Co-expression of Defective Luteinizing Hormone Receptor Fragments Partially Reconstitutes Ligand-induced Signal Generation*

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Gonadotropin receptors are unique members of the seven-transmembrane (TM), G protein-coupled receptor family with a large extracellular (EC) sequence forming the high-affinity ligand binding domain. In a patient with Leydig cell hypoplasia, we identified a mutant LH receptor that is truncated at TM5. This protein retains limited ligand binding ability but cannot mediate cAMP responses. To study interactions between receptor fragments defective in either ligand binding or signal transduction, we co-expressed this truncated receptor together with a chimeric receptor containing the EC region of the FSH receptor and the TM region of the LH receptor. Although the chimeric receptor could not respond to human chorionic gonadotropin in producing cAMP, co-expression with the truncated LH receptor allowed partial restoration of ligand signaling through intermolecular interactions. In addition, co-expression of the same truncated LH receptor with an N-terminally truncated LH receptor that lacked the EC ligand binding domain also partially restored ligand signaling. Further shortening of the TM region in the mutant receptor found in the patient indicated that the EC domain and TM1 were sufficient for interactions with the N terminally truncated receptor. In contrast, co-expression of the N terminally truncated receptor together with cell-associated or soluble EC region of the LH receptor did not allow ligand signaling. Unlike thrombin receptors, co-expression of the anchored EC region of the LH receptor together with the N-terminally truncated receptor did not allow ligand signaling despite moderate levels of human chorionic gonadotropin binding in transfected cells. These studies demonstrate that the co-expression of binding (+)/signaling (−) and binding (−)/signaling (+) receptor fragments partially restores ligand-induced signal generation and indicate the importance of TM1 of the LH receptor in the proper orientation of the EC ligand binding domain.

Luteinizing hormone (LH),1 FSH, and thyrotropin receptors belong to the large guanine nucleotide regulatory protein (G protein)-coupled protein family (1–5). Molecular cloning analysis indicated that proteins in this family share a common structure, consisting of seven α-helical hydrophobic putative transmembrane (TM) regions, joined by three extra and intracellular loops. Agonist occupancy of these G protein-coupled receptors leads to the activation of different G proteins, which, in turn, modulate the activity of different effector enzymes or ion channels. The receptors for LH, FSH, and thyrotropin represent a small subclass of this superfamily that has a large extracellular (EC) amino-terminal region responsible for high affinity binding of their large (28–38-kDa) ligands (6–10). The EC region of these receptors is encoded by multiple exons and contains leucine-rich repeat sequences important for ligand binding (11–14), whereas the C-terminal half of the receptor endodomain is encoded by a single exon and represents the signal-transducing component (9). Occupancy of glycoprotein hormone receptors by specific ligands allows stimulation of G proteins and activation of the cAMP-protein kinase A pathway (15, 16).

Earlier studies using β2-adrenergic, muscarinic, and angiotensin II receptors and rhodopsin indicate that the TM regions of these heptahelical molecules are composed of independent functional units and that the co-expression of receptor fragments allows partial reconstitution of functional proteins (17–22). In artificial membrane preparations, fragments of bacteriorthodopsin also refold into stable TM helices with partial restoration of protein function (23–25). In addition, co-expression of receptor peptides leads to the functional rescue of mutant V2 vasopressin receptors found in patients with nephrogenic diabetes insipidus (26). For receptors used in these studies, their TM regions are important for both ligand binding and signal transduction, thus rendering it difficult to separate the two important functions of these proteins.

For gonadotropin and TSH receptors, their ligand binding and signal transduction domains can be separated (6, 7, 11, 27, 28). The thrombin receptor, a protein evolutionarily related to these glycoprotein hormone receptors, also has a large N-terminal domain and is activated by proteolytic cleavage within its EC region to unmask a new N-terminal peptide sequence capable of binding to and activating the TM region (29). Of interest, an N-terminally truncated thrombin receptor defective in thrombin signaling can be rescued by co-expression with the N-terminal EC region of the thrombin receptor anchored to the cell surface by the TM domain of CD8 (30), suggesting intermolecular interactions.

We have recently found a mutant LH receptor truncated at TM5 in a patient with Leydig cell hypoplasia. The defective LH-binding protein; LHR, LH receptor.

