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Research Article

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Cloning, Characterization, and Expression of a Human Calcitonin Receptor from an Ovarian Carcinoma Cell Line

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Abstract

A human ovarian small cell carcinoma line (BIN-67) expresses abundant calcitonin (CT) receptors (CTR) (143,000 per cell) that are coupled, to adenylate cyclase. The dissociation constants (K_d) for the CTRs on these BIN-67 cells is ~ 0.42 nM for salmon CT and ~ 4.6 nM for human CT. To clone a human CTR (hCTR), a BIN-67 cDNA library was screened using a cDNA probe from a porcine renal CTR (pCTR) that we recently cloned. One positive clone of 3,588 bp was identified. Transfection of this cDNA into COS cells resulted in expression of receptors with high affinity for salmon CT ($K_d = \sim 0.44$ nM) and for human CT ($K_d = \sim 5.4$ nM). The expressed hCTR was coupled to adenylate cyclase. Northern analysis with the hCTR cDNA probe indicated a single transcript of ~ 4.2 kb. The cloned cDNA encodes a putative peptide of 490 amino acids with seven potential transmembrane domains. The amino acid sequence of the hCTR is 73% identical to the pCTR, although the hCTR contains an insert of 16 amino acids between transmembrane domain I and II. The structural differences may account for observed differences in binding affinity between the porcine renal and human ovarian CTRs. The CTRs are closely related to the receptors for parathyroid hormone-related peptide and secretin; these receptors comprise a distinct family of G protein-coupled seven transmembrane domain receptors. Interestingly, the hCTR sequence is remotely related to the cAMP receptor of *Dictyostelium discoideum* (21% identical), but is not significantly related to other G protein-coupled receptor sequences now in the data bases. (*J. Clin. Invest.* 1992. 90:1726–1735.) **Key words:** osteoclast • cAMP • G protein • giant cell tumor of bone • parathyroid hormone receptor • secretin receptor

Introduction

Calcitonin (CT)¹ is a peptide hormone comprising 32 amino acids first identified as a hypocalcemic factor secreted by the

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1. Abbreviations used in this paper: CT, calcitonin; CTR, CT receptor; hCTR, human CTR; pCTR, porcine CTR; CGRP, CT gene-related

parafollicular cells of the thyroid gland in response to elevations in serum calcium levels (1). The hypocalcemic effect of CT is mediated predominantly by direct inhibition of osteoclast-mediated bone resorption (2, 3), although CT also enhances renal calcium excretion (3, 4). High affinity CT binding has been demonstrated in bone and kidney as well as other tissues, such as the central nervous system (5), testes and spermatozoa (6, 7), placenta (8), and lung (9). The presence of calcitonin receptors (CTR) has also been reported in cells derived from lung (10) and breast (11) carcinomas, as well as certain lymphoid (12) and myeloid cell lines (13). Although the physiological role of CT in many of these tissues has not been established, its actions clearly extend beyond calcium homeostasis (14).

The unique ability of CT to inhibit osteoclast-mediated bone resorption has led to its use in the treatment of disorders of bone remodeling, including osteoporosis, Paget's disease of bone, and some forms of hypercalcemia of malignancy. In addition, CT has been used to treat pancreatitis (15) and peptic ulcer disease (16) and to produce centrally mediated analgesia (17). It is possible that some of the pharmacological effects of CT may be indirect and attributable to the cross-reaction of CT with receptors for other hormones that are structurally similar, such as α - or β -CT gene-related peptide (CGRP) (18, 19) or amylin (20). α -CGRP is a product of the CT gene produced by differential RNA slicing (21). β -CGRP is a product of a separate gene but differs from α -CGRP in only 3 of the 37 amino acids (21–23). These related ligands most likely interact primarily with their own high affinity receptors to produce hormone-specific effects, but at very high concentrations may also cross-react with the receptors for the other peptides (18–20).

We recently used an expression strategy to clone the cDNA for a CTR from LLC-PK₁ cells, a porcine renal epithelial cell line (24). Transfection of the cDNA into COS cells resulted in expression of high affinity CTRs that were functionally coupled to adenylate cyclase. Although previous studies (25–27) indicated that the effects of CT in most tissues are mediated via coupling to guanine nucleotide regulatory proteins (G proteins), analysis of the deduced amino acid sequence of the cloned porcine CTR (pCTR) revealed an unusual structure that was not similar to the sequences of other G protein-coupled receptors (receptors that transduce signals via G proteins and span the membrane seven times) available in the data bank at that time (24). Subsequently, comparison of the pCTR with the recently cloned receptors for parathyroid hormone-

peptide; PTHrp, PTH-related peptide; CAR, *Dictyostelium discoideum* cAMP receptor; bROD, bovine rhodopsin receptor; β AR, hamster β -adrenergic receptor; PAM, accepted point mutation.

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parathyroid hormone-related peptide (PTH-PTHrp) and secretin revealed an unexpected similarity in amino acid sequence and predicted receptor structure (24, 28, 29). The unique structure of these related receptors suggests that they may represent examples of a new family of peptide-binding G protein-coupled receptors.

In this report we describe the cloning of a human CTR (hCTR) cDNA from a eukaryotic expression library prepared from an ovarian small cell carcinoma cell line (BIN-67). The BIN-67 cells had been shown to respond to CT with increases in content of cellular cAMP (30). We also describe the binding of human and salmon CTs to the receptor on these cells and on COS cells transfected with the cloned human ovarian CTR cDNA. The functional analysis of the cloned hCTR after expression in COS cells confirms that its binding kinetics differ from those of the porcine renal receptor. Analysis of the deduced amino acid sequence predicted from the hCTR cDNA and comparison to the pCTR sequence provides potential insights into the structural basis for these differences. Comparison of the hCTR receptor sequence to protein sequences in the available data bases suggests that the receptors for CT may be evolutionarily related to a chemoattractant receptor from the primitive eukaryote, *Dictyostelium discoideum* (31).

Methods

Cultured cells and tissue. The BIN-67 cell line was isolated from a trypsin digest of a metastatic pelvic nodule derived from a primary ovarian small cell carcinoma, a rare tumor composed of poorly differentiated cells of uncertain developmental origin (30, 32, 33). The cultured cell line preserves the mixed character of the primary tumor with both large and small cell components. The BIN-67 cells respond to CT with increases in cAMP content (30). These cells were maintained from frozen stock and cultured in DMEM supplemented with 20% (vol/vol) fetal calf serum (Sigma Chemical Co., St. Louis, MO) and enriched with 20% Ham's F12 medium (Sigma Chemical Co.), as previously described (30). LLC-PK₁ cells were maintained from frozen stock of those strains originally described to be CT responsive (34). T47D cells, a line derived from a human breast carcinoma that expresses CTRs (35, 36), were obtained from the American Type Culture Collection, Rockville, MD. Fresh specimens of human giant cell tumor of bone (37) were provided by Dr. H. J. Mankin, Massachusetts General Hospital, Boston, MA. COS-M6 cells, a subclone of COS-M7 cells, were obtained from Dr. Brian Seed, Massachusetts General Hospital.

