Extracellular Domain of Lutropin/Choriogonadotropin Receptor Expressed in Transfected Cells Binds Choriogonadotropin with High Affinity*

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The lutropin-choriogonadotropin (LH/CG) receptor is a cell surface receptor comprised of two domains of roughly equivalent size. The amino-terminal half of the receptor is relatively hydrophilic and is located extracellularly, whereas the carboxyl-terminal half of the receptor shares amino acid homology with other receptors that couple to G proteins and is similarly thought to span the plasma membrane seven times, ending with a relatively short carboxyl-terminal tail. In order to test the role of the extracellular domain in binding hormone, we constructed a mutated rat luteal LH/CG receptor cDNA (termed pCLHR-D2), which encodes for only the extracellular domain, and used it to transiently transfect human kidney 293 cells. Here we report that the expressed extracellular domain of the LH/CG receptor is capable of binding human CG with a high affinity, comparable with that of the full-length receptor. Thus, not only is the extracellular domain of the glycoprotein hormone receptors involved in binding hormone, but it alone is capable of conferring high affinity binding. Unexpectedly, it was also found that this truncated receptor is not secreted into the culture media but remains trapped within the cells.

The LH/CG receptor recognizes the large (28–38 kDa) glycoprotein hormones LH and hCG. In response to binding either LH or hCG, the receptor increases adenylyl cyclase activity (1–3) via coupling to a G protein. Biochemical studies had shown that the LH/CG receptor is a single glycoprotein with a molecular mass (on sodium dodecyl sulfate-polyacrylamide gels) of 93 kDa (see Ref. 4 for a review). Cloning of the rat luteal and porcine testicular LH/CG receptor cDNAs (5, 6) has revealed that the carboxyl-terminal half of the LH/CG receptor is related to the family of G protein-coupled receptors exemplified by rhodopsin (7, 8) and the adrenergic receptors (9, 10) and, therefore, is thought to similarly span the plasma membrane seven times. Unlike other G protein-coupled receptors, however, the LH/CG receptor contains a large (341 amino acids) amino-terminal domain which we have shown to be located extracellularly (11). The extracellular domain has six potential sites for N-linked glycosylation, and it contains a repetitive leucine-rich motif that is observed in several other leucine-rich glycoproteins (5).

Since the cloning of the LH/CG receptor cDNA, the cDNAs encoding receptors for the other glycoprotein hormones, follicle-stimulating hormone and thyroid-stimulating hormone, have also been cloned (12–16). The three glycoprotein hormone receptors have been shown to be related to each other and to share the same overall structural design. Thus, together, they represent a unique subclass of G protein-coupled receptors in that they possess large extracellular domains.

Binding of the small catecholamine ligands to the adrenergic receptors has been shown to occur by interactions of these ligands with amino acids within the transmembrane helices (17–20). By mechanisms not yet understood, the interactions of agonists within the transmembrane bundle permit the coupling of the receptor, via regions in the intracellular loops and amino-terminal portion of the carboxyl-terminal tail (21), to G. In contrast, the presence of large extracellular domains in the glycoprotein hormone receptors has led investigators to postulate that the binding of the glycoprotein hormones to these receptors would occur through the extracellular domains. Although it appeared likely that the extracellular domain would be involved in the initial recognition of the glycoprotein hormone, it could not be presumed that it alone was sufficient to confer high affinity binding. For example, one might envision a scenario where both the extracellular amino terminal domain and the extracellular loops of the carboxyl-terminal half of the receptor were necessary for recognition and high affinity binding of the ligand. Alternatively, the extracellular amino-terminal domain might bind hormone with low affinity but would subsequently need to associate with regions in the COOH-terminal half of the molecule to lock the binding into a high affinity state.

