The Ectodomain of the Luteinizing Hormone Receptor Interacts with Exoloop 2 to Constrain the Transmembrane Region

STUDIES USING CHIMERIC HUMAN AND FLY RECEPTORS

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The receptors for lutropin (LH),1 follitropin (FSH), and thyrotropin (TSH) belong to a group of leucine-rich repeat-containing, G protein-coupled receptors (LGRs) found in vertebrates and flies. We fused the ectodomain of human LH or FSH receptors to the transmembrane region of fly LGR2. The chimeric human/fly receptors, unlike their wild type counterparts, exhibited ligand-independent constitutive activity. Because ectodomains likely interact with exoloops to constrain the receptors, individual exoloops of the chimeric receptor containing the ectodomain of the LH receptor and transmembrane region of fly LGR2 was replaced with LH receptor sequences. Chimeric receptors with the ectodomain and exoloop 2, but not exoloop 1 or 3, from LH receptors showed decreased in constitutive activity, but ligand treatment stimulated cAMP production. Furthermore, substitution of key residues in the hinge region of fly LGR2 with LH receptor sequences led to constitutive receptor activation; however, concomitant substitution of the homologous exoloop 2 of the LH receptor decreased Gs coupling. These results suggest that the hinge region of the LH receptor interacts with exoloop 2 to constrain the receptor in an inactive conformation whereas ligand binding relieves this constraint, leading to Gs activation.

Received for publication, October 4, 2001, and in revised form, November 21, 2001
Published, JBC Papers in Press, November 26, 2001, DOI 10.1074/jbc.M109617200

The Journal of Biological Chemistry
Vol. 277, No. 6, Issue of February 8, pp. 3958–3964, 2002
Printed in U.S.A.

Lutropin (LH) and follitropin (FSH) receptors belong to a group of leucine-rich repeat-containing, G protein-coupled receptors (LGRs) found in vertebrates and flies. We fused the ectodomain of human LH or FSH receptors to the transmembrane region of fly LGR2. The chimeric human/fly receptors, unlike their wild type counterparts, exhibited ligand-independent constitutive activity. Because ectodomains likely interact with exoloops to constrain the receptors, individual exoloops of the chimeric receptor containing the ectodomain of the LH receptor and transmembrane region of fly LGR2 was replaced with LH receptor sequences. Chimeric receptors with the ectodomain and exoloop 2, but not exoloop 1 or 3, from LH receptors showed decreased in constitutive activity, but ligand treatment stimulated cAMP production. Furthermore, substitution of key residues in the hinge region of fly LGR2 with LH receptor sequences led to constitutive receptor activation; however, concomitant substitution of the homologous exoloop 2 of the LH receptor decreased Gs coupling. These results suggest that the hinge region of the LH receptor interacts with exoloop 2 to constrain the receptor in an inactive conformation whereas ligand binding relieves this constraint, leading to Gs activation.

The receptors for lutropin (LH),1 follitropin (FSH), and thyrotropin (TSH) belong to the large G protein-coupled receptor (GPCR) family with seven-transmembrane (TM) helices, but are unique in having a large N-terminal extracellular (ecto-) domain containing leucine-rich repeats important for interaction with the glycoprotein ligands (1, 2). Recent studies indicate the evolution of a large family of the leucine-rich repeat-containing, G protein-coupled receptors (LGRs) with at least seven members in mammals, including the well studied glycoprotein hormone receptors (3, 4) and LGR4–7 (5, 6). In addition, homologous LGRs were found in fly (LGR1 and LGR2) (7, 8), nematode (nLGR) (9), sea anemone (10), and snail (11). These genes can be divided into three subgroups, each with unique structural characteristics (6). Understanding of the evolution-
Ectodomain of LH Receptor Interacts with the Exoloop 2

RESULTS

Chimeric Receptors with the Ectodomain from Human LH or FSH Receptors and the TM Region from Fly LGR2 Showed Constitutive Activity—Although both wild type human LH receptor and fly LGR2 showed minimal basal cAMP production when overexpressed in human 293T cells, the chimeric receptor LDR, with the ectodomain from the LHR receptor and the TM region from fly LGR2, showed major increases in basal cAMP production (Fig. 1). Due to variations in receptor expression levels, all cAMP data for this and subsequent experiments were normalized based on cell surface M1 antibody binding (Table I). For determination of cAMP data without normalization were also presented (Table II). Of interest, the chimeric LDR showed high constitutive activity, which was increased by hCG treatment with further increases in cAMP production (Fig. 1). Due to variations in receptor expression levels, all cAMP data for this and subsequent experiments were normalized based on cell surface M1 antibody binding (Table I). For determination of cAMP data without normalization were also presented (Table II). Of interest, the chimeric LDR showed high constitutive activity, which was increased by hCG treatment with further increases in cAMP production (Fig. 1). Due to variations in receptor expression levels, all cAMP data for this and subsequent experiments were normalized based on cell surface M1 antibody binding (Table I). For determination of cAMP data without normalization were also presented (Table II). Of interest, the chimeric LDR showed high constitutive activity, which was increased by hCG treatment with further increases in cAMP production (Fig. 1). Due to variations in receptor expression levels, all cAMP data for this and subsequent experiments were normalized based on cell surface M1 antibody binding (Table I). For determination of cAMP data without