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1 The abbreviations used are: LH, luteinizing hormone; TM, transmembrane; EC, extracellular; hCG, human chorionic gonadotropin; FSH, follicle-stimulating hormone; G protein, GTP-binding protein; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; LBP,

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receptor retained limited ligand binding ability but was incapable of mediating cAMP responses (31). Taking advantage of the unique separation of ligand binding and signal transduction domains of gonadotropin receptors, we have studied the interactions between this binding (+) /signaling (−) mutant receptor and several receptor mutants defective in ligand binding but retaining their C-terminal transmembrane endodomain. We have also tested if the anchored receptor approach used for the related thrombin receptor could allow restoration of ligand signaling for anchored LH receptors. Our findings suggest that ligand signaling can be partially restored when defective LH receptors are co-expressed, but, unlike thrombin receptors, TM1 is essential for interactions between gonadotropin receptor fragments.

EXPERIMENTAL PROCEDURES

Hormones and Reagents—Purified hCG (CR-129) and FSH (I-3) were supplied by the National Hormone and Pituitary Program (NIDDK, National Institutes of Health). 125I-Sodium was purchased from Amersham Corp. Human LH and FSH receptor cDNAs have been cloned and characterized as described previously (32, 33). Fetal bovine serum was obtained from Gemini (Calabasas, CA).

Construction of Mutant Receptor cDNAs—PCR-based mutagenesis was performed using overlapping primers to construct cDNAs for mutant LH receptors and chimeric FSH/LH receptors (Fig. 1) as described previously (34). PCR was performed with deep VENT® DNA polymerase (New England Biolabs, Beverly, MA). L(EC-TM1–7), L(EC-TM1–3), L(EC-TM1), and L(EC) represent LH receptor mutants with truncation at amino acids 546, 462, 388, and 363, respectively. L(TM1–7) was constructed according to Ji and Ji (35) to express the endodomain of the rat LH receptor and encodes parts of exons 1 and 10 of the human LH receptor. Junctional amino acid sequences of chimeric receptors are listed below together with the amino acid number for each receptor at the junctional site (represented by a slash): FLR (FSH receptor EC region fused to the TM region of LH receptor), . . . RALRE27/359YDFLR . . . ; Ref. 32); NPCED355/372FLR . . . ; Ref. 32); RVL364LIWLI . . . ; Refs. 32 and 33); L(TM1–7) (TM region plus 10 amino acids at the N-terminal region, . . . RLARLE27/359YDFLR . . . ; Ref. 32); L(EC)CD8 (EC region of LH receptor fused to the CD8 TM region, . . . YDFLR29/38/359DIY W . . . ; Ref. 36); L(EC)CD8 (EC region of LH receptor fused to the CD8 TM region through a thrombin cleavage site present in the thrombin receptor EC region, . . . NPCED29/38ATLDF . . . NESGL/372/38/359/372YIWA . . . ; Ref. 29).

All cDNAs were subcloned into the expression vector pcDNA3 (Invitrogen, San Diego, CA). When PCR was used to generate plasmids, two or three clones derived from different PCR were prepared for each construct and used for expression studies. Both the fidelity of PCR-amplified regions and the junctional sequences were confirmed by sequencing on both strands using the dideoxy chain termination method (37) as well as by digestion with appropriate restriction enzymes. All plasmids were purified using a Maxi plasmid preparation kit (Qiagen, Chatsworth, CA) (37) as well as by digestion with appropriate restriction enzymes. All plasmids were transfected using a Maxi plasmid preparation kit (Qiagen, Chatsworth, CA). DNA concentration and plasmid purity were estimated by reading optical density at 260/280 nm and confirmed using ethidium bromide staining following agarose gel electrophoresis.

Transfection of Cells and Analysis of Signal Transduction—293 cells derived from human embryonic kidney fibroblast were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Before transfection, 2 × 10^6 cells were seeded in 10-cm dishes (Nunc, Naperville, IL). When cells were 70–80% confluent, transient transfection was performed using up to 30 μg of expression vector with or without cDNA inserts by the calcium phosphate precipitation method (34, 38). Cells transfected with the empty plasmid (mock) served as negative controls. In cells co-transfected with a single construct, 15 μg of DNA containing insert cDNA was mixed with same amounts of empty plasmid. After 12–16 h of incubation with the calcium phosphate-DNA precipitates, media were replenished with Dulbecco’s modified Eagle’s medium/Ham’s F-12, 10% fetal bovine serum. 12–36 h after transfection, cells were washed twice with PBS, harvested from culture dishes, and centrifuged at 400 × g for 5 min. Cell pellets were then resuspended in Dulbecco’s modified Eagle’s medium/Ham’s F-12 supplemented with 0.1% bovine serum albumin. 200,000 cells in 300 μl were placed on 24-well tissue culture plates (Corning, Corning, NY) and preincubated at 37 °C for 30 min in the presence of 0.25 mM 3-isobutyl-1-methyl xanthine (Sigma) before treatment with or without hormones for 3 h. At the end of incubation, cells and medium in each well were frozen and thawed once and then collected and boiled at 95 °C for 3 min to inactivate phosphodiesterase activity. Total cAMP in each well was measured in triplicates by specific radioimmunoassay (39). All experiments were repeated at least three times using cells from independent transfections. Statistical analysis was performed using Student’s t test.