Cloning of a hCTR cDNA. A size-fractionated cDNA library with inserts > 2 kb in length consisting of $\sim 17 \times 10^6$ recombinants was constructed from BIN-67 cells. Polyadenylated RNA was prepared by the proteinase K/SDS method (38) and oligo-dt cellulose chromatography (Collaborative Research Inc., Bedford, MA). The mRNA was converted to double-stranded cDNA (39) and size fractionated over a potassium acetate gradient (5%:20%). The cDNA was ligated into the plasmid eukaryotic expression vector pcDNA-1 (Invitrogen, San Diego, CA) and an aliquot was electroporated into MC1061/P3 *Escherichia coli* using a gene pulser (Bio-Rad Laboratories, Richmond, CA) (pulse conditions: 200 Ω , 2.5 kV, and 2.5 μ F) in 0.2-cm gap cuvettes (Bio-Rad Laboratories). Bacteria were diluted and grown on 15-cm diameter selective agar plates and two nylon filters (ICN Nutritional Biochemicals, Cleveland, OH) were sequentially imprinted with plasmid-containing clones by placing them in contact with the bacterial colonies on the agar. The imprinted filters were screened by colony hybridization as follows. The filters were placed face up in 5% SDS with 2 \times standard saline citrate (SSC) and then transferred to a microwave oven (650 W for 2.5 min) followed by washes in 5 \times SSC/0.1% SDS. After prehybridization (40), a radiolabeled cDNA probe was prepared

from a $\sim 1,100$ bp NarI and XmaI digested restriction fragment from the pCTR open reading frame (24) using the Klenow fragment of DNA polymerase I (Pharmacia, Uppsala, Sweden), in the presence of random hexamer primers and [α -³²P]dCTP (New England Nuclear/Dupont, Boston, MA). Hybridization to this probe was carried out at 42°C in a 40% formamide solution according to established procedures (40). The filters were washed briefly in 2 \times SSC, 0.2% SDS followed by extensive washing in 0.5 \times SSC, 0.2% SDS at 52°C with multiple buffer changes. Autoradiography was performed using film (XAR-5; Eastman Kodak Co., Rochester, NY) exposed for 12 to 72 h at -70°C with an intensifying screen. Colonies that hybridized with the labeled restriction fragment probe on both filters from a matching filter pair were isolated from the original agar plate and the cloned plasmid DNA isolated from an overnight growth using alkaline lysis plasmid preparation procedures (40). Positive clones were verified by an additional Southern hybridization of the cloned cDNA inserts after restriction enzyme digestion of plasmid polycloning sites and agarose electrophoresis (40). The cDNA from the positive clone was then chosen for transient expression in COS cells, and the transfected cells evaluated for radioligand binding to ¹²⁵I-labeled salmon CT.

Sequencing and analysis of the CTR cDNA. Restriction fragments of a positive cDNA clone were subcloned into M13 phage vectors mp18 and mp19 (Boehringer Mannheim Biochemicals, Indianapolis, IN) and sequenced using both strands of template by the dideoxynucleotide chain termination procedure with modified T7 DNA polymerase (Sequence kit; United States Biochemical Corp., Cleveland, OH). Double-stranded template sequencing using the pcDNA-1/hCTR cDNA as a template was also used in some reactions. Oligonucleotides complementary to sequenced DNA were synthesized for use as sequencing primers.

The hCTR sequence was analyzed using computer programs in version 7.0 of the University of Wisconsin Genetics Computer Group (UWGCG) (41). The program BESTFIT was used for pairwise comparisons and the generation of randomized control comparisons using the RAND qualifier. The assigned BESTFIT parameter for Gap Weight was 3.0 and for Length Weight was 0.1. Similar amino acids were determined from the BESTFIT program assignments. Multiple sequence comparisons employed the program PILEUP. The sequences were then manually aligned using the putative transmembrane domains and conserved residues that characterize G protein-coupled receptors (31, 42, 43) as parse points (44). Accepted point mutation (PAM) values were derived from the final alignments and the tables of Dayhoff (45). PAM values reflect the total number of amino acid interchanges (some superimposed) that are necessary to produce the observed difference in sequence. The BLAST network service statistical analysis program (46) was used to compare the hCTR peptide sequence to other sequences in the database.

Transfection of COS-M6 (COS-7 subclone) with the hCTR cDNA. "Miniprep" plasmid DNA prepared by alkaline lysis or "maxiprep" plasmid DNA purified by cesium chloride banding (40) was used to transfect COS-M6 cells grown in 10-cm plastic dishes (Falcon Plastics, Lincoln Park, NJ) using the DEAE-Dextran/chloroquine procedure as previously described (47). CT binding or cAMP responses were measured 48 h after transfection.

Binding of radiolabeled salmon and human CT to cultured cells. Radioligand binding assays were performed in triplicate as follows. Cells were grown in 10-cm diameter plastic tissue culture dishes (Falcon Plastics), as described above. Before assay, cells were washed, trypsinized, and counted using an automated cell counter (Coulter Electronics, Inc., Hialeah, FL) and aliquot portions of 5×10^5 cells were placed into 12 \times 75 mm glass tubes in a volume of 200 μ l of binding buffer (PBS, pH 7.4, 11 mM glucose, 1% bovine serum albumin) plus 200 pmol of either ¹²⁵I-salmon CT (Peninsula Laboratories, Inc., Belmont, CA) or ¹²⁵I-human CT (Amersham Corporation, Arlington Heights, IL) in the presence of appropriate amounts of unlabeled ligand (Peninsula Laboratories, Inc.). Incubation time was from 14 to 16 h at 4°C. The cells were washed by layering 100 μ l of cell suspension

over 200 μ l of 10% sucrose (wt/vol) in a minimicrofuge tube (Bio-Rad Laboratories) and spinning at maximum speed for 5 min to pellet the cells. The sucrose and incubation buffer was then removed by aspiration and the tip of the centrifuge tube containing only the cell pellet was cut off and assayed for radioactivity in a gamma scintillation counter (TM Analytic Inc., Elk Grove Village, IL). Ligand binding to COS-M6 cells transfected with the hCTR was performed using the same technique.

cAMP assay. BIN-67 cells or COS-M6 cells were passaged in 10-cm diameter plastic culture dishes and 48 h before cAMP assay the COS-M6 cells were transfected with either hCTR cDNA or with β -galactosidase cDNA (control). After 24 h the transfected COS-M6 cells or the BIN-67 cells were trypsinized and transferred to 24-well plastic trays (Falcon Plastics) at an initial plating density of 2×10^5 cells per well. To test for hormone-induced cAMP responses, cells in triplicate wells were washed in PBS with Ca^{2+} and Mg^{2+} and incubated for 20 min at 37°C with either test buffer alone (PBS with Ca, Mg, and 0.2% bovine serum albumin, 11 mM glucose, and 1 mM 3-isobutyl-1-methyl-xanthine [IBMX]) or with 4 mM isoproterenol or appropriate concentrations of peptide hormone in test buffer. Reactions were stopped by transferring the culture plates to a water bath at 100°C and evaporating to dryness. 1 ml of 50 mM sodium acetate buffer, pH 6.2, was added to each well and the contents were then scraped with a plastic spatula and transferred to a glass tube for centrifugation (500 g for 10 min). Aliquot portions of each supernatant were assayed for cAMP using a radioimmunoassay kit (cAMP [^{125}I] radioimmunoassay kit, New England Nuclear/Dupont).

Northern blot analysis. Polyadenylated RNA prepared from BIN-67 cells (5 μ g), T47D cells (5 μ g), human giant cell tumor of bone tissue (5 μ g), and LLC-PK₁ cells (1 μ g) was electrophoresed on a 1% agarose gel containing formaldehyde and transferred by capillary action using 10 \times SSC to a supported nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH) that was then heated for 90 min at 80°C under vacuum. After prehybridization (40), the blots were hybridized overnight at 42°C in a 40% formamide solution containing a 950-bp probe prepared by digestion of the hCTR cDNA with SacI followed by labeling with [^{32}P]dCTP (New England Nuclear/Dupont) by the random hexamer primer method. The blots were washed two times with 2 \times SSC, 0.2% SDS at room temperature followed by four 20-min washes with 0.2 \times SSC, 0.2% SDS at 60°C. Autoradiography was performed with Kodak X-AR film exposed for 24 to 72 h at -70°C.

Results

Characterization of [^{125}I]-salmon and human CT binding and cAMP responses in BIN-67 cells. Scatchard analysis of binding of radiolabeled salmon CT is consistent with a single class of high affinity CT-binding sites with a $K_d \sim 0.42$ nM (Fig. 1 A) and an average number of specific binding sites per cell of 143,000. Scatchard analysis of binding of radiolabeled human CT is consistent with a single class of receptors with a K_d of ~ 4.6 nM, 10-fold higher than that of salmon CT (Fig. 1 B).

