk and 0.2% Tween-20, and then were incubated with 1D4 antibody for 1 h at room temperature (RT). A HRP conjugated anti-mouse IgG (1:10,000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) then was added and membranes were developed using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Inc., Boston, MA). The concentration of hPTH1R was determined by interpolation from the intensities of the serially diluted standard.

**Sequence analysis of hPTH1R.** Purified hPTH1R was concentrated 25-fold on Microcon YM-50 membranes and subjected to SDS polyacrylamide (10%) gel electrophoresis. The ~80 kD protein then was electrotransferred to a polyvinylidiene difluoride (PVDF) membrane (Millipore, Bedford, MA) and stained with Ponceau S. The receptor band was excised, sequenced directly using a gas-phase protein sequenator (Applied Biosystems, Model 477A), and the phenylthiohydantoin derivatives of each amino acid were identified.

**Radioreceptor assay.** PTH (1-34) was labeled with $^{125}$I using chloramine T as catalyst, and purified by HPLC as described previously (25,83). Moniodinated $^{125}$I-PTH(1-34) (2,000 Ci/mmol) was used within 3 weeks of labeling. Radioreceptor assays with intact COS-7 cells that express the hPTH1R were conducted as described previously (1,25). To assess binding to membrane-embedded hPTH1R, $^{125}$I-PTH (1-34) (5x10$^4$-10x10$^4$ cpm) and unlabeled peptides were added to 0.1 ml of Buffer B, pH 7.4, with 1mM PMSF, 10
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µg/ml leupeptin and 0.3% BSA. The reaction then was initiated by addition of 2 µg of membrane protein. After a 1-h incubation at 21°C, tubes were centrifuged at 16,000 x g for 4 min at 21°C (Eppendorf centrifuge 5415C, Brinkmann Instrument Co., Westbury, NJ). The pellets were washed once with 200 µl of Buffer B, the tips of the microfuge tubes containing the membranes were excised, and radioactivity was counted in a Wallac 1470 Wizard gamma counter.

Binding to purified receptor was assessed both prior to and after dissociation from the affinity support. The solubilized receptor fraction derived from 80 µg of crude membrane was added to siliconized Eppendorf tubes containing 30 µl of Sepharose 4B to which 1D4 had been coupled by the CNBr method. After incubation (1 h, 4°C), the Sepharose was washed (x3) with 250 µl of Buffer B containing 0.05% DM, 125I-PTH (1-34) (8-10 x 10^4 cpm) and unlabelled peptides were added in 100 µl of buffer B with 10% FBS and 0.05% DM. The reactants were incubated at 21°C for varying periods of time. Separation of bound from free radioligand was achieved by applying samples to 25 mm PVDF filters (0.45 µm, Millipore Co., Bedford, MA), which had been pre-soaked with Buffer B containing 10% FBS before mounting them on a 12-port vacuum manifold (Millipore Co., Bedford, MA). Filters were washed with 3 ml of chilled buffer B (x2), transferred to tubes, and radioactivity counted with a gamma counter.

The affinity of purified hPTH1R also was assessed after elution from the affinity support. Ninety-six well plates pre-coated with protein G by the manufacture (Pierce, Rockford, IL) were reacted with a 1:10 dilution of G48 (100 µl, 16 h, 4°C) that had been immunodepleted of antibodies that recognize the epitope in the hPTH1R N-terminal ectodomain. The wells were then washed with 100 µl of Buffer B (x2), and unreacted sites on the wells were blocked by incubation with Buffer B, which contained 3% heat-inactivated BSA (100 µl, 1 h, RT). After washing the wells with Buffer B (100 µl, x2), a
solution containing purified hPTH1R was added in 50 µl of Buffer B containing 0.05% DM. After 1-h incubation at RT, unlabeled PTH (1-34) at various concentrations (5 µl) and 125I-PTH (1-34) (7-10 x 10^4 cpm, 50 µl) were added in Buffer B containing 20% FBS and 0.05% DM and incubated for an additional hour. The contents of each well were aspirated, the wells were washed with Buffer B (100 µl, x2), and bound radioactivity was recovered by incubation with 1N NaOH (100 µl, 30 min, at RT). The contents of each well were transferred to a glass tube and the radioactivity was counted.

