Communication

Full Activation of Chimeric Receptors by Hybrids between Parathyroid Hormone and Calcitonin

EVIDENCE FOR A COMMON PATTERN OF LIGAND-RECEPTOR INTERACTION*

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Calcitonin (CT) and parathyroid hormone (PTH), whose receptors belong to the same family of G protein-coupled receptors, share no amino acid sequence homology and selectively activate either CT or PTH receptors. We now show, however, that reciprocal hybrid ligands (CT/PTH and PTH/CT), which do not activate the "wild-type" receptors, activate PTH/CT and CT/PTH receptors, respectively. Our findings indicate that PTH and CT share a similar architecture with at least two functional, receptor-specific domains. These domains are sufficiently independent to permit synthetic hybrid ligands to efficiently activate appropriate receptor chimeras. Therefore, both ligands follow, despite their very different primary sequences, a common pattern of ligand-receptor interaction.

The isolation of cDNAs encoding the receptors for secretin (1), calcitonin (CT)1 (2), and parathyroid hormone (PTH)/PTH-related peptide (PTHrP) (3, 4) established a new family of G protein-coupled receptors (GPRs) (5, 6). Members of this family, including some invertebrate receptors (7)2,3 share, in addition to their overall structure with seven membrane-spanning helices, approximately 50 strictly conserved amino acids, including 8 important cysteines, an almost invariant amino acid sequence of the seventh membrane-spanning domain, and a similar intron/exon organization (10–16). These findings suggest that this family of GPRs may have evolved from a common ancestral precursor. The ligands which activate these GPRs are similar in length, but lack, with the exception of the first 13 residues in PTH and PTHrP, any amino acid sequence homology. For both PTH(1–34) and CT(1–32), the importance of the amino terminus for bioactivity has been recognized, whereas the carboxyl-terminal portion contributes predominantly to receptor binding (17–23). Based on the limited data available, we and others had previously proposed that the carboxy-terminal portion of these ligands determines specificity for the amino-terminal, extracellular receptor domains, while the amino termini of most ligands functionally interact with the membrane-embedded receptor region (23–29). We have now tested this hypothesis more directly by constructing reciprocal chimeric ligands composed of portions of PTH and CT, and reciprocal chimeric receptors composed of portions of the PTH/PTHrP and CT receptor. These chimeras were designed such that the carboxy-terminal portion of the ligand would match the amino-terminal, extracellular domain of the receptor, while the amino-terminal portion of the hybrid ligand would correspond to the membrane-embedded domains of the receptor and connecting loops. Our functional analysis of these hybrid ligands and chimeric receptors strengthens the proposed model of ligand-receptor interaction. Moreover, our studies imply that the receptors and their ligands are composed of functionally independent domains.

MATERIALS AND METHODS

Peptides and Chemicals—The hybrid peptides and [Nle23,24,Tyr32][bPTH(1–34)-NH2] (PTH(1–34)) were synthesized by the MGH Biopolymer Facility using Fmoc (9-fluorenylemethoxycarbonyl) technology (30). All peptides were HPLC-purified and analyzed by mass spectroscopy, amino-terminal sequencing, and acid hydrolysis. Hybrid peptides (C(1–11)/P(15–34) is sCT(1–11)/[Nle18,Tyr34]bPTH(15–34)-NH2 and hybrid peptide P(1–13)/C(12–32) is [Nle6]bPTH(1–13)/sCT(12–32)-NH2. Salmon calcitonin (sCT(1–32)-NH2) and human calcitonin (hCT(1–32)-NH2) were purchased from Bachem, Torrance, CA. Peptides were radiolabeled by the chloramine-T method using Na125I (2,000 Ci/mmol, DuPont NEN) and were HPLC-purified using a 30% acetonitrile gradient (31). All other chemicals and reagents were of the highest available grade.

Cell Culture—COS-7 African green monkey kidney cells were cultured in Dulbecco’s modified Eagle’s medium (Mediatech, Washington, D.C.) supplemented with 10% fetal bovine serum (Sigma), 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate (Life Technologies, Inc.) at 37 °C in a humidified 95% air, 5% CO2 atmosphere.

Construction of Chimeric Receptors and Expression in COS-7 Cells—Expression cloning of the rat PTH/PTHrP receptor (PR) and the porcine calcitonin receptor (CR) using the mammalian expression vector pcDNAI (Invitrogen, San Diego, CA) was described previously (2–4). The human influenza virus hemagglutinin (HA) epitope tag (32) which had been introduced into the PR (replacing residues 93–101) (33) was also inserted into the CR (between residues 66 and 67, FVDVDPVA) using site-directed mutagenesis (34). To distinguish them from chimeric receptors, these receptors are referred to as "wild-type." Corresponding regions of the PR and CR were identified by aligning the complete amino acid sequences of the two receptors using the GCG Pile-up program (Genetics Computer Group, Madison, WI).