Measurement of hormone binding using competition-dissociation analysis after incubation of BIN-67 cells with ^{125}I -human CT in the presence of increasing concentrations of unlabeled salmon CT revealed an apparent 50% inhibitory concentration (IC_{50}) of ~ 0.6 – 0.7 nM (Fig. 2 A). Parallel assays using ^{125}I human CT with increasing concentrations of unlabeled human CT confirmed the lower affinity for human CT ($\text{IC}_{50} \sim 3$ – 7 nM), ~ 0.1 – 0.2 that of salmon CT (Fig. 2 A). In other experiments (not shown) the peptide hormones secretin and PTH did not displace radiolabeled salmon CT or human CT binding even at concentrations up to 10 μM . Calcitonin bind-

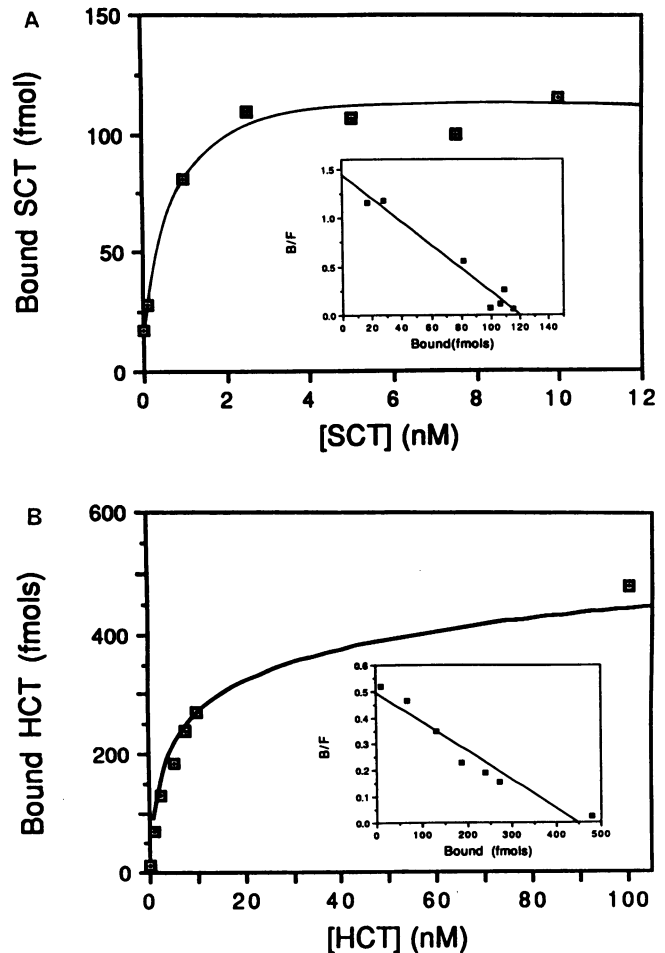


Figure 1. Salmon and human CT binding to native BIN-67 cells. (A) Salmon CT (SCT) binding to BIN-67 cells. Maximal binding averaged 1.89×10^4 cpm per aliquot. Binding in the presence of 1 μM unlabeled salmon CT averaged 289 cpm per aliquot. Calculated K_d by Scatchard analysis (inset) was ~ 0.42 nM with an average of 143,000 receptors per cell. (B) Human CT (HCT) binding to BIN-67 cells. Maximal binding averaged 1.92×10^4 cpm per aliquot. Binding in the presence of 1 μM human CT averaged 1.19×10^3 cpm per aliquot. Calculated K_d by Scatchard analysis (inset) was ~ 4.6 nM.

ing sites were saturable; maximal binding at 4°C was observed at ~ 12 h (data not shown).

As shown in Fig. 2 B, there was a concentration-dependent increase in cAMP levels in BIN-67 cells in response to salmon CT or human CT. The 50% maximal effective concentration (EC_{50}) for salmon CT was ~ 0.7 nM and for human CT was ~ 3 nM. These data are consistent with the results of the binding studies.

Cloning of the BIN-67 ovarian carcinoma hCTR cDNA. A ^{32}P -labeled probe prepared from a restriction fragment of the pCTR cDNA was used to screen a size-fractionated BIN-67 cell cDNA library by colony hybridization. Approximately 55,000 colonies were transferred to nylon filters and screened to yield one positive clone that contained an insert of 3,588 bp. The functional characteristics of this putative hCTR cDNA clone were then examined after DEAE-Dextran/chloroquine transfection into COS-M6 cells that had previously been shown not to bind CT (24).

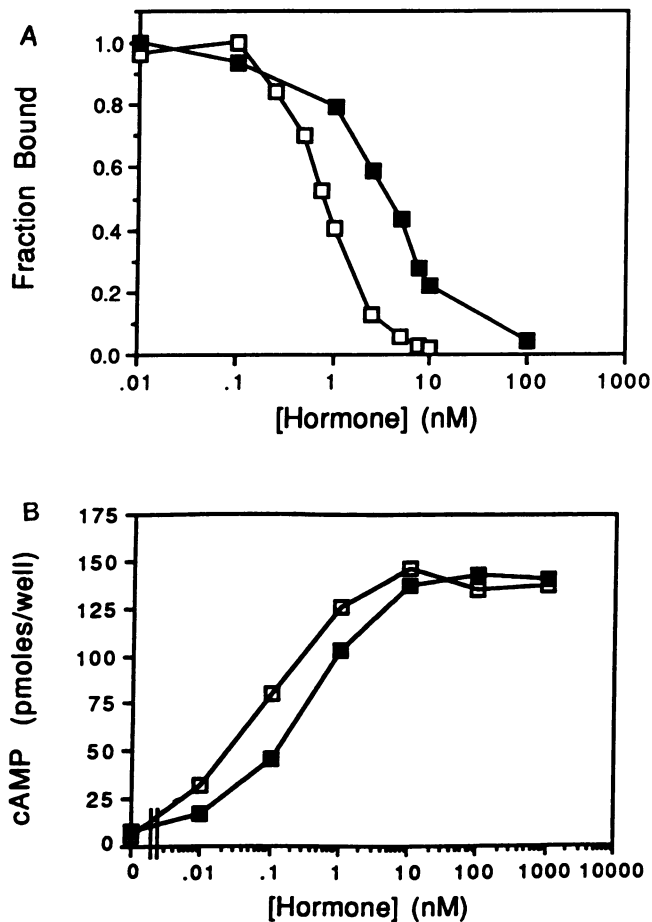


Figure 2. Salmon and human CT competition-dissociation binding curves and cAMP responses in BIN-67 cells. (A) Competition-dissociation binding curves for ^{125}I -human CT competed with unlabeled human CT (\blacksquare) or with unlabeled salmon CT (\square). (B) Cellular content of cAMP in BIN-67 cells incubated with salmon CT (\square) or human CT (\blacksquare). Note the greater potency of salmon CT.