**Measurement of intracellular cAMP and inositol phosphate (IP) accumulation.**

Intracellular cAMP and IP were measured as described previously (19,25).

**Preparation of membrane fraction from Sf9 cells.** Recombinant Gαs-, β1- and γ2-subunits were generated in Sf9 cells and purified by minor modifications of previously described methods (84). The concentration of each G-protein subunit was estimated by Coomassie blue staining using BSA as standard. Purified G-protein subunits were stored in aliquots (10-50 ng/µl) at –80°C.

**Reconstitution of hPTH1R in phospholipid vesicles.** Two mg of phospholipids, POPC, POPE and DMPA at a molar ratio of 6:3:1, were mixed and the chloroform was evaporated overnight in the Speed-Vacuum Concentrator (SVC200H-115) (Savant Instrument Inc., Hicksville, NY), a minor modification of the methods of Mirabekov et al. (85). The lipid was resuspended in buffer B (1 ml) without glycerol, [3H] DPPC (0.05 µCi/) was added, and mixture was sonicated (1 min, Sonifier 450, Branson Ultrasonic Co., Danbury, CT). The sonicated solution was repeatedly passed (x20) through a polycarbonate membrane (100 nm, pore size) mounted on Liposo Fast-Basic extrusion device (Avestin Inc., Ottawa, Canada). The purified, concentrated hPTH1R (7.6 pmol in 5 µl of Buffer B containing 0.05% DM) was mixed with phospholipid vesicles (50 µl of Buffer B containing 0.1% DM), incubated for 30 min at RT. The mixture was then added
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gently to a polyethylene tube containing a discontinuous sucrose gradient (10, 20, 30, and 50%; 400 µl, 400 µl, 400 µl, and 300 µl, respectively). The tubes were centrifuged in SW-60 rotor at 160,000 x g for 16 h at 4°C and incorporation of hPTH1R into phospholipid vesicles was assessed. Samples (120–170 µl) were collected by puncturing the bottom of the tube with a 21-gauge needle. Aliquots from each fraction were counted for [³H], and hPTH1R was assessed by immunoblotting with 1D4 antibody.

Incorporation of GTPγS into Gs after reconstitution with hPTH1R and G protein subunits. Purified hPTH1R (1 pmol) was reconstituted with Gαs- (1.3 pmol) and βγ-subunits (5.3 pmol) in 50 µl of phospholipid vesicles by incubation for 20 min at RT. The mixture was dialyzed against 2 liters of Buffer B for 16 h at 4°C. Dialyzed samples were then mixed with 150 µl of TED/BSA buffer (20 mM Tris-HCl, pH 7.4 with 1% BSA, 5 mM MgCl₂, 1 mM EDTA, 50 nM GDP) without or with various concentrations of PTH (1-34). The reaction was initiated by addition of 2 µl of 100 nM [³S] GTPγS. After incubation at 21°C for 60-90 min, the reaction was terminated by the addition of 500 µl of ice-cold TEM buffer (50 mM Tris-HCl pH 7.4, 5 mM MgCl₂, 0.1 % BSA). The mixture was immediately loaded onto Whatman GF/C glass fiber filters (Whatman Inc, Fairfield, NJ) in the Millipore manifold. The filters were washed with 1 ml of ice-cold TEM buffer (x2), air-dried, and then placed in 5-ml vials with 4 ml of a scintillant (ULTIMA-FLO AF, Packard Instrument Co., Meriden, CT) for 20 min at 21°C with moderate shaking. Filters were counted on a Beckman LS 6000IC (Beckman Instruments, Inc).