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1 The abbreviations used are: CT, calcitonin; PTH, parathyroid hormone; PTHrP, PTH-related peptide; GPR, G protein-coupled receptor; HPLC, high performance liquid chromatography; s, salmon; b, bovine; h, human; PR, PTH/PTHrP receptor; CR, calcitonin receptor; HA, hemagglutinin; PCF, CR(1–66)/PR(98–175)/CR(141–482); PCP, PR(1–97)/CR(67–142)/PR(178–591); PC1R, PR(1–177)/CR(143–482); PC2R, PR(1–97)/CR(67–482); PC1R, CR(1–142)/PR(178–591); PC2R, CR(1–66)/PR(98–591).

2 J. Sulston, Z. Du, K. Thomas, R. Wilson, L. Hillier, R. Staden, N. Wilm, and D. C. time 2 J. D. Reagan (1995) GenBank™ accession number U15959.
A PstI restriction site in the CR, engineered into the PR at the cor- responding location (residue 177) by site-directed mutagenesis, was used to construct the hybrid receptor PCR1 that contains residues 1–177 of the PR, joined to residues 143–482 of the CR. The reciprocal receptor hybrid CPR1 (amino terminus of the CR (residues 1–142) annealed at position 178 to the carboxyl terminus of the PR) was constructed by introducing an XmaI site into the CR; the introduction of this site into the PR was reported previously (23). A second set of reciprocal receptor hybrids was constructed at a BspEI site within the HA-tag: PCR2 contains residues 1–97 of the PR, joined to residues 67–482 of the CR; CPR2 contains the corresponding amino terminus of the CR (residues 1–66), joined to residues 98–519 of the PR. The described restriction sites and a novel BspH1 site in CR (residue 140) were also used to construct the receptors CR-(1–66)/PR-(98–175)/CR-(141–482)/(PCR) and PR-(1–97)/CR-(67–482)/PR-(178–591)/(CPR).

Plasmids encoding the wild-type and hybrid receptor constructs were transfected into COS-7 cells, grown in 10-cm dishes, using the diethylaminoethyl (DEAE)-dextran method (23, 33). The cells were subcultured into 24-well plates 18–24 h after transfection, and functionally evaluated after 72 h.

**Evaluation of Receptor Cell Surface Expression**—The use of an HA-epitope to assess receptor surface expression in intact COS-7 cells was described previously (32, 33). Briefly, transfected COS-7 cells were incubated at 15 °C for 2 h with binding buffer (50 mM Tris·HCl (pH 7.5), 100 mM NaCl, 5 mM KCl, 2 mM CaCl2, 5% heat-inactivated horse serum, 0.5% heat-inactivated fetal bovine serum) containing 5 μg/ml anti-HA monoclonal antibody (12CA5, Boehringer Mannheim). After extensive washing, the anti-HA antibody binding was detected by incubation with a 125I-labeled sheep anti-mouse (Fab′)2 fragment (NEX-162, DuPont NEN) (200,000 cpm/well) which was diluted in binding buffer for an additional 2 h at 15 °C. The cells were then rinsed twice, lysed with 1 N NaOH, and the entire lysate was counted for γ-irradiation. The cell surface expression levels of the different receptors, as determined by specific anti-HA antibody binding, were equalized by varying the amount of transfected plasmid DNA (Fig. 1A). The amounts transfected per 10-cm dish were: PCR1 = 0.14 μg; PCR2 = 150 μg; CPR1 = 80 μg; CPR2 = 2 μg; CPC = 1.1 μg; PR = 0.8 μg; CR = 0.17 μg.

**Cyclic AMP Assay**—24-well plates containing transfected COS-7 cells were chilled on ice, the cells were rinsed with phosphate-buffered saline, and stimulated in a CAMP assay buffer (DuBecco’s modified Eagle’s medium, 2 mM 3-isobutyl-1-methylxanthine, 0.1% bovine serum albumin, 20 mM HEPES (pH 7.4)) containing various concentrations of peptide for 15 min at 37 °C. The reaction was then stopped by removing the buffer and chilling the plates on dry ice. Intracellular CAMP was extracted with 50 mM HCl and measured by radioimmunooassay as described (3).