To address these questions directly, we have constructed a cDNA which encodes for the amino-terminal extracellular domain of the rat luteal LH/CG receptor and have expressed it in human kidney 293 cells. The results presented demonstrate that the extracellular domain of the LH/CG receptor binds hormone with a high affinity comparable with that of the full-length receptor. However, hormone binding activity can only be detected in detergent-solubilized cells.

**MATERIALS AND METHODS**

Construction of the cDNA Encoding the Extracellular Domain—pCLHR, the expression vector pCIS containing the rat luteal LH/CG receptor cDNA, has been previously described (5). A 2546-base pair ClaI/NheI restriction fragment (containing the full coding region of the receptor plus portions of the 5'- and 3'-untranslated regions) was subcloned into a modified PGEMZ (Promega). The modification consisted of replacing the HindIII-BamHI region within the polylinker site with a synthetic oligonucleotide containing HindIII, ClaI, BspHI, Ncol, NheI, and BamHI restriction sites. The resulting plasmid, pGXLHR, was then used as the template for creating a mutated cDNA in which the transmembrane and cytoplasmic tail regions of the receptor were deleted. As such this cDNA would encode for a...
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The amino-terminal extracellular domain of the LH/CG receptor was truncated after the alanine 338. The polymerase chain reaction (PCR) was used to splice out nucleotides 1093-2100 by overlap extension as described by Pease and colleagues (22). The conditions used for the PCR consisted of a 2-min preheating at 94 °C followed by 25 cycles consisting of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. Using synthetic oligonucleotides (24-26 nucleotides in length) and plasmids containing a 5' overhang corresponding to nucleotides 429-1092 and containing a 3' overhang corresponding to nucleotides 1080-1092. Restriction sites for Bgl II and for Nhe I were present near the 5' end of Fragment I (nucleotide 440) and the 3' end of Fragment II (nucleotide 2141), respectively. Fragments I and II were annealed together and amplified by PCR using oligonucleotide primers corresponding to the 5' end of Fragment I and the 3' end of Fragment II, and the product was digested with Bgl II and Nhe I. This yielded a 1015-base pair fragment (designated D2) containing the desired deletion and flanked by Bgl II and Nhe I restriction sites. The 204-base pair Bgl II/Nhe restriction fragment of plasmid pGXLHR was then replaced with D2, yielding pGXLHR-D2. A Clal/Nhel digest of pGXLHR-D2 yielded a fragment containing the D2 deletion, which was subcloned into pCLHR in which the Clal/Nhel fragment had been excised. The identity of the resulting plasmid, called pCLHR-D2, was confirmed by restriction enzyme analysis. The fidelity of the regions that had been amplified by PCR were confirmed by direct sequencing (23).

Transfections — Human embryonic kidney 293 cells (ATCC CRL 1678) were maintained in growth medium (Dulbecco's modified Eagle's medium containing 50 μg/ml gentamicin and 10% heat-inactivated horse serum) in a humidified atmosphere containing 5% CO2. Transient transfections were initiated when the cells were 50-70% confluent, following the procedure of Chen and Okayama (24). Eighteen hours later the cells were washed two times with Waymouth MB752/1 containing 20 μg/ml gentamicin and 1 mg/ml bovine serum albumin, the growth medium was replaced, and the cells were incubated at 4 °C. The assay was finished as described above for binding to solubilized cells.

125I-hCG Binding to Detergent-solubilized Extracts — The cells were placed on ice and were washed three times with ice-cold Buffer A (150 mM NaCl, 20 mM Hepes, pH 7.4). The cells were then scraped into cold Buffer A containing protease inhibitors, (25-27), collected by centrifugation (1900 x g, 15 min), and solubilized as previously described except that Nonidet P-40 was used rather than Triton X-100 (28).

Equilibrium binding constants for the detergent-solubilized receptor were performed by incubating aliquots of the receptor (in Buffer A containing 0.1% Nonidet P-40, 20% glycerol, and protease inhibitors) overnight on ice with increasing concentrations of 125I-hCG in the absence or presence of an excess of crude hCG (final concentration, 50 IU/ml). The assay was finished by filtration (29). All measurements were performed in duplicate and corrected for nonspecific binding. Data were analyzed by the methods of Scatchard, and lines were calculated by linear regression.