Miscellaneous Methods. Protein concentration was measured by intrinsic fluorescence using BSA as standard. Protein was excited at 280 nm and the emission at 350 nm was recorded.
Statistical analysis. Data were calculated from 2-5 independent experiments and expressed as mean +/- S.E. of duplicate or triplicates samples. Statistical significance was determined by ANOVA with Fisher’s PLSD.
Results and Discussion

**Characterization of the modified hPTH1R.** COS-7 cells were transiently transfected with either wild-type hPTH1R or hPTH1R, which had been modified by substitution of five corresponding amino-acids from the rat PTH1R sequence encoded by exon 2 to improve recognition by the G48 antiserum, and by addition of the rhodopsin epitope at the C-terminus. Wild-type and modified hPTH1R were equally well expressed, as assessed by ligand binding (data not shown). The IC$_{50}$s for binding of PTH (1-34), an agonist, to the two receptors were indistinguishable (wild type, 27.3 +/- 7.6 nM vs modified hPTH1R, 28.8 +/- 14.2 nM (n=4)), as were the IC$_{50}$s for binding of PTHrP (5-36), an antagonist (wild type, 41.7 +/- 10.8 nM vs modified hPTH1R, 39.7 +/- 7.5 nM (n=4)) (Fig 1A, B). PTH stimulation of cells expressing wild-type and modified hPTH1R resulted in accumulation of cAMP and generation of inositol phosphates that also were indistinguishable [cAMP (EC$_{50}$)-wild type-0.29 +/- 0.04 nM vs modified hPTH1R-0.29 +/- 0.05 nM, n=2, inositol phosphates (EC$_{50}$)-wild type-43.7 +/- 1.6 nM vs modified hPTH1R-40.3 +/- 2.1 nM, n=2, Fig 1 C, D].

**Solubilization and Purification of the Recombinant hPTH1R.** COS-7 cell membranes expressing modified hPTH1R were suspended in Buffer B and solubilized (45 min, 4°C) by addition of an equal volume of Buffer B, which contained 0.5% DM. This concentration maximized recovery of functionally intact receptors as assessed by binding of $^{125}$I-PTH (1-34) to 1D4-bound receptors and by Western blotting of eluted receptors using 1D4 antibody. Compared with receptors solubilized in 0.25% DM, recovery of receptors was inefficient at a final concentration of 0.125% DM and receptors recovered after solubilization at a final concentration of 0.5% DM did not efficiently bind the
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radioligand (data not shown). Additionally, DM at a final concentration of 0.25% was more efficacious for solubilizing functional receptors that other detergents we assessed—digitonin, CHAPSO, \( n \)-hexyl-, \( n \)-septyl-, \( n \)-octyl-, \( n \)-nonyl- or \( n \)-decyl-\( \beta \)-D-glucopyranoside (data not shown). Fig 2A shows a Coomassie blue stained gel of proteins recovered from the membrane extract and after affinity chromatography; the purified hPTH1R appears as a broad ~80 kD band, free of detectable contaminating proteins.

Recovery of receptor protein and functional receptors was estimated, respectively, by comparing the intensity of the purified receptor band detected by immunoblotting with 1D4 against a serially-diluted standard of hPTH1R that had first been quantified by amino acid analysis and by Scatchard analysis of equilibrium ligand binding. Two preparations of crude COS-7 cell membrane extract, each harvested from twenty 15-cm dishes, contained respectively, 4.85 and 5.50 mg of protein, 1.56 nmol and 1.70 nmol of receptor protein, and 1.21 nmol and 1.30 nmol of functional receptors (Table I). Thus, approximately 75% of the membrane-embedded receptors were functional. DM (0.25%) solubilized 1.44 nmol and 1.50 nmol of receptor protein in the two experiments, or approximately 90%. The eluate after 1D4-affinity-chromatography contained 1.09 nmol and 1.15 nmol of receptor protein (75%), giving an overall receptor recovery of about 69%. Scatchard analysis revealed minimal, if any, loss of functional receptors after solubilization and purification. Human PTH1Rs comprised about 2% of the protein in the crude membrane preparations and were purified about 42-fold, with a resultant increase in specific activity from 0.25 nmol/mg to 10.5 nmol/mg.