**Radioceptor Assays**—For radioligand studies, transfected COS-7 cells cultured in 24-well plates were rinsed with binding buffer and then incubated at 4 °C for 8 h in binding buffer containing 125I-PTH-(1–34) (800,000 cpm/well, except 200,000 cpm/well for the PR), 125I-sCT-(1–32) (800,000 cpm/well, except 200,000 cpm/well for the CR), or 125I-P (1–13)/C(12–32) (800,000 cpm/well, except 200,000 cpm/well for the CR), or 125I-C (1–13)/C(12–32) (800,000 cpm/well, except 200,000 cpm/well) at 15 °C for 4 h with 125I-CT (1–11) (15–34) (800,000 cpm/well) and various concentrations of unlabeled competing peptides (31). Ligand association studies demonstrated that under these conditions binding reached equilibrium. For subsequent determination of cell-associated radioactivity, cells were rinsed twice with binding buffer, lysed with 1 N NaOH, and the entire lysate was counted for γ-irradiation. Specific binding was determined by subtracting nonspecific binding that was obtained in the presence of maximal competing peptide (10−6 to 10−3 M).

**Data Calculation—EC50 values and IC50 values were determined from plots of log(S/Smax − S) versus log[peptide] and calculation of the x-intercept. S represents the CAMP accumulation or the specific binding at a given peptide concentration [peptide], respectively. The data shown represent the mean ± S.E. of at least three independent experiments that were performed in duplicate.**

**RESULTS AND DISCUSSION**

The reciprocal exchange of portions of the amino-terminal, extracellular domain of the rat PTH/PTHrP receptor (PR) and the porcine calcitonin receptor (CR) resulted in several receptor constructs (PCR1, PCR2, CPR1, and CPR2) that were efficient expressed in COS-7 cells (Fig. 1A). To evaluate their biological properties, these receptors were first tested with maximal doses (10−6 to 10−5 M) of sCT, PTH-(1–34), and the two hybrid peptides, C(1–11)/P(15–34) and P(1–13)/C(12–32).

The hybrid peptide C(1–11)/P(15–34) selectively activated cells expressing the chimeric receptor CPR1, but not or only slightly activated cells expressing the reciprocal receptor chimera CPR1 or the two wild-type receptors (Fig. 1B). The maximal cAMP accumulation with C(1–11)/P(15–34) was similar to that observed with PTH-(1–34) and sCT-(1–32) activating the wild-type receptors, PR and CR, respectively. The reciprocal hybrid peptide, P(1–13)/C(12–32), was a potent and selective agonist when tested with cells expressing CPR1 (Fig. 1C), but did not activate PCR1 or PR, and activated CR only slightly.

The EC50 for the activation of PCR1 by C(1–11)/P(15–34) was 12 ± 1.7 nM, while cells expressing the chimeric receptor CPR1 were activated by P(1–13)/C(12–32) with an EC50 of 27.0 ± 4.4 nM (Fig. 2, B and D). The chimeric receptors are, therefore, efficiently and specifically activated by hybrid ligands which contain a carboxyl-terminal portion that matches the amino-terminal, extracellular domain of the receptor and an amino-terminal portion that corresponds to the membrane-embedded domains of the receptor and the connecting loops.

Interestingly, PTH-(1–34) activated not only the wild-type PR, but also the chimeric receptor CPR1 (Fig. 1D), while it did not activate PCR1 or CR. However, the EC50 of PTH-(1–34)-
activated cAMP accumulation was 420-fold higher with cells expressing PCR1 than with those expressing the wild-type PR (EC_{50}: 460 ± 83 and 1.1 ± 0.28 nM, respectively) (Fig. 2, C and D). Similarly, sCT-(1–32) stimulated cAMP accumulation not only in COS-7 cells expressing the CR, but also in cells expressing the chimeric receptor PCR1, while cells expressing CPR1 or PR showed no response (Fig. 1E). Comparable to the findings with PTH-(1–34), the EC_{50} of sCT-(1–32)-activated cAMP accumulation was 40-fold higher with cells expressing the PCR1 than that observed for the native receptor CR (EC_{50} 19 ± 1.3 and 0.5 ± 0.13 nM, respectively) (Fig. 2, A and B). These results indicate that the membrane-embedded portion and associated loops of each receptor are sufficient to mediate, albeit with reduced potency, the biological activity of sCT-(1–32) and PTH-(1–34). The relatively high potency of sCT-(1–32) with the chimeric receptor PCR1 may be due to its exceptionally low dissociation constant (37, 35). Indeed, human CT-(1–32)-NH_{2}, which does not share this low dissociation constant, was only weakly active with the PCR1 chimera (EC_{50} 58 ± 17 nM for CR, EC_{50} >10,000 nM for PCR1, Fig. 2, A and B).

For the hybrid ligands, the position at which the peptide domains are recombined influences their potency. A hybrid peptide that contains a shorter amino-terminal portion of CT, sCT-(1–9)/PTH-(7–34)-NH_{2}, while selectively activating cells expressing the PCR1, was less potent than C(1–11)/P(15–34) (data not shown). Furthermore, a hybrid peptide with a shorter amino-terminal portion of PTH, PTH-(1–6)/sCT-(9–32)-NH_{2}, was completely inactive as were the fragments sCT-(1–9)/NH_{2} (circular and linear form), [Nle^{8}]bPTH-(1–13)-NH_{2}, sCT-(12–32)-NH_{2}, and [Nle^{8,14,15},Tyr^{32}]bPTH-(7–34)-NH_{2}.

