Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein hormone consisting of an α and a β subunit that stimutates intracellular levels of cAMP via a G protein-coupled receptor. Herein we report the engineering and characterization of a novel molecule in which the receptor and its heterodimeric ligand were covalently linked in a single polypeptide chain. The hormone-receptor complex was expressed in cells transfected with this construct, but the cells were unable to bind significant amounts of exogenous hCG. However, cleavage of the hormone with a site-specific protease rendered the receptor accessible to exogenously added hormone. Cells transfected with the hCG-receptor construct contained elevated basal levels of cAMP; moreover, addition of hormone had no significant effect. These results are consistent with a strong and stable interaction between the single-chain hormone and its covalently linked receptor that results in a constitutively active complex.

Human chorionic gonadotropin (hCG) plays a central role in reproductive physiology. Its synthesis by the syncytiotrophoblast is crucial for corpus luteum rescue and the stimulation of progesterone production, which is required for sustaining early pregnancy. hCG is a member of the glycoprotein hormone family, which also includes pituitary-derived luteinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone. These hormones are structurally related, and each has two subunits, α and β. The α subunit is common to all members of the glycoprotein family, whereas each hormone has a unique β subunit that determines the receptor specificity (1, 2). The noncovalent association of the two subunits into a heterodimer is required for hormonal activity (1). A distinct feature of hCGβ is the presence of a C-terminal extension of about 30 amino acid residues (CTP) that is not essential for hormone activity (3, 4).

In gonadal cells, hCG binds to its cell surface receptor (LH/CG-R), which belongs to the superfamily of G protein-coupled receptors (5). Binding of hCG to LH/CG-R leads to the activation of Gα, resulting in an increase in the concentration of intracellular cAMP (6). The LH/CG-R contains a relatively large N-terminal extracellular domain, a seven-transmembrane domain, and a short cytoplasmic tail (5). The extracellular domain is characterized by a motif of imperfect leucine-rich repeats, which contributes largely to the high affinity hormone binding of the receptor (7–11). The crystal structure of anhydro-drous hydrofluoric acid-treated hCG is known (12, 13), and several models exist for the extracellular domain of the receptor (14–17); however, the nature of the interaction of the extracellular domain with the hormone is poorly understood, as is the mechanism of transmembrane signaling.

We (18) and others (19) have previously constructed a functional single-chain yoked hCG in which the α subunit is fused to the C terminus of the β subunit via its CTP. Compared to wild-type hCG under in vitro conditions, this yoked hCG displayed the same binding affinity to the LH/CG-R and steroidogenic activity indicating the correct assembly of the two subunits when covalently linked. Herein, we report a unique construct of a hormone-receptor complex, denoted yoked hormone-receptor (YHR), where the yoked hCG is fused to the N terminus of the mature LH/CG-R sequence via the CTP sequence in a single polypeptide chain. Expression of this construct in COS-7 and HEK 293 cells resulted in high basal levels of intracellular cAMP; demonstrating a stable interaction between the receptor and the single chain covalently linked hormone that results in a constitutively active hormone-receptor complex.

EXPERIMENTAL PROCEDURES

Construction of YHR—The yoked hCG cDNA was amplified by PCR from the baculovirus transfer vector pVL1393 (18) using primers containing BamHI and AflIII sites to generate the entire coding sequence of yoked hCG followed by the first half (amino acid residues 116–132) of the CTP sequence. Ser-132 on the CTP was changed to Thr in order to create an AflIII site. The rat LH/CG-R sequence was amplified by PCR from the baculovirus transfer vector pVL1393 (20) using primers containing AflIII and BamHI sites to generate a product containing the second half of the CTP sequence (amino acid residues 133–145) and a Factor Xa recognition sequence, Ile-Glu-Gly-Arg (21), upstream of the coding sequence of the mature receptor. The two PCR products were ligated at the AflIII site and subcloned into the BamHI site of the eukaryotic expression vector pcDNA3 (Invitrogen). The clone was sequenced in both directions to ensure that no errors had occurred during the PCR amplification.

Cell Culture and DNA Transfections—African green monkey kidney epithelial (COS-7) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicilllin, and 100 μg/ml streptomycin. HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 4.5% glucose, 10% horse serum, 100 units/ml penicilllin, 100 μg/ml streptomycin, and 10 mM HEPES, pH 7.0. COS-7 cells were transiently transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s directions. To generate the stable cell line, HEK 293 cells were transfected as described above. Forty-eight hours after transfection, cells were washed and plated in growth medium containing 500 μg/ml geneticin. Surviving cells were pooled and maintained in the growth medium containing 500 μg/ml geneticin.