Characterization of [^{125}I]CT binding to COS-M6 cells transfected with the hCTR cDNA. COS-M6 cells were transfected with the putative hCTR cDNA and binding of ^{125}I -salmon CT or ^{125}I -human CT assayed. Scatchard analysis of CT binding is consistent with the presence of a single class of high affinity CT-binding sites (Fig. 3). Assuming 10% transfection efficiency (on the basis of previous estimations with the pCTR [24]), the receptor number per transfected cell is $\sim 1.4 \times 10^6$. The apparent K_d for salmon CT in the transfected COS cells is ~ 0.44 nM (Fig. 3 A), similar to the K_d for salmon CT in the native BIN-67 cells (~ 0.42 nM, Fig. 1 A). The K_d for human CT of the expressed hCTR is ~ 5.4 nM (Fig. 3 B), similar to that in the native BIN-67 cells (~ 4.6 nM, Fig. 1 B), and ~ 10 times that for salmon CT. PTH and secretin did not compete for binding of ^{125}I -salmon CT or ^{125}I -human CT in COS cells transfected with the hCTR cDNA, indicating the specificity of binding to CT (data not shown). Competition-dissociation binding curves for ^{125}I -salmon CT competed with unlabeled salmon CT in COS cells transfected with either the hCTR cDNA or the pCTR cDNA (24) confirm the relative lower affinity of the pCTR ($\text{IC}_{50} \sim 1.5$ nM) compared with the hCTR ($\text{IC}_{50} \sim 0.3$ nM) (Fig. 3 C). The K_d values for the hCTR

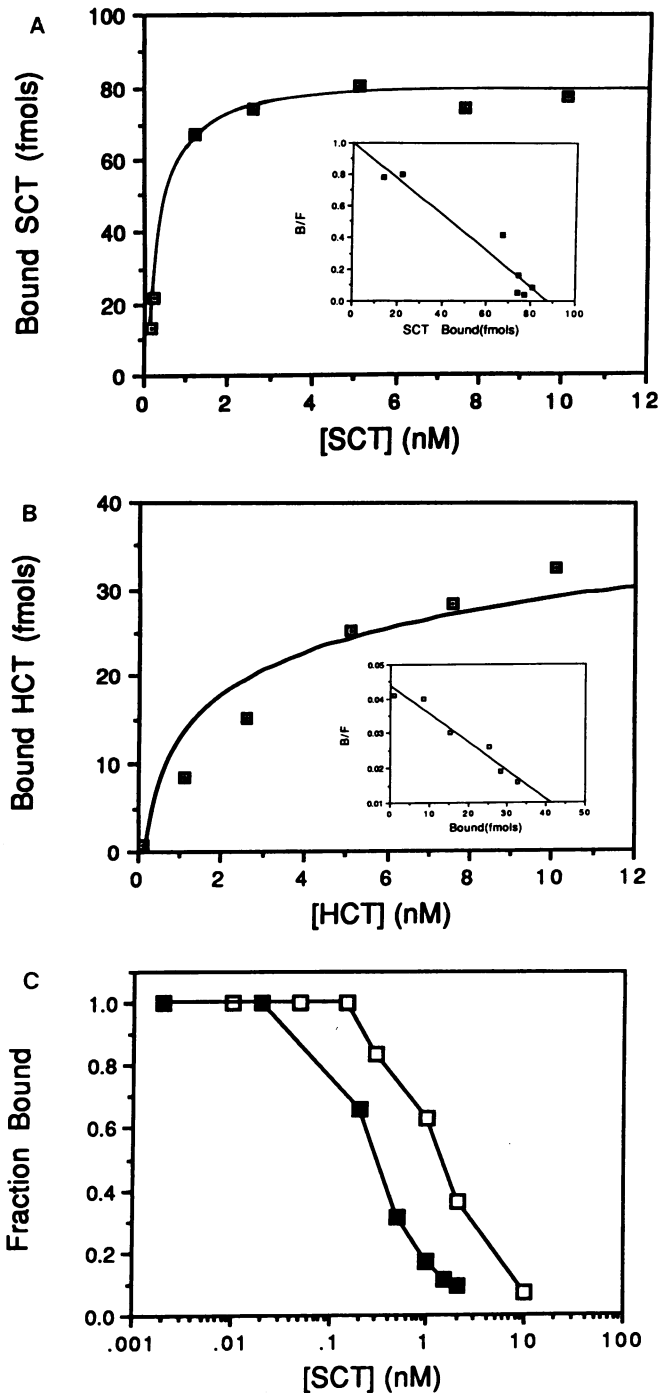


Figure 3. CT binding to COS cells transfected with the human and porcine CTRs. (A) Salmon CT binding to COS cells transfected with the hCTR cDNA clone. Maximal binding averaged 1.47×10^4 cpm per aliquot. Binding in the presence of $1 \mu\text{M}$ unlabeled salmon CT averaged 260 cpm per aliquot. Calculated K_d by Scatchard analysis (insert) was ~ 0.44 nM. (B) Human CT binding to COS cells transfected with the hCTR cDNA clone. Maximal binding averaged 3.62×10^3 cpm per aliquot. Binding in the presence of $1 \mu\text{M}$ unlabeled salmon CT averaged 833 cpm per aliquot. Calculated K_d by Scatchard analysis (insert) was ~ 5.4 nM. (C) Competition-dissociation binding curves for ^{125}I -salmon CT competed with unlabeled salmon CT in COS cells transfected with the hCTR cDNA clone (\blacksquare) or the pCTR cDNA clone (\square) (24). Note the lower affinity of the pCTR compared with the hCTR. Data represent the means of triplicate measurements and are representative of two separate experiments.

(Fig. 3 A and B) and the pCTR (~ 6 nM for salmon CT; reference 24) were calculated by Scatchard analysis.

Characterization of hormone-induced cAMP responses in COS-M6 cells transfected with the hCTR cDNA. To determine if the hCTR cDNA encodes a CT-binding protein that could be coupled to adenylate cyclase, COS-M6 cells were transfected with the hCTR cDNA and then incubated with CT for 20 min in the presence of the phosphodiesterase inhibitor, IBMX. As shown in Fig. 4 B, there was an approximate fourfold increase in cAMP levels in cells transfected with the hCTR that were incubated with maximal stimulatory concentrations of salmon CT (range: ~ 1.6 – 4.3 -fold in five experiments). There was no increase in cAMP levels in COS-M6 cells mock-transfected with a β -galactosidase cDNA that were incubated with salmon CT (Fig. 4 A). PTH did not alter cAMP levels in the hCTR-transfected COS cells (Fig. 4 B). As expected, isoproterenol, an agonist of the β -adrenergic receptor, increased cAMP levels in the β -galactosidase cDNA-transfected (control) (Fig. 4 A) as well as the hCTR-transfected COS cells (not shown).

Analysis of the CTR cDNA predicted amino acid sequence. Analysis of the 3,588-bp sequence of the hCTR cDNA (Fig. 5) reveals an open reading frame beginning at position 250 that encodes a putative peptide of 490 amino acids. Comparison of this deduced amino acid sequence to that of the pCTR reveals 73% identity and 89% similarity. The hCTR is eight amino acids longer than the pCTR. The hCTR cDNA contains an

additional in-frame AUG at position 195, 55 bp upstream from the assigned start site. Both AUG codons have an A at the minus 3 position consistent with and sufficient for a consensus start site, although neither fits the strict consensus CC (A,G) CC AUG G (48) established for translational initiation. The NH_2 -terminal domain encoded by the assigned start site includes a hydrophobic domain flanked by polar regions consistent with the general outline of a signal peptide (49). The most likely cleavage site for this putative signal peptide is between residues 22 and 23 (49). The assignment of the hCTR cDNA start site to the downstream AUG at position 250 is strongly supported by the alignment of identical and similar amino acid sequences, including a homologous NH_2 -terminal hydrophobic sequence, encoded by the open reading frame of the pCTR cDNA (24). The potential upstream start site is not conserved in the pCTR cDNA, which possesses an in-frame stop 27 nucleotides upstream from the AUG.

The deduced structure of the hCTR has many of the features exhibited by the pCTR. A hydropathy plot (not shown) (50) of the hCTR indicates the presence of seven hydrophobic regions flanked by several charged residues consistent with models for multiple membrane-spanning domains (51). The 22-residue putative signal sequence precedes an extracellular domain of 124 amino acids that contains four potential *N*-linked glycosylation sites. Both hCTR and pCTR contain an unusual alanine-rich hydrophobic sequence near the COOH terminus. This sequence in the hCTR (amino acids 442–451) is shorter than in the pCTR (amino acids 423–439) and is not long enough to form a membrane-spanning domain (51). Both CTRs possess an unusually short cytosolic loop between helices V and VI. In other G protein-coupled receptors this region is thought to couple to G_{α} .

A major area of divergence between the human and porcine CTRs is in the intracellular loop between the first and second transmembrane hydrophobic domains where the hCTR contains an inserted sequence of 16 consecutive amino acids not found in the pCTR (amino acids 176–191 in the hCTR). This insert provides the hCTR with a longer intracellular loop between the first and second predicted transmembrane helices.