Immunoblotting with 1D4 of the SDS-PAGE, after electrotransfer to Immobilon P, showed an apparently homogeneous broad band with a molecular size of ~80 kD, consistent with a glycosylated receptor protein. In some gels, an ~180 kD protein band
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also was detected, regardless of the presence of reducing reagent, which may represent dimers (Fig 2B).

The modified hPTH1R also was expressed in Sf9 cells. However, under a variety of detergent conditions that solubilized as little as 10-15% of the receptors, including conditions used by Ohtaki et al. for the PACAP receptor (79), receptors no longer bound 125I-PTH (1-34) (data not shown).

The SDS-PAGE purified receptor band was electrotransferred to PVDF membrane and subjected to sequence analysis for 10 cycles, which yielded YALVDADDVM. Y23 at the amino-terminus is consistent with algorithms that predict removal of a 22 amino-acid signal peptide from the receptor’s N-terminus. Signal peptide sequences are highly conserved among all cloned PTH1Rs, including opossum, rat, canine, murine, human, porcine and zebrafish, and thus tyrosine is the likely N-terminus in these mature receptors.

Purified and membrane-embedded hPTH1R have similar binding properties. Ligand association to the high concentration of receptors in both membrane-embedded and purified hPTH1R, when still attached to the affinity gel was rapid; 1/2 maximal binding was achieved in less than 3 min at 21°C; equilibrium binding was achieved within 10 min (Fig 3A and B). Ligand dissociation was measured after initially binding 125I-PTH (1-34) to the purified receptors for 1h at RT and then measuring residual binding at various times after addition of 10^-6M PTH (1-34). Dissociation was rapid and biexponential; the half-time of the rapid component was approximately 3 min (Fig 3C). This compares favorably with dissociation of ligand bound to endogenous canine renal PTH1R and also to hPTH1R overexpressed in COS-7 cells after addition of GTPγS, 1-2 min (86) and 5 min (87), respectively. In the absence of added GTPγS, less than half of the PTH radioligand dissociated from endogenous receptors by 200 min (86), which contrasts
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strikingly with a half-time of only 6.5 min when radioligand is bound to exogenously-expressed receptors on COS-7 cells (87).

Equilibrium binding to membrane-embedded receptors had a slightly lower affinity constant, $K_d = 11.9 \pm 1.9 \text{nM}$, specific binding $10.7 \pm 1.4 \%$, $n=8$) compared with binding to immobilized, purified hPTH1R ($K_d = 16.5 \pm 1.3 \text{nM}$, specific binding $18.6 \pm 4.7 \%$, $n=4$) (Fig 4A), and was indistinguishable from the apparent affinity, $K_d = 17 \text{nM}$, of ligand bound to canine renal membranes after treatment with GTP$_\gamma$S. In contrast, the apparent $K_d$ of canine PTH1R in the absence of added GTP$_\gamma$S is 2-5 nM (86,88). The data are consistent with the notion that most of the extraordinarily large number of receptors expressed in COS-7 cells are not coupled to G proteins, and thus are in a low affinity state (87). Because purified receptors are not associated with G proteins, they retain binding properties closely similar to those of membrane-embedded receptors in COS 7 cells.

The affinity of solubilized receptors also was measured after they had been eluted from the affinity gel and added to wells containing immobilized G48, which had been depleted of antibodies that recognize the epitope in the N-terminal ectodomain. Specific binding at equilibrium was $7.6 \pm 0.5 \%$ and the apparent $K_d$ by Scatchard analysis was $69.5 \pm 16.9 \text{nM}$ ($n=3$) (Fig 4B). The higher affinity constant of these receptors may well reflect altered receptor conformation attendant to binding to the immobilized G48 antiserum. We cannot exclude, however, that receptors were slightly denatured upon elution from the 1D4 resin.