Ligand Binding Analysis—Purified hCG was iodinated by the lactoperoxidase method (40) and characterized by radioligand receptor assay using human LH receptors stably expressed in 293 cells. Specific activity and maximal binding of the labeled hCG were 100,000–150,000 cpm/ng and 40–50%, respectively. To estimate ligand binding on the cell surface, cells were washed twice with PBS and collected in PBS before centrifugation at 400 × g for 5 min. Pellets were resuspended in PBS containing 0.1% bovine serum albumin and incubated at 37 °C for 3 h. Conditioned media were concentrated 250-fold using DIAFLO ultrafiltration membrane XM50 and Centricon 30 (Amicon, Bradford, MA). Aliquots of the concentrated media were incubated with labeled hCG (5 ng/ml, final concentration) for 6 h at room temperature. Complexes formed between labeled hCG and the soluble EC region of the LH receptor were cross-linked using diisuccinimidyl carbonate (2 mM) for 1 h, and the reaction was terminated with the addition of 3.6 mM Tris-HCl, pH 7.4. After the addition of Laemmli buffer without reducing reagents, cross-linked complexes were visualized following fractionation using SDS-polyacrylamide (10%) gel electrophoresis and autoradiography. In duplicate experiments, complexes between nonlabeled hCG and the soluble EC region of the LH receptor were cross-linked using diisuccinimidyl carbonate (2 mM) for 1 h, and the reaction was terminated with the addition of 3.6 mM Tris-HCl, pH 7.4. After the addition of Laemmli buffer without reducing reagents, cross-linked complexes were visualized following fractionation using SDS-polyacrylamide (10%) gel electrophoresis and autoradiography. In duplicate experiments, complexes between nonlabeled hCG and the soluble EC region of the LH receptor similarly prepared were incubated with 293 cells expressing L(TM1–7) for 3 h at 37 °C to estimate cAMP stimulation. For studies using cells transfected with the plasmid encoding L(ECD), similar ligand binding and cross-linking analyses were performed using intact cells followed by solubilization with buffer containing 0.1% Nonidet P-40 and 20% glycerol or by using total cell homogenates to estimate intracellular and cell surface ligand binding.
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**RESULTS**

Co-expression of LH Receptors Truncated at Different TM Domains Together with a Chimeric Receptor FLR, but Not with the Wild Type FSH Receptor, Partially Restores Ligand Signaling—A mutant receptor identified in a patient with Leydig cell hypoplasia was found to have a stop codon at TM5. Although low levels of high-affinity ligand binding to this truncated receptor L(EC-TM1–5) (Fig. 1) could still be found, cAMP stimulation by hCG was impaired (31). We have generated a chimeric receptor FLR containing the EC region of the FSH receptor and the TM region of the LH receptor (34). In cells expressing FLR, FSH but not hCG stimulated cAMP production. We tested if ligand signaling could be restored in cells co-transfected with the truncated LH receptor L(EC-TM1–5) together with FLR. As shown in Fig. 2, no stimulation of cAMP production by hCG was found in cells transfected with plasmids encoding either L(EC-TM1–5) or FLR. In contrast, cells co-transfected with both plasmids responded to hCG treatment and showed dose-dependent increases in cAMP production. Significant stimulation of cAMP production was found at 30 ng/ml hCG (p < 0.01). Furthermore, hCG treatment (1 μg/ml) did not stimulate cAMP production in cells expressing the wild type FSH receptor (FLR + hCG) together with L(TM1–7). As shown in Fig. 3, treatment with 1 μg/ml of hCG did not stimulate cAMP production in cells co-transfected with L(EC) together with L(TM1–7) as compared with a major stimulation of cAMP by hCG (100 ng/ml) in cells co-expressing L(TM1–7) and L(EC-TM1–3). These data suggested that the presence of TM1 is sufficient to partially restore ligand signaling.