Searches of nucleic acid data banks (Genbank and European Molecular Biology Laboratory) and protein sequence databases (Genbank Translated Databases, PIR, and Swiss-Prot) identified the rat secretin receptor and the recently cloned PTH-PTHrp receptor as the only published sequences exhibiting high percentages of identity to both the hCTR and pCTR. A statistical analysis, using the BLAST network service to compare the hCTR with the other sequences in the database, also identified one distantly related protein possessing $\sim 21\%$ identity and $\sim 47\%$ similarity. This protein is the cAMP receptor (CAR) from the slime mold, *D. discoideum*, which gave a Highest Scoring Hit Extension of 73 histogram units ($P = 0.024$), compared to 163 units ($P = 1.8 \times 10^{-16}$) for the secretin receptor. CAR binds cAMP, which acts as a chemoattractant to induce aggregation and differentiation of individual *D. discoideum* cells into a primitive multicellular organism (31). CAR is $\sim 19\%$ identical to the PTH-PTHrp and secretin receptors. In contrast, the hCTR is 34% identical (58% similar) to the PTH-PTHrp receptor and 30% identical (54% similar) to the secretin receptor. The PTH-PTHrp and secretin receptors are even more closely related with $\sim 43\%$ identity (see Fig. 6). In comparison, the overall sequence identity among the receptors for CT, PTH-PTHrp, and secretin is appreciably

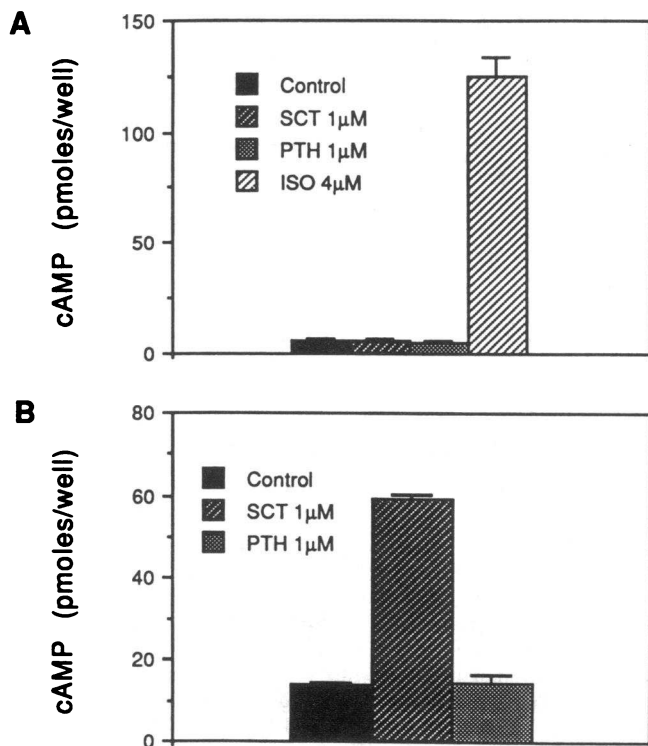


Figure 4. cAMP responses in COS cells transfected with the hCTR cDNA. (A) Mock (β -galactosidase)-transfected COS cells (Control). No change in cAMP content in response to salmon CT or PTH was detected, although the expected response to isoproterenol, an agonist of the β -adrenergic receptor, was observed. (B) hCTR-transfected COS cells. cAMP responses ranged from ~ 1.6 - to 4.3 -fold in five separate experiments. No response to PTH was detected. Values represent the mean \pm SEM for three separate wells.

TCGCCACTCCACCTGGGCTGCATCTCTCCGCCAGAGATGGGGCTGAACTTTGAGGGCTGAGTCCCTGGAGCCACACCTGGACACCTTTGCTTCTATTGAGCTGTGCCAG 128
CCGCCAGTGCAGATTCAGAAZAAAGATTCOCACACTGCCACCCACTTTTGCCACCCAGGATGCAATTTTCGGAGAGAGATZAGTGGCAAGAGATCTTCAAAAATCAAAA 247

ATG AGG TTC ACA TTT ACA AGC OGG TGC TTG GCA CTG TTT CTT CTT CEA AAT CAC CCA ACC CCA ATT CTT OCT GCC TTT TCA AAT CAA ACC 337
Met Arg Phe Thr Phe Thr Ser Arg Cys Leu Ala Leu Phe Leu Leu Leu Leu Asn His Pro Thr Pro Ile Leu Pro Ala Phe Ser Asn Gln Thr 30



TAT CCA ACA ATA GAG OCC AAG CCA TTT CTT TAC GTC GEA GGA CGA AAG AAG ATG ATG GAT GCA CAG TAC AAA TGC TAT GAC CGA ATG CAG 427
Tyr Pro Thr Ile Glu Pro Lys Pro Phe Leu Tyr Val Val Gly Arg Lys Lys Met Met Asp Ala Gln Tyr Lys Cys Tyr Asp Arg Met Gln 60



CAG TTA CCC GCA TAC CAA GGA GAA GGT CCA TAT TGC AAT CGC ACC TGG GAT GGA TGG CTG TGC TGG GAT GAC ACA CCG GCT GGA GEA TTG 517
Gln Leu Pro Ala Tyr Gln Gly Glu Gly Pro Tyr Cys Asn Arg Thr Trp Asp Gly Trp Leu Cys Trp Asp Asp Thr Pro Ala Gly Val Leu 90



TCC TAT CAG TTC TGC CCA GAT TAT TTT CCG GAT TTT GAT CCA TCA GAA AAG GTT ACA AAA TAC TGT GAT GAA AAA GGT GTT TGG TTT AAA 607
Ser Tyr Gln Phe Cys Pro Asp Tyr Phe Pro Asp Phe Asp Pro Ser Glu Lys Val Thr Lys Tyr Cys Asp Glu Lys Gly Val Trp Phe Lys 120



CAT CCT GAA AAC AAT CGA ACC TGG TCC AAC TAT ACT ATG TGC AAT GCT TTC ACT OCT GAG AAA CTG AAG AAT GCA TAT GTT CTG TAC TAT 697
His Pro Glu Asn Asn Arg Thr Trp Ser Asn Tyr Thr Met Cys Asn Ala Phe Thr Pro Glu Lys Leu Lys Asn Ala Tyr Val Leu Tyr Tyr 120



TTG GCT ATT GFG GGT CAT TCT TTG TCA ATT TTC ACC CEA GTG ATT TCC CTG GGG ATT TTC GTG TTT TTC AGA AAA TTG ACA ACT ATT TTT 787
Leu Ala Ile Val Gly His Ser Leu Ser Ile Phe Thr Leu Val Ile Ser Leu Gly Ile Phe Val Phe Phe Arg Lys Leu Thr Thr Ile Phe 180

I

OCT TTG AAT TGG AAA TAT AGG AAG GCA TTG AGC CTT GCC TGC CAA AGG GEA ACC CTG CAC AAG AAC ATG TTT CTT ACT TAC ATT CTG AAT 877
Pro Leu Asn Trp Lys Tyr Arg Lys Ala Leu Ser Leu Gly Cys Gln Arg Val Thr Leu His Lys Asn Met Phe Leu Thr Tyr Ile Leu Asn 210

TCT ATG ATT ATC ATC ATC CAC CTG GTT GAA GEA GEA CCC AAT GGA GAG CTC GTG CGA AGG GAC CCG GTG ACC TGC AAG ATT TTG CAT TTT 967
Ser Met Ile Ile Ile Ile His Leu Val Glu Val Val Pro Asn Gly Glu Leu Val Arg Arg Asp Pro Val Ser Cys Lys Ile Leu His Phe 240



II

TTC CAC CAG TAC ATG ATG GCC TGC AAC TAT TTC TGG ATG CTC TGT GAA GGG ATC TAT CTT CAT ACA CTC ATT GTC GTG GCT GTG TTT ACT 1057
Phe His Gln Tyr Met Met Ala Cys Asn Tyr Phe Trp Met Leu Cys Glu Gly Ile Tyr Leu His Thr Leu Ile Val Val Ala Val Phe Thr 270