**Purified PTH1R have functional N-terminal and juxtamembrane binding domains.**

Binding of PTH and PTHrP to the PTH1R is consistent with a “two-site” model in which the C-terminal portion of the ligands interacts with the N-terminal ectodomain of the
Characterization of Purified, Functional PTH1Receptors

receptor and N-terminal portion of the ligands binds to juxtamembrane domains (13,16,17,37-40,89). We therefore sought to establish the integrity of these apparently distinct binding domains by assessing competition between $^{125}$I-PTH (1-34) and four PTH or PTHrP fragments, PTH (1-34), PTHrP (5-36), Aib-PTH (1-21) and Aib-PTH (1-14), whose binding properties with wild-type hPTH1R has been previously characterized on transfected intact COS-7 cells (89). As with intact cells, PTH (1-34) and PTHrP (5-36) completely displaced the $^{125}$I-PTH (1-34), whereas Aib-PTH (1-21) and Aib-PTH (1-14) maximally displaced 80% and 10% of the ligand, respectively. The IC$_{50}$s of PTH (1-34) and PTHrP (5-36) were nearly identical, 17.0 +/- 2.8 (n=5) and 20.8 +/- 2.0 nM (n=5), respectively, whereas the IC$_{50}$ of Aib-PTH (1-21) was estimated to be ~50 nM (n=5) (Fig 4C). Thus, N-terminal and juxtamembrane binding determinants are both functional in the purified hPTH1R.

*PTH treatment of purified hPTH1R reconstituted in phospholipid vesicles with G proteins stimulates incorporation of $[^{35}S]GTP_\gamma S$ into Gs.* We first determined that 0.1% DM was sufficient to solubilize the phospholipids (data not shown). Purified hPTH1R (7.6 pmol) in Buffer B containing 0.05% DM was then mixed with the solubilized phospholipids containing $[^{3}H]$-DPPC, applied to a discontinuous sucrose gradient, and centrifuged. Fractions including the interface of the 20-30% sucrose layers contained the highest concentration of $[^{3}H]$-DPPC and hPTH1R, as assessed by immunoblotting with 1D4 (Fig. 5A).

Purified hPTH1R (1 pmol), G$\alpha$s-, $\beta$1- and $\gamma$2- subunits (1.3, 5.3, and 5.3 pmol, respectively) were reconstituted into the phospholipids, and dialyzed overnight. PTH (1-34) (1 $\mu$M) treatment increased $[^{35}S]$ GTP$\gamma S$ incorporation into Gs by 4-fold, compared to incorporation in the absence of PTH. Half-maximal incorporation was at 14.4 +/- 1.1 min
Characterization of Purified, Functional PTH1 Receptors

(Fig 5B). Incorporation of $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ by PTH was dose-dependent, with an EC$_{50}$ of 64.3 +/- 3 nM (Fig 5C). The antagonist peptide, PTHrP (5-36) did not increase incorporation of $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ intoGs (data not shown). We were unable to assess ligand binding to hPTH1R in phospholipid vesicles under the conditions tested, because non-specific binding was too high to permit interpretation of the data with confidence.

In summary, we have purified hPTH1R to apparent homogeneity, demonstrated that the signal peptide is removed, leaving Y23 as the N-terminus of the mature receptor. The purified receptors retain ligand-binding properties closely similar to those of membrane-embedded receptors, including functional N-terminal ectodomain and juxtamembrane ligand-interactive domains. PTH-stimulated GTP$^\gamma$S incorporation into G$\alpha$s, when receptors are reconstituted in phospholipid vesicles with recombinant G protein subunits, demonstrates that the purified receptors couple to and activate G$s$. In studies to be published elsewhere, we have replaced all unpaired cysteine residues with functionally neutral amino acids, mutated native amino acids to cysteine one at a time, and have begun to identify structural features of the hPTH1R, using thiol-specific reagents and site-directed spin labeling/electron paramagnetic resonance. Transient expression of hPTH1R mutants in COS-7 cells also yields sufficient receptors to readily enable analysis of protein structure using fluorescent probes. The synthesis of sufficient hPTH1R for crystallographic and NMR studies obviously will require considerable “scale-up” and may best be achieved through recovery of receptors expressed stably in mammalian cells adapted for growth in suspension culture. The use of 1D4-affinity chromatography should efficiently purify other functional class II GPCR as well as other proteins, which have been difficult to purify by more conventional methods.
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Figure legends