To further analyze the requirements for the interactions between hybrid ligands and chimeric receptors, we tested the reciprocal receptor chimeras PCR2 and CPR2, in which smaller portions of the respective amino termini were exchanged (Fig. 3). The hybrid ligand C(1–11)/P(15–34), which had shown potent activation of the chimeric receptor PCR1, showed no activity when tested with PCR2. Similarly, the hybrid ligand P(1–13)/C(12–32), which had shown potent activation when tested with CPR1, revealed no activity with cells expressing the chimeric receptor CPR2. These results suggest that the extracellular residues 98–177 of the PR in PCR1 and residues 67–142 of the CR in CPR1 contain sites that are important for efficient interaction with the hybrid peptides. This was further supported by findings with the chimeric receptor CPC, where the region 67–140 in the CR was replaced by the PR residues (1–66)/PR-(98–175)/CR-(141–482), was activated by the hybrid ligand C(1–11)/P(15–34) (EC_{50} 139 ± 36 nM). The wild-type ligand sCT-(1–32), however, had a reduced potency with CPC (EC_{50}: 120 ± 17 nM), when compared to the wild-type CR (EC_{50}: 0.5 ± 0.13 nM). The reciprocal receptor PCP was not expressed in COS-7 cells and thus was uninformative (data not shown).

Interestingly, in comparison to CR, sCT-(1–32) had reduced, but comparable potency on PCR1 and CPR2. Similarly, PTH-(1–34) was equally active with CPR1 and CPR2, but less active than with the PR. Determinants for ligand binding are, therefore, likely to be located at least in two distinct portions of the amino-terminal, extracellular domain of each receptor (25, 33).

The conclusions from the above activation studies were also supported by radioreceptor binding studies. Selective and specific binding of radiolabeled C(1–11)/P(15–34) to COS-7 cells expressing PCR1 (0.5 ± 0.06% of total radioactivity) could be detected. Similarly, radiolabeled P(1–13)/C(12–32) preferentially bound to CPR1 (1.6 ± 0.3% of total radioactivity). The radiolabeled wild-type ligands ^{125}I-PTH-(1–34) and ^{125}I-sCT-(1–32) bound selectively to PR and CR (6.0 ± 1.4% and 14 ± 4.6% of total radioactivity, respectively).

In competition binding experiments, specific binding of ^{125}I-PTH-(1–34) and ^{125}I-sCT-(1–32) to cells expressing PR or CR was inhibited only weakly, or not at all, by the hybrid ligands (Fig. 4, A and C). In contrast, binding of radiolabeled C(1–11)/P(15–34) to PCR1 was inhibited fully and potently by unlabeled C(1–11)/P(15–34). This binding was also inhibited to a lesser degree by PTH-(1–34), but not by sCT-(1–32) (IC_{50} values: 338 ± 159, 1,895 ± 213, and >10,000 nM, respectively) (Fig. 4B). Analogous findings were made when using the radiolabeled hybrid ligand P(1–13)/C(12–32) (Fig. 4D). Binding of this radioligand to cells expressing the chimeric receptor CPR1 was fully inhibited by unlabeled P(1–13)/C(12–32) and to a lesser extent by sCT-(1–32) and PTH-(1–34) (IC_{50} values: 13 ± 3.1, 1.407 ± 0.354, and >10,000 nM, respectively). Hybrid ligand binding is thus inhibited, at least partially, by the wild-type ligand that contributes the carboxyl-terminal portion of the hybrid ligand. This finding is consistent with an interaction of...
the carboxyl terminus of the hybrid ligand with the amino-terminal, extracellular domain of the receptor. Specific binding of either radiolabeled hybrid peptide to either PCR2 and CPR2 could not be detected (data not shown). Therefore, a substantial component for the binding energy of the hybrid ligands is likely to be derived from an interaction with the regions PR-(98–177) and CR-(67–142), respectively.

The members of the secretin/PTH/CT receptor family share significant amino acid sequence homology, and the precise spacing of conserved residues predicts that these GPRs all have a common pattern of functional, if the interacting domains are appropriately exchange is possible; the resulting pairs of chimeras may be members of this receptor family and their ligands. Domains with ligands and receptors that interact with each other appear to be sufficiently independent, such that their modular exchange is possible; the resulting pairs of chimeras may be functional, if the interacting domains are appropriately matched. This approach may prove to be useful for the further analysis of ligand-receptor interactions.

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