Preparation of Detergent-soluble Extracts—Membrane fractions were prepared by homogenizing cells from a 150-cm² flask at 4 °C in 15 ml of 50 mM Tris-HCl, pH 7.5, 250 mM sucrose, and protease inhibitors (1 mM PMSF). The paper is available on line at http://www-jbc.stanford.edu/jbc/
phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, and 2 mM EDTA), followed by centrifugation at 50,000 x g for 1.5 h. The pellet was resuspended in 1 ml of solubilization buffer containing 0.15 M NaCl, 20 mM HEPES, pH 7.4, 20% glycerol, 1.5% CHAPS, and protease inhibitors and incubated at 4 °C for 1 h, followed by centrifugation at 15,000 x g for 30 min. The protein concentration in the supernatant (soluble fraction) was determined by the bicinchoninic acid (BCA) assay (Pierce).

**Western Blot Analysis—** Membrane fractions were resolved on 10% SDS-polyacrylamide gels under reducing conditions and transferred to Immobilon P (Millipore). Western blots were performed as described in detail previously (22, 23), except that incubation with rabbit anti-hCGβ antibody, raised against amino acid residues 109–145 of hCGβ, was performed for 1 h at room temperature, and the blots were developed with SuperSignal Substrate Working Solution (Pierce) for 5 min followed by exposure to Kodak X-OMAT film.

**In Vitro Bioassays—** Assays with transiently transfected COS-7 cells were performed 48 h after transfection. Direct cell surface binding, using 1.5 ng (50 pmol) of 125I-hCG in a final volume of 1 ml was measured as described (22, 23). Nonspecific binding was measured in the presence of 2 µg of unlabeled hCG. Cells were washed, solubilized with 1 N NaOH and counted in a γ counter. For competitive binding experiments, cells were incubated for 3 h with 3 ng (100 pmol) of 125I-hCG and various dilutions of unlabeled hCG in a final volume of 1 ml. Binding to detergent-soluble extracts was performed as follows. Soluble extracts (500 µg) were dialyzed against 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 1 mM CaCl2 for 4 h at 4 °C and then incubated with 2 µg of Factor Xa (New England Biolabs) or without enzyme for 4 h at room temperature. The samples were then diluted 10-fold in 0.15 M NaCl, 20 mM HEPES, pH 7.4, containing 20% glycerol, and 50 µg of extract was incubated with 100 pmol of 125I-hCG overnight at 4 °C in a final volume of 500 µl. Nonspecific binding was determined in the presence of excess unlabeled hCG. Bound radioactivity was separated from free by filtration through Whatman GF/B filters that had been soaked in 0.3% polyethyleneimine in 10 mM Tris-HCl, pH 9.1. The filters were then extensively washed with 0.1 M NaCl, 10 mM Na2CO3, 1 mg/ml bovine serum albumin in phosphate-buffered saline, air-dried, and counted. For determination of intracellular levels of cAMP, cells were incubated with Waymouth’s MB752/1 medium containing 1% bovine serum albumin and 0.8 mM isobutyl-myristate at 37 °C for 15 min, and then incubated with the same medium containing 0 or 50 ng/ml hCG at 37 °C for 30 min. The medium was removed, and the cells were lysed by incubation with 100% ethanol at −20 °C overnight. The lysed cells were collected by centrifugation at 2,000 × g for 15 min. The pellets were air-dried and dissolved in 0.1 M NaOH for determination of the protein concentration by the BCA assay. The supernatants were dried using flowing nitrogen gas and resuspended in 200 µl of cAMP assay buffer. cAMP concentrations were determined with the 125I-cAMP radioimmunoassay kit (DuPont NEN). For the time course of cAMP stimulation, transiently transfected COS-7 cells were replated into 6-well plates 2–4 h after transfection and assayed as above.

**Results**

Expression of YHR in Transfected Cells—The YHR construct in which the yoked hCG is covalently linked to the N terminus of the mature LH/CG-R sequence via the CTP sequence encodes a single polypeptide chain consisting of 949 amino acids (Fig. 1). The signal sequence of hCG is present at the N-terminus, and the recognition sequence for Factor Xa protease was introduced between the hormone and receptor sequences. This construct was transiently and stably transfected into COS-7 and HEK 293 cells, respectively.