III

GAG AAG CAA CCC TTG CCG TGG TAT TAT CTC TTG GGC TGG GGG TTC CCG CTG GTG CCA ACC ACT ATC CAT GCT ATT ACC AGG GCC GTG TAC 1147
Glu Lys Gln Arg Leu Arg Trp Tyr Tyr Leu Leu Gly Trp Gly Phe Pro Leu Val Pro Thr Thr Ile His Ala Ile Thr Arg Ala Val Tyr 300

IV

TTC AAT GAC AAC TGC TGG CTG AGT GTG GAA ACC CAT TTG CTT TAC ATA ATC CAT GGA CCT GTC ATG GCG GCA CTT GTG GTC AAT TTC TTC 1237
Phe Asn Asp Asn Cys Trp Leu Ser Val Glu Thr His Leu Leu Tyr Ile Ile His Gly Pro Val Met Ala Ala Leu Val Val Asn Phe Phe 330



V

TTT TTG CTC AAC ATT GTC CCG GTG CTT GTG ACC AAA ATG AGG GAA ACC CAT GAG GCG GAA TCC CAC ATG TAC CTG AAG GCT GTG AAG GCC 1327
Phe Leu Leu Asn Ile Val Arg Val Leu Val Thr Lys Met Arg Glu Thr His Glu Ala Glu Ser His Met Tyr Leu Lys Ala Val Lys Ala 360

ACC ATG ATC CTT GTG CCC CTG CTG GGA ATC CAG TTT GTC GTC TTT CCC TGG AGA CCT TCC AAC AAG ATG CTT GGG AAG ATA TAT GAT TAC 1417
Thr Met Ile Leu Val Pro Leu Leu Gly Ile Gln Phe Val Val Phe Pro Tyr Arg Pro Ser Asn Lys Met Leu Gly Lys Ile Tyr Asp Tyr 390

VI

GTG ATG CAC TCT CTG ATT CAT TTC CAG GGC TTC TTT GTT GCG ACC ATC TAC TGC TTC TGC AAC AAT GAG GTC CAA ACC ACC GTG AAG CGC 1587
Val Met His Ser Leu Ile His Phe Gln Gly Phe Phe Val Ala Thr Ile Tyr Cys Phe Cys Asn Asn Glu Val Gln Thr Thr Val Lys Arg 420

VII

CAA TGG GCC CAA TTC AAA ATT CAG TGG AAC CAG CGT TGG GGG AGG CGC CCC TCC AAC CGC TCT GCT CGC GCT GCA GCC GCT GCT GCG GAG 1597
Gln Trp Ala Gln Phe Lys Ile Gln Trp Asn Gln Arg Trp Gly Arg Arg Pro Ser Asn Arg Ser Ala Arg Ala Ala Ala Ala Ala Glu 450

GCT GGC GAC ATC CCA ATT TAC ATC TGC CAT CAG GAG CCG AGG AAT GAA CCA GCC AAC AAC CAA GGC GAG GAG AGT GCT GAG ATC ATC CCT 1687
Ala Gly Asp Ile Pro Ile Tyr Ile Cys His Gln Glu Pro Arg Asn Glu Pro Ala Asn Asn Gln Gly Glu Glu Ser Ala Glu Ile Ile Pro 480

TTG AAT ATC ATA GAG CAA GAG TCA TCT GCT TGA ATGTGAACCAACACAGTATCGTATCACTGACCCATCAITTCCTGGGAGAAAGACCATGCAITTAAGATATCT 1795
Leu Asn Ile Ile Glu Gln Glu Ser Ser Ala End 490

CCATCTCCACGAAACCAEAETCAITTTGGAAGAAETATTCAGTGAATTTTCCATTTGAAATCTGAGAAAGTATTTCTTGGTACTGTGCTTTGGGGACAGCTCAGGAATGGA 1914
GTCTCCCACTGCCACTTGTGAACTCCACATTCATCCAGGACTGAGATGCAAAATGTACACGTAATGCAAGCAAAAGTATCAAGAAAACAATGMAATGACCTAGTTCAGATACAGGGT 2033
GCTCCTTGTCAADACTGAGCCATTTAATCTTTGAAATZATAAAATCACTGTCAATATTTTATTTTAACTCTGGATTTGAAATAGATATTTCTGTATTGGCTATGGATCTGATT 2152
TTGAAATTTTTAAATTCAGTCAATTCGATGTACTGAGATGTTTACCATCTTACAAATGAAACCATGAACTAGCTGACCTCTGCAAGCAAAAGGGCTTTCTATATAGAGAGA 2271
TTAGTAAATATGTGAAAGAAAGACCTGCAITTTGGCAGGAGATGTATGCTTTGAAATGCAAAAGAAATTTAGATCAATTTGCTGAAACATTAACATGCTCAGCTTGTTTGGACAG 2390
CTGTCCATTTGGCAGGACCTAGCTGTGTAAAGAAATGCTTAAATCTGAAATGATTTTGTCTGATCTTTAACTGAGAGGTACAAAGAAATCTATCACTAAAAATTTTAC 2509
AAACTGCCAAAATAZAAATCTZAGTGGAGCAATACTCCCTTAAAGAAAGAGATTTGCCACTCCCTAACTCCAGGATTAZAAAGCAAAATTAATCCAGGTTTATAAAGCAG 2628
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GAGCTCAGGCTTTTCCAGGAAATCCAGGAGGCTAAATAGAAATCAACTTGTGGTTCAGCCGCTTGTCTTGTATTACCAAAACAGGAGGGGAAAAAATTAATCTGCTCAAAATTTAA 2866
CCATAAATCAATCAATTTAAAGTTTCTCATTAATAAATCCAGTATTAATATCATATCTCTCTTTACTTCCAGTATAAGATTTTGAAATCTGAAATAAACAGTATGCTACTG 2985
CACTGAAATTAATTTGTGAAATTTGCAACAGTAATCAGNTTACCAITTAATTTGATGCTAAATGAGGAGTACATTTGAAACCTCCAAATCTCCAGTCTCATATGCTCAAT 3104
TTGCCACTGCTTTCAGAAAGTATTTAGTGTGGAAGATAAATTAATTTGATTTGTTATGTTTACATTAATTCAGCCACCCAGAAATTAATTAATTTTACAGAGAAAGAAATTTTAC 3223
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CAGAGTATTTCTGGCCAGTGTGGTACAAAGTGAZATATATGCTAAATATTTAGATGTTATATGCTAATATAGTAGGGGTTGAGAAACAAAATAGCTTATATAGAAATTTGCA 3461
CATAGTCTGGCCAAAATATGTGAATGCTTATGCTTGTGTATATGATAAATAATACAGAGTACGTTAAAGCAAAAAGATGATATATTTGCATATTTTCTAAAGAAATATATAT 3588
CATCTTTT...