125I-hCG Binding to Intact Cells and Culture Media — Cells were placed on ice, and aliquots of culture media were collected and diluted 2-fold into cold Buffer A containing 20% glycerol and protease inhibitors. 125I-hCG binding activity was assayed by incubating the media samples overnight on ice at a saturating concentration (final concentration, 100 ng/ml) of 125I-hCG in the absence or presence of an excess of crude hCG (final concentration, 50 IU/ml). The assay was finished as described above for binding to solubilized cells.

The amino-terminal extracellular domain of the LH/CG receptor could bind hormone, we constructed a cDNA (pCLHR-D2) that encodes for a receptor in which the transmembrane and cytoplasmic domains were deleted. This cDNA contains LH/CG receptor 5'-untranslated sequence, the coding sequences for the signal peptide and the extracellular domain, and 3'-untranslated sequence. The mature truncated receptor expected from expression of pCLHR-D2 is shown in Fig. 1. Not shown in Fig. 1 is the 26-amino acid signal sequence (5) that is not in the mature receptor.

Human embryonic kidney 293 cells were transiently transfected with an expression vector containing the full-length cDNA of the rat luteal LH/CG receptor (pCLHR) (5) or with pCLHR-D2, which was constructed with the same expression vector. Since pCLHR-D2 encoded for a truncated receptor which now lacked the membrane-spanning domains and since the encoded extracellular domain was preceded by a signal sequence, we had predicted that the truncated receptor encoded for by pCLHR-D2 would be secreted into the culture medium. However, no specific 125I-hCG binding activity was observed in conditioned culture media (data not shown) under conditions in which as little as 6 fmol of receptor/ml of culture medium could have been detected. These data suggest that either the truncated receptor is not secreted from the cells, that it is secreted but degraded too rapidly to detect, or that...
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it is secreted but in a form unable to bind hormone. We next tested whether $^{125}$I-hCG binding activity could be detected on the cell surface. Although cell surface high affinity $^{125}$I-hCG binding was observed in cells transfected with pCLHR (see Fig. 2), no cell surface binding (at any concentration of $^{125}$I-hCG tested) was detected in cells transfected with pCLHR-D2 (data not shown).

Finally, we tested whether $^{125}$I-hCG binding activity could be observed in detergent-solubilized extracts of cells transfected with pCLHR-D2. As a control, pCLHR was used to transfect cells, and $^{125}$I-hCG binding was assayed on both the intact cells and in detergent-solubilized extracts of the cells. Solubilization of the cells had no effect on the affinity of the LH/CG receptor for $^{125}$I-hCG. Cells were then transfected with either pCLHR or pCLHR-D2, and $^{125}$I-hCG binding was assayed in detergent-solubilized extracts of the cells (Fig. 2). As shown in Fig. 3, binding activity was indeed detected in the detergent extracts of cells transfected with pCLHR-D2. Moreover, the extracellular domain encoded by pCLHR-D2 binds $^{125}$I-hCG with a high affinity ($K_d = 194$ PM), similar to that of the full-length receptor encoded by pCLHR.

**DISCUSSION**

The glycoprotein hormone receptors have been shown to be structurally related to each other and to consist of single polypeptides which both bind hormone and activate adenylyl cyclase when occupied by agonist (5, 6, 12–16). Whereas the carboxyl-terminal halves of these receptors share significant amino acid homology with other G protein-coupled receptors, these receptors also contain large amino-terminal domains, shown to be situated extracellularly (11). The data presented herein demonstrate that the extracellular domain of the LH/CG receptor, expressed in 293 cells from an expression vector containing a mutated LH/CG receptor cDNA (pCLHR-D2), is capable of binding $^{125}$I-hCG with a high affinity comparable with that of the full-length receptor. Thus, an interaction of hCG with the extracellular loops or membrane-spanning regions of the LH/CG receptor does not appear to be necessary to achieve high affinity binding.

It has been shown for the $\beta$-adrenergic receptors that the affinity of this receptor for agonist is dependent upon its association with $G_\text{s}$, such that a high affinity state is observed when the receptor is coupled to $G_\text{s}$ and a low affinity state generated when this complex is dissociated. This is reflected as a GTP-dependent shift from high to low affinity of the receptor-agonist interaction (see Ref. 31 for a review). Most investigators, however, cannot document a similar GTP-de-
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Independent shift in the affinity of LH/CG receptor for agonist (see Ref. 22, however). Given our current understanding of the structures and binding domains of the LH/CG and β-adrenergic receptors, it is possible to put forth a hypothesis that would reconcile this apparent discrepancy. Thus, since the ligands for the β-adrenergic receptor intercalate within the transmembrane helices, it is possible to envision how a physical interaction of the receptor with G, (occuring through the intracellular loops and cytoplasmic tail of the receptor) could effect a conformational change in the transmembrane helices that would in turn alter (increase) the affinity of the binding of the agonist. Although a physical interaction of the LH/CG receptor with G might invoke a similar conformational change within the transmembrane helices, since LH and hCG bind to the extracellular domain of the receptor, this change may not lead to an alteration in the affinity of the receptor for agonist. From the studies presented herein we conclude that, unlike the β-adrenergic receptor, the high affinity binding of agonist to the LH/CG receptor does not require a physical interaction of the receptor with G,.

Our results, showing that high affinity binding of hCG can occur solely to the extracellular domain of the LH/CG receptor, do not preclude a subsequent interaction of a region (s) of the extracellular domain and/or hormone with the transmembrane helices. In fact, this hypothesis seems plausible since it is unlikely that the general mechanisms underlying the translation of ligand binding to receptor-G protein coupling would be fundamentally different between different G protein-coupled receptors. Clearly, more work is required to address the mechanism by which LH or hCG binding to the LH/CG receptor leads to receptor-G coupling and activation.

The growth hormone receptor has been shown to express its extracellular domain in a water-soluble form which binds hormone with high affinity (33). This binding protein, present in serum, may have important physiological functions. The cloning of the rat luteal and porcine testicular LH/CG receptors revealed cDNAs encoding for truncated forms of the receptor containing only the extracellular domain (6, 34). These data raised the possibility that a LH/CG binding protein, consisting of the extracellular domain of the receptor, might be expressed by gonadal cells. Although this possibility cannot be ruled out, data thus far suggest that even if the mRNA for such a truncated receptor is present in gonadal cells, it does not appear to be expressed (or expressed into a stable protein). Furthermore, the data presented herein show that a truncated LH/CG receptor protein representing the extracellular domain is not secreted from 293 cells.

The finding that the extracellular domain encoded for by pCLHR-D3 was not secreted from the cells was unexpected. The mechanism of this was considered was that it might be anchored to the plasma membrane. In this respect, it should be noted that expression of a cDNA encoding a truncated platelet-derived growth factor receptor resulted in the extracellular domain of that receptor being attached to the plasma membrane through a lipid linkage (35). We did not, however, detect 125H-hCG binding activity to intact cells transfected with pCLHR-D2. From the experiments shown, we can conclude that the truncated receptor remains trapped intracellularly; however, we do not yet know where it is located and whether it is glycosylated or not. Studies are currently under way to address these questions.

In summary, an extracellular domain of the rat luteal LH/CG receptor has been expressed in transfected cells and shown to bind hCG with a high affinity comparable with the full-length receptor. These results clearly demonstrate that this region of the glycoprotein hormone receptors is not only involved in the initial recognition and binding of hormone, but it is entirely capable of conferring high affinity binding.

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