To first determine if the YHR complex was expressed in the transfected cells, the membrane fraction was isolated from both COS-7 and HEK 293 cells, and membrane proteins were detergent-solubilized. The presence of the YHR complex was demonstrated by Western blot analysis of the solubilized proteins using an antibody directed against amino acid residues 109–145, which includes the CTP sequence (amino acid residues 116–145). As shown in Fig. 2, three bands with apparent molecular size of 125, 97, and 53 kDa, specific to cells transfected with the YHR cDNA, were detected. These bands are absent in mock-transfected cells. As expected, no bands were detected in cells transfected with wild-type LH/CG-R since it lacks the CTP. In contrast, yoked hCG expressed in baculovirus-infected insect cells and containing the CTP could be readily detected with an apparent size of 42 kDa.

We next determined if exogenous hCG could bind COS-7 cells transfected with YHR cDNA. Cells transfected with wild-type LH/CG-R and empty vector were included as positive and negative controls, respectively. No specific binding of 125I-hCG was detected in YHR-transfected cells, while cells transfected with wild-type LH/CG-R were able to bind 125I-hCG (Fig. 3A). To ascertain if the lack of binding of 125I-hCG to intact cells expressing the YHR complex is due to improper folding of the complex such that it cannot recognize the exogenous hCG or because the receptor in the YHR complex is already occupied by the covalently linked hormone, we took advantage of the Factor Xa cleavage site that was engineered between the hormone and receptor sequences. Attempts to detect cell surface binding after incubating intact cells with Factor Xa were unsuccessful. Therefore, detergent-solubilized extracts from COS-7 cells transfected with the YHR complex were treated with Factor Xa protease. Specific binding of 125I-hCG was then observed in protease-treated samples (Fig. 3A). Similarly, HEK 293 cells stably transfected with wild-type LH/CG-R bound 125I-hCG, whereas cells transfected with the YHR complex displayed <20% of wild-type binding (Fig. 3B). Treatment of detergent-solubilized extracts with Factor Xa increased the amount of 125I-hCG that was able to bind receptor when compared to untreated extracts.

Western blot analysis of control and Factor Xa-treated samples with antibody against the CTP indicated that about 50–60% of the YHR complex was cleaved by the protease (Fig. 3C). The 125- and 97-kDa bands are not well resolved on this gel and migrate as a doublet. Surprisingly, the cleaved yoked hCG with an apparent size of 49 kDa exhibits a much higher affinity for the antibody than the YHR complex, possibly because both CTP sequences are now accessible to the antibody compared to...
only one in the YHR complex.

Since the YHR-expressing HEK 293 cells exhibited low but measurable binding of exogenous $^{125}$I-hCG to mock-transfected cells (MT), cells expressing the LH/CG-R (LHR) and YHR, normalized to protein content, were presented as an average of two independent experiments and are represented by solid bars. The hatched bars represent specific binding of $^{125}$I-hCG to solubilized extracts of YHR-expressing cells that were either treated with Factor Xa (+) or untreated (−). C, Western blot analysis of untreated and Factor Xa-treated samples (20 μg) from COS-7 and HEK 293 cells. The blot was probed with an antibody against the CTP. YhCG represents crude media from insect cells infected with the recombinant yoked hCG baculovirus. The sizes of the molecular mass standards are indicated. The film was densitometrically scanned to quantify band intensities.

FIG. 3. hCG binding in transfected COS-7 (A) and HEK 293 cells (B). Specific cell surface binding of $^{125}$I-hCG to mock-transfected cells (MT), cells expressing the LH/CG-R (LHR) and YHR, normalized to protein content, are presented as an average of two independent experiments and are represented by solid bars. The hatched bars represent specific binding of $^{125}$I-hCG to solubilized extracts of YHR-expressing cells that were either treated with Factor Xa (+) or untreated (−). C, Western blot analysis of untreated and Factor Xa-treated samples (20 μg) from COS-7 and HEK 293 cells. The blot was probed with an antibody against the CTP. YhCG represents crude media from insect cells infected with the recombinant yoked hCG baculovirus. The sizes of the molecular mass standards are indicated. The film was densitometrically scanned to quantify band intensities.

FIG. 4. Competitive binding to HEK 293 cells expressing wild-type LH/CG-R and YHR. Cells were incubated with $^{125}$I-hCG and increasing concentrations of unlabeled hCG. The specific binding in the absence of unlabeled competitor is normalized to 100%.

increases in cAMP levels in COS-7 and HEK 293 cells, respectively, in response to hCG. In contrast, the YHR-expressing cells exhibited a high basal level of cAMP that was not altered in the presence of exogenous hCG. The basal level of cAMP in YHR-expressing COS-7 cells was 48-fold higher than the basal level in mock-transfected cells and slightly higher than the stimulated level in wild-type LH/CG-R expressing cells. Similar results were obtained with HEK 293 cells, the basal level being stimulated 58-fold when compared with mock-transfected cells. The basal and stimulated intracellular level of cAMP in HEK 293 cells was higher than in COS-7 cells.