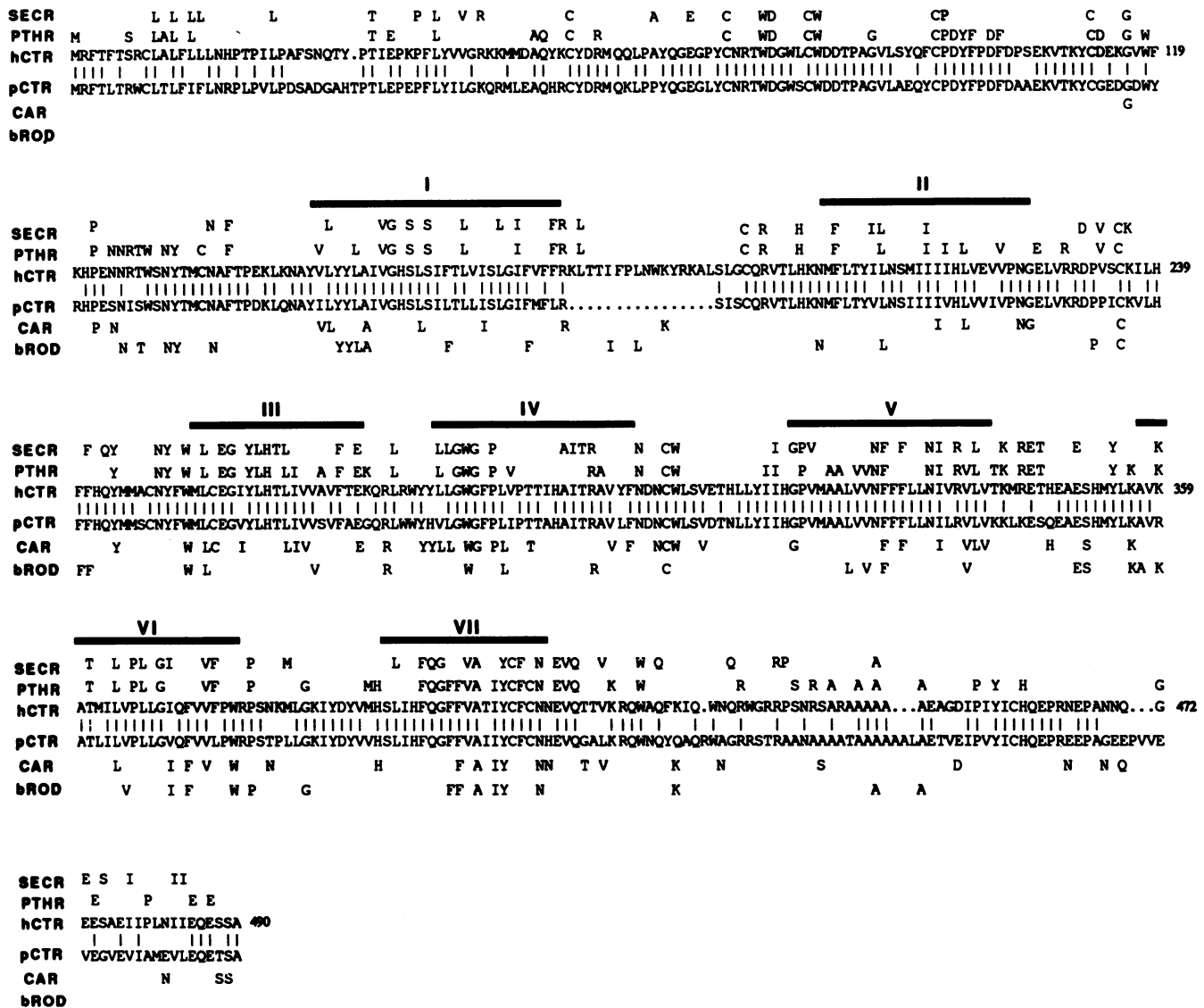


Figure 6. Alignment of the hCTR to the pCTR and to other G protein-coupled receptors. The complete hCTR and pCTR sequences are compared; gaps for this alignment are indicated by dots and identical residues indicated by vertical lines. Identities of the hCTR and the closely related opossum PTH-PTHrp (PTHR) and rat secretin receptor (SECR) are indicated above the CTR sequences. Identities of the hCTR with the *D. discoideum* CAR and bROD are indicated below the CTR sequences. The alignment of the CAR NH₂ terminus relative to the longer hCTR sequence begins at hCTR residue 115 and the alignment ends with CAR residue 362 aligned with the hCTR COOH terminus (hCTR residue 490). The bROD NH₂ terminus alignment begins with hCTR residue 123 and the alignment ends with bROD residue 349 aligned with hCTR residue 449. The solid lines above the sequences indicate the proposed transmembrane domains for the hCTR, labeled I-VII.

higher than the 12–20% identity found among the other principal families that comprise the superfamily of G protein-coupled receptors (42).

Each of the closely related peptide-binding receptors for CT, PTH-PTHrp and secretin possess homologous signal peptide-like NH₂-terminal domains. The six cysteines in the first extracellular domain of the hCTR and pCTR are conserved and require no gap insertions for their alignment. The positions

of the five cysteines closest to the carboxy-terminal end are conserved in the PTH-PTHrp and CT receptors, but only four of these cysteines are conserved in the secretin receptor. In addition, two other extracellular cysteines are notably conserved at sites in the putative second and third extracellular domains of all three receptor types. Three of four potential N-linked glycosylation sites in the first extracellular domains are preserved in the hCTR and pCTR; the two sites closest to

Figure 5. Nucleotide and predicted amino acid sequence of the hCTR cDNA. The underlined nucleotide triplet indicates a potential initiation codon upstream of the assigned putative translation start site (see text). The arrow indicates a potential cleavage site (between amino acids 22 and 23) for a hydrophobic leader sequence. Four potential N-linked glycosylation sites are indicated by shaded circles. Open circles indicate cysteines in the first extracellular loop and the conserved cysteines in the second and third extracellular loops. The seven putative hydrophobic membrane-spanning domains are also underlined.

the carboxy terminal are also conserved in the CT and PTH-PTHrp receptors. Only the *N*-linked glycosylation site of the hCTR nearest to the first transmembrane domain is preserved in the secretin receptor and this site is displaced one amino acid toward the NH₂ terminus relative to the transmembrane domain. Despite these conserved extracellular glycosylation sites and cysteine residues, the major areas of sequence divergence among these receptors are in the NH₂-terminal extracellular and COOH-terminal cytoplasmic regions where gaps are required to align the CTR and secretin receptor sequences relative to the longer PTH-PTHrp sequence. The most strikingly conserved regions of these receptors, not surprisingly, lie in the membrane-spanning domains.

CAR is also most homologous to the hCTR in the transmembrane domains, with an optimal alignment (44) matching each of the seven transmembrane domains and several conserved discriminator residues (31, 50, 53) (Fig. 6). This cAMP receptor also shares regions of homology with members of other G protein-coupled receptor families (31). The most conserved region between the hCTR and CAR is an area of transmembrane domain IV around the universally conserved tryptophan (42), which includes a conserved proline three residues carboxyl to the tryptophan. Over this region (hCTR residues 279–289), there are eight identical and two conserved amino acids in CAR. In this region of the hamster β -adrenergic (β AR) and bovine rhodopsin (bROD) receptors, only the tryptophan and a leucine are identical to the residues in hCTR or CAR and

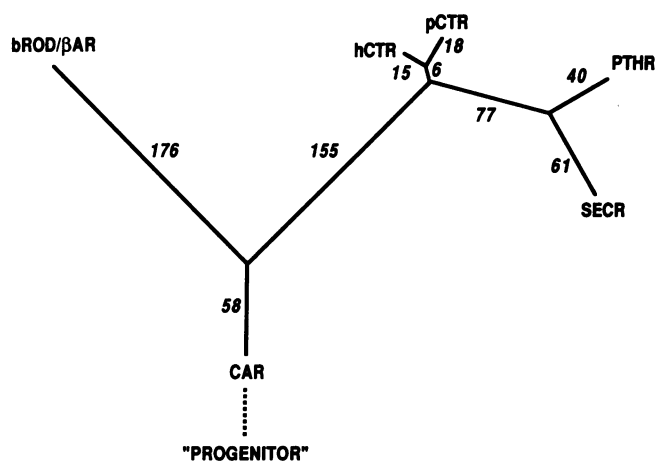


Figure 7. Phylogenetic and evolutionary relationships among selected members of the G protein-coupled seven transmembrane domain receptor superfamily, including β AR. The branchings of this evolutionary tree indicate the relative order in which these proteins diverged from one another assuming constant mutation rates for all species. The numerical values indicate phylogenetic relatedness and are expressed in units of PAMs per 100 residues (45). PAMs reflect the total number of amino acid interchanges (some superimposed) necessary to produce the observed difference in sequence. Numerical values between CAR and bROD/ β AR and CAR and the CTR/PTHR/SECR family of receptors reflect $\pm 20\%$ uncertainty resulting from distant relationships. Specifically, the PAM value for the relationships between hCTR and CAR is 234, whereas that between PTHR or SECR and CAR is 259. The uncertainty in the numerical values among the closely related CTR/PTHR/SECR family members is $< 4\%$. The broken line indicates the unknown early evolution of the superfamily progenitor, which likely involved numerous gene duplication events.