Fig. 1. Comparison of PTH binding, and PTH-stimulated intracellular cAMP and inositol phosphate accumulation in transiently transfected COS-7 cells between wild-type hPTH1R (closed triangles) and hPTH1R, which has been modified by 5 amino acid substitutions in exon 2 and addition of the rhodopsin epitope at the C-terminus (open circles). A: binding using $^{125}$I-PTH (1-34) as a tracer and unlabeled PTH (1-34) as a competitor (n=4). B: binding using $^{125}$I-PTH (1-34) as a tracer and unlabeled PTHrP (5-36) as a competitor (n=4). C: PTH (1-34)-stimulated intracellular cAMP accumulation (n=2). D: PTH (1-34)-stimulated intracellular inositol phosphate accumulation (n=2).
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Fig. 2. Solubilization and purification of modified hPTH1R. A: Coomassie blue staining; B: Western blot analysis using 1D4 antibody. Lane 1: crude membrane fraction. Lane 2: solubilized membranes. Lane 3: fraction that did not bind to Sepharose-4B immobilized 1D4 immuno-affinity column. Lane 4: wash fraction. Lane 5: purified receptor eluted by competition with the 9-amino acid rhodopsin peptide. M: molecular size marker. The percentage of the original extract loaded in A, lanes 1-5 was 0.18%, 0.14%, 0.05%, 0.04% and 43%. In B, with respect to the original extract, 0.25% was loaded in each lane.

Fig. 3. Association and dissociation of $^{125}$I-PTH (1-34) binding to membrane-embedded and solubilized hPTH1R. A: association to membrane-embedded hPTH1R. B: association to purified hPTH1R. Total binding (open circle); binding in the presence of 1 µM PTH (1-34) or non-specific binding (closed triangle). C: dissociation of $^{125}$I-PTH (1-34) from purified, modified hPTH1R after addition of 1 µM of PTH (1-34) at time 0.

Fig. 4. Specific binding to hPTH1R using $^{125}$I-PTH (1-34). A: Competition by unlabeled PTH (1-34) to modified hPTH1R in COS-7 cell membranes (open circle, n=8), to purified receptors when immobilized on the 1D4-affinity resin (--X--, n=4) and to hPTH1R after elution (filled square, n=4). B: Scatchard analysis of data in panel A. C: Competition by PTH (1-34) (open circle), PTHrP (5-36) (closed triangle), Aib-PTH (1-21) (closed square) and Aib-PTH (1-14) (X) to modified purified, immobilized hPTH1R (n=5).

Fig. 5. Discontinuous sucrose gradient centrifugation of purified hPTH1R in phospholipid vesicles. A: Quantification of $[^3]$H DPPC (open circles) and immunoblotting for modified hPTH1R with 1D4 antibody (dot blot) in aliquots after
centrifugation. B: Time-dependence of PTH-stimulated GTPγS incorporation into Gs in phospholipid vesicles with reconstituted hPTH1R and Gα- and βγ-subunits [with 1 μM PTH (1-34) (open circles) and without PTH (1-34) (filled triangles) (n=3)]. C: Dose-dependent stimulated of GTPγS incorporation into Gs by PTH (1-34) in phospholipid vesicles with reconstituted modified hPTH1R and Gα- and βγ-subunits (n=3).

Table I. *Purification of the modified hPTH1R from twenty 15-cm plates of COS-7 cells.*

Results are from two different batches of cells.
<table>
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<th>Crude membrane</th>
<th>Solubilized receptors</th>
<th>Solubilized, purified receptors</th>
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<tr>
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Purification and characterization of a receptor for human parathyroid hormone and parathyroid hormone-related peptide
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