Because an earlier report suggested that co-expression of the EC region and endodomain of the porcine LH receptor could allow hCG stimulation of cAMP production (41), we constructed the plasmid L(EC) encoding the EC region of the human LH receptor but lacking the endodomain (Fig. 1). As shown in Fig. 3C, treatment with 1 μg/ml of hCG did not stimulate cAMP production in cells co-transfected with L(EC) together with L(TM1–7) as compared with a major stimulation of cAMP by hCG (100 ng/ml) in cells co-expressing L(TM1–7) and L(EC-TM1). To demonstrate that L(EC) could still bind hCG, ligand cross-linking experiments were performed. As shown in Fig. 3D (left panel), formation of high molecular mass complexes (87 kDa) between labeled hCG and L(EC) was found in the total cell extract from cells co-transfected with plasmids encoding L(EC) and L(TM1–7), and the complex formation could be competed by nonlabeled hCG. However, cross-linking of labeled hCG to plasma membrane proteins in the same cells did not lead to complex formation in direct contrast to the formation of high molecular mass, computable complexes (130 kDa) between labeled hCG and wild type LH receptor (Fig. 3D, right panel). These data suggest minimal restoration of receptor function when cells were co-transfected with plasmids encoding L(TM1–7) and L(EC) under the present experimental conditions and the importance of TM1 in ligand signaling.

Co-expression of the Anchored EC Region of LH Receptor with the LH Receptor Endodomain Did Not Restore Ligand Signaling—Earlier studies demonstrated that the ligand-binding EC region of the thrombin receptor, anchored on the cell surface through the single TM region of CD8, interacted efficiently with the TM segments (endodomain) of the thrombin receptor to restore ligand signaling (30). We also anchored the EC re-
region of the LH receptor to the single TM domain of CD8 to facilitate ligand binding to the cell surface (Fig. 1). As shown in Fig. 4A, moderate levels of hCG binding were found in cells expressing the anchored chimeric receptor L(EC)CD8. Although hCG binding to L(EC)CD8 was lower than that of the wild type LH receptor, it was much higher than for cells expressing L(EC-TM1–5). In addition, co-expression of cells with plasmids encoding L(TM1–7) and L(EC)CD8 did not increase hCG binding above that in cells expressing L(EC)CD8 alone. Signal transduction of cells expressing these mutant receptors was also analyzed. As shown in Fig. 4B, hCG stimulation of cAMP production was found in cells co-expressing L(EC-TM1–5) (Fig. 1). However, no stimulation of cAMP production by hCG could be detected in cells co-expressing L(EC-TM1–7). These data suggested that the LH receptor is different from the related thrombin receptor in that co-expression of its TM endodomain together with its anchored EC region fused to a foreign TM domain could not restore ligand signaling.

**Inability of the Soluble EC Region of the LH Receptor (LH-binding Protein; LBP) Complexed with hCG to Activate the TM Endodomain of the LH Receptor**—We further investigated whether the soluble EC region of the LH receptor complexed with its ligand hCG could activate the TM endodomain. We constructed a chimeric anchored receptor, L(EC)tCD8 (Fig. 1), by fusing the EC region of the LH receptor to the single TM region of CD8 through the thrombin cleavage site found in the thrombin receptor to allow proteolytic cleavage. Following expression of this anchored receptor in 293 cells, thrombin was added to the culture media to allow proteolytic cleavage. We concentrated large amounts of the conditioned media containing the LH receptor EC region and incubated the media with 125I-hCG for 6 h at 23 °C before cross-linking using disuccinimidyl suberate. As shown in Fig. 5A, the EC region of the LH receptor cleaved after thrombin treatment formed complexes with labeled hCG. In SDS-polyacrylamide gel, a lower band showed the migration pattern of labeled hCG.
the presence of a complex between hCG and the EC region of the LH receptor. Although the exact amount of this soluble LBP was unknown, the concentrated, conditioned media allowed the binding of 95% of hCG to form the high molecular mass complexes (left lane). Stimulation of cAMP by hCG in cells co-expressing L(EC-TM1–5) and L(TM1–7) is also shown. Values shown are mean ± S.E.

FIG. 4. Inability of hCG to activate cells co-expressing the anchored EC region of the LH receptor together with the endodomain. A, moderate levels of hCG binding to the anchored EC region of the LH receptor L(EC)CD8 as compared with low hCG binding to cells expressing L(EC-TM1–5). Specific 125I-hCG binding to 293 cells (10^6 cells/group) transfected with different receptor plasmids is shown. B, lack of cAMP stimulation by hCG in cells co-transfected with plasmids encoding L(EC)CD8 and L(TM1–7). Stimulation of cAMP by hCG in cells co-expressing L(EC-TM1–5) and L(TM1–7) is also shown. Values shown are mean ± S.E.