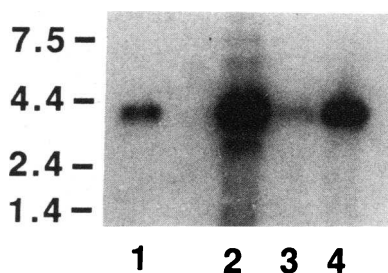


Figure 8. Northern analysis of poly A⁺-selected RNA from cells and tissues hybridized to a ³²P-labeled fragment of the hCTR cDNA. Lane 1: 1 μ g of RNA from LLC-PK₁ cells. Lane 2: 5 μ g of RNA from BIN-67 human ovarian carcinoma

cells. Lane 3: 5 μ g of RNA from T47D cells. Lane 4: 5 μ g of RNA from a human giant cell tumor of bone.

a gap is required for alignment (reference 31 and Fig. 6). Overall, CAR has approximately the same modest but significant degree of identity to the bROD receptor (21%), for example, as it does to the hCTR (21%). A dendrogram shown in Fig. 7 depicts the phylogenetic and evolutionary relationships among the hCTR, pCTR, PTH-PTHrp receptor, secretin receptor, CAR, bROD and hamster β AR receptors. This scheme depicts the approximately equal distances between CAR and the bROD/ β AR receptors and between CAR and the CT receptors. This relationship suggests that CAR represents a phylogenetic branch point linking these receptor families to a remote common ancestor. The much closer, and therefore more certain, relationship among the CT, PTH-PTHrp, and secretin receptors is also shown.

RNA analysis. Northern analysis of mRNA from BIN-67 cells, LLC-PK₁ cells, and T47D cells is shown in Fig. 8. Also included in Fig. 8 (lane 4) is mRNA from a human giant cell tumor of bone. Giant cell tumors are characterized by the presence of large numbers of multinucleated giant cells, which express phenotypic features of osteoclasts, including CTRs (37). The analysis was performed on the same blot under conditions of moderately high stringency (wash conditions: 60°C, 0.2 \times SSC). A single transcript of ~ 4.2 kb was identified in each of these samples. The high levels of CTR mRNA in the porcine LLC-PK₁ cells (which express $\sim 3 \times 10^5$ CTRs per cell) (24) resulted in a labeled band of moderate intensity even though only 1 μ g of polyA⁺-selected RNA was analyzed compared with 5 μ g of RNA from the human cells and tissue. In the human samples, the abundance of the CTR mRNA was higher in the samples from the BIN-67 cells and the giant cell tumor than the T47D cells.

Discussion

We report here the presence of a high affinity CTR on cells from a human ovarian small cell carcinoma line, BIN-67, and the cloning of the cDNA for this CTR. Transfection of this cDNA into COS cells resulted in expression of a receptor that has hormone-binding kinetics similar to the native CTR and is functionally coupled to adenylate cyclase.

The hCTR has 73% identity to the pCTR (24). The CTRs are closely related to the recently cloned receptors for PTH-PTHrp and secretin with ~ 34 and 30% identity, respectively. The PTH-PTHrp and secretin receptors have $\sim 43\%$ identity. These receptors do not have significant sequence identity to the other G protein-coupled receptors from higher organisms described in the current databases. The ~ 30 –40% sequence identity noted among the receptors for CT, PTH-PTHrp, and

secretin is, therefore, consistent with the existence of a separate G protein-coupled receptor family to which these proteins belong. These receptors are more distantly related to a CAR from the primitive eukaryote, *D. discoideum* (31), which has 21% amino acid identity to the hCTR. CAR is also remotely related to other G protein-coupled receptor families (31), suggesting that all of these receptor families may be linked to a remote common ancestor.

Although the receptors for CT, secretin, and PTH-PTHrp have little overall sequence identity to the other major G protein-coupled receptor families, they do share certain basic structural features and a few key residues characteristic of this protein superfamily (42). Common features include amino-terminal potential *N*-linked glycosylation sites and a cysteine residue in the second and third extracellular domains. There is evidence that these two extracellular cysteines form a disulfide bond essential for ligand binding (43, 52). These cysteines and some other key residues are also found in the *D. discoideum* CAR. The most conserved regions among the CT, PTH-PTHrp, and secretin receptors (and to a lesser extent, the CAR) lie in the membrane spanning domains, which are notably different from the transmembrane domains of the other members of the G protein-coupled receptor superfamily.

The unique structural features of the CTR family of receptors could account for several of the unusual functional properties of these receptors. For example, the CT receptor may couple to at least two distinct G proteins, resulting in activation of different signal-transduction pathways (26, 27). In the LLC-PK₁ cells, differential G protein coupling to the CT receptor appears to be dependent upon the stage of the cell cycle (27). So far, Northern analysis using the hCTR cDNA indicates the presence of a single transcript size in the cells from which RNA was probed.

Despite the highly conserved sequences of the two cloned CTRs there are differences in ligand-binding affinity to human CT and salmon CT. Binding affinity for salmon CT is higher for the hCTR than the pCTR when both receptor cDNAs are expressed in COS cells. The K_d values for both cloned CTRs fall within the range reported for the majority of CTRs naturally expressed on their native cells. Structural differences in these two receptors may account for the differing binding characteristics for salmon CT since the K_d values for the receptors in the transfected COS cells and in the native cells are indistinguishable. This suggests that coupling to different G proteins is not responsible for the observed differences in binding affinities of the two receptors. Possible regions of structural divergence that may relate to the differences in binding characteristics include the region between the predicted first and second transmembrane domains where the hCTR contains a 16-amino acid sequence not present in the pCTR. Presumably, the differences in binding affinities for human and salmon CT between the human and porcine CTRs also relate to the distinct structural features of the human and salmon CT ligands (14).

Photo emulsion autoradiography performed on BIN-67 cells incubated with ¹²⁵I-salmon CT shows these cells to be heterogeneous with respect to the expression of CT receptors. The larger cells that tend to spread out over the culture substrate express the CTR in greatest abundance (Goldring, S. and Gorn, A., unpublished). The function of CTRs in the BIN-67 ovarian carcinoma cells or in the ovary itself is unknown. The developmental origin of the BIN-67 carcinoma cells is also un-

certain (30, 32, 33). The CT receptor may be a marker for an undifferentiated stage in the malignant transformation of the ovarian cells that give rise to the BIN-67 cells. The most likely explanation for the presence of the CTR on the BIN-67 cells, however, is that the CTR reflects the persistent expression of a normal ovarian cell phenotype. CTRs have in fact been demonstrated on Leydig cells of the normal testes (53). We have also observed by Northern analysis, using the pCTR cDNA as a probe, the presence of an appropriately sized CTR transcript in mRNA from normal porcine ovaries (A. Gorn and S. Goldring, unpublished). CT stimulates zinc transport in the testis (54) and increases testosterone secretion and the concentration of sex-steroid receptors in Leydig cells (55). Although the actions of CT in the ovaries are undefined, high circulating levels of CT have been measured during pregnancy, lactation, and in women taking oral contraceptives (56-58).

The cloning of the hCTR offers new opportunities to explore the role of CT in human physiology. The availability of two cloned CTRs that exhibit distinct functional properties should help define the structural features responsible for these properties. The homology of the hCTR with the CAR from *D. discoideum* may provide clues to explain the evolutionary relationships among the CT, PTH-PTHrp, and secretin family of receptors and the other families comprising the G protein-coupled receptor superfamily. Current evidence suggests the existence of a remote common ancestor for the entire class, or superfamily, of G protein-coupled receptors.

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