A time course of cAMP stimulation in YHR-transfected COS-7 cells shows high basal levels as early as 24 h that reach a maximum at 36–48 h post-transfection (Fig. 5C). The cAMP levels then declined, although they were still higher than levels in mock-transfected cells at 72 h after transfection. The time course and levels of cAMP in cells stimulated with 50 ng/ml hCG were similar to those of unstimulated cells. Cells expressing the wild-type LH/CG-R also demonstrated elevated levels of cAMP upon addition of hCG as early as 24 h after transfection, and the time course was similar to that of YHR-expressing cells.

The dose-response of cAMP production in LH/CG-R and YHR-expressing HEK 293 cells is shown in Fig. 6. The intracellular cAMP levels in LH/CG-R expressing cells increased from a basal level of 4 pmol/mg of protein to saturating levels of about 90 pmol/mg of protein with an EC$_{50}$ value of 6.5 ng/ml (0.2 nM). In contrast, the basal cAMP level in YHR-expressing cells was 45 pmol/mg of protein and showed a negligible increase with increasing concentrations of hCG.

DISCUSSION

We have designed a unique molecule in which a seven-transmembrane G protein-coupled receptor can interact with its covalently linked ligand, itself a covalently linked holoprotein, to generate a stable complex competent in signal transduction. Our results clearly demonstrate that the hormone-receptor complex is constitutively activated in two mammalian cell types. Since this activation is observed in intact cells, the YHR complex must be anchored on the cell surface with the receptor in an activated conformation. Compared to the wild-type receptor, the amount of radiolabeled hCG that can bind YHR is low, presumably because a large fraction of the receptor is already occupied by the covalently linked hormone in a stable interaction. Alternatively, the level of the YHR complex on the cell surface may be much lower than that of the wild-type receptor, although we have no independent determination of the total number of YHR complexes per cell. Interestingly, the small
amount of radiolabeled ligand that is bound in YHR-expressing HEK 293 cells can be competed by unlabeled ligand with an apparent higher affinity than wild-type receptor. However, in these studies, it is important to recognize that the labeled hormone can be competed not only by exogenously added hCG but also by the covalently linked hCG. Since it is not possible to distinguish displacement of labeled ligand by exogenous hCG from displacement by covalently bound hCG, of which the concentration is unknown, the dissociation constant of the covalently linked ligand in YHR cannot be accurately measured.

Additional evidence that the hormone and receptor in the YHR complex form a stable interaction is provided by both the high basal levels of intracellular cAMP and the lack of response of the complex to additional ligand. Interestingly, in transiently transfected COS-7 cells, the basal cAMP levels are elevated as early as 24 h after transfection and rapidly decline at 60 and 72 h after transfection suggesting a decrease in the expression, internalization, or desensitization of the YHR complex.

Western blot analysis of extracts from YHR-expressing cells using an antibody against the CTP revealed two protein bands that most likely represent the mature form (125 kDa) and an immature form (97 kDa) of the YHR complex, e.g., with incomplete carbohydrate processing. Particularly intriguing is the presence of the third 53-kDa protein band that is present in untreated extracts from both COS-7 and HEK 293 cells. Since this species is slightly larger than the yoked hCG, it is likely that it contains a small part of the N terminus of the receptor in addition to the hormone. It is tempting to speculate that a membrane-associated site-specific protease may be responsible for the generation of this purported fragment.

Significantly, these studies demonstrate that the CTP of hCG is competent not only in linking the α and β subunits of hCG but also in joining the hormone to its receptor. The flexible nature of the CTP (12) must be the contributing factor that allows the hormone and receptor to fold correctly and interact with each other. In addition, the CTP sequence is not involved in holoprotein formation, receptor binding, or signal transduction (3, 4), and its only known biological role is to increase the in vivo half-life of hCG (4). Therefore, the CTP may be an effective universal peptide for linking polypeptides in a single chain (18, 19, 24), as well as imparting stability to the resulting protein, and this design may be applied to construct other productive ligand-receptor complexes.

To the best of our knowledge, this is the first report of a covalently linked ligand-receptor complex that is capable of constitutively activating cells. This particular single chain hormone-receptor complex will be quite useful in structure-function studies of this complex ligand and its receptor. We have previously identified several amino acid residues in hCG that are important in receptor binding (25–27) and several in the receptor that are important for signal transduction (17, 22, 23, 28). It will be of interest to determine if the hormone mutants can bind the covalently linked receptor and if the receptor mutants will render the constitutively active hormone-receptor complex inactive.

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