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FIG. 5. Inability of the soluble EC region of the LH receptor complexed with hCG to activate the endodomain of the LH receptor. A, cross-linking of 125I-hCG to soluble EC region of the LH receptor. The EC region of the LH receptor anchored on the cell surface was cleaved following thrombin treatment. Conditioned media containing the soluble EC region (LBP) were concentrated and incubated with 3 μg/ml of hCG before cross-linking analysis. The complexes formed between the ligand and the EC region were separated from the free ligand using SDS-polyacrylamide gels. B, lack of cAMP stimulation by hCG in L(TM1–7)-expressing cells incubated with complexes of hCG and the EC region of the LH receptor (LBP). Cells expressing L(TM1–7) and L(EC-TM1–5) were treated with 100 ng/ml hCG to serve as positive controls. Values shown are mean ± S.E.

DISCUSSION

TM helices of G protein-coupled receptors are believed to represent independent folding units and form a tightly packed channel-like structure (5). Our study indicated that co-transfection of cells with LH receptor fragments or chimeric gonadotropin receptors defective in either ligand binding or signal transduction led to functional complementation and ligand-activated signal generation (Fig. 6A). Studies using the EC region of the LH receptor alone, the EC region co-expressed through the heterologous single TM domain of CD8 to the cell surface, or as soluble complexes with its ligand, further suggested that the TM1 region of the LH receptor is important for receptor function. The large EC region of the LH receptor, when connected to one or several of the TM domains, can be reconstituted into functional proteins after co-expression with its own endodomain. The observed interactions between receptor fragments took place with TM1 connected to the EC region.
in cells co-expressing L(EC-TM1) together with L(TM1–7) or with FLR. This interaction is receptor-specific, because co-expression of L(EC-TM1–5), together with the wild type FSH receptor, was ineffective in restoring ligand signaling. Our results are consistent with earlier studies showing that the function of truncated β-adrenergic, vasopressin V2 and muscarinic M3 receptors could be reconstituted when co-expressed with the missing TM folding domains (reviewed in Ref. 21).

The molecular basis of the interactions between different mutant LH receptors is not clear. Transient (collisional) oligomer formation at the fluid cell surface could allow ligand signaling. Alternatively, co-expression of mutant receptors could lead to the “rescuing” of misfolded receptor fragments through receptor complementation and proper trafficking of functional complexes to the plasma membrane (21). Although our early studies indicated that the majority of the L(EC-TM1–5) protein was trapped intracellularly (31), co-expression of both L(EC-TM1–5) and L(TM1–7) did not lead to higher cell surface binding despite partial restoration of ligand signaling. Dimerization of adrenergic receptors has been proposed to be important for ligand signaling (42). It is, however, unclear whether receptor dimerization is required for all G protein-coupled receptors. The metabotropic glutamate receptor, which has a large EC domain similar to the gonadotropin receptors, forms disulfide-linked dimers through its EC domain (43). The observed functional complementation between L(EC-TM1–5) and L(TM1–7), which lacks the EC region, indicated that interactions between EC domains are not obligatory for LH receptor function.

Our attempts to restore LH receptor function by expressing ecto- and endodomains separately did not lead to ligand signaling, unlike an earlier study using porcine LH receptor fragments (41). Although the exact reason for the observed discrepancies is unclear, ligand cross-linking analysis indicated that the expression levels for the EC ectodomain are low and the proteins formed are trapped inside transfected cells. The intracellular form of the EC fragent of LH receptor is probably not fully glycosylated because it is smaller than the cleaved EC region (LBP) derived from the anchored chimeric receptor L(EC)CD8.

Activation of the LH receptor appears to require proper orientation between the EC and TM segments, and the integrity of the EC/TM1 junction is a prerequisite for a functional protein (Fig. 6B). Although efficient formation of complexes could be demonstrated between hCG and the soluble EC domain or hCG and the EC region anchored to the TM domain of CD8, these complexes could not activate L(TM1–7) that lacked the EC region. Based on the proportion of complexes formed between hCG and the soluble EC region of the LH receptor, up to 3 μg/ml hCG binding equivalent of the complexes was incubated with the cells expressing L(TM1–7), but no stimulation of cAMP production was evident. Moreover, co-expression of
L(TM1–7) and L(TM1–7) partially restore ligand signaling suggest that overexpression of L(TM1–7) in testis cells could form the basis of gene therapies to rescue genetic defects found in these patients. A similar approach has allowed the restoration of the function of defective vasopressin V2 receptors found in patients with nephrogenic diabetes insipidus (26). The present finding extends the co-expression strategy in the treatment of diseases caused by inactivating mutations in the seven-TM receptor family.

In conclusion, the observed complementation of LH receptor fragments defective in either signal transduction or ligand binding provides a unique model to study the bifunctional receptor molecule and indicates the unique role of TM1 and/or the EC/TM1 junction of gonadotropin receptors in signal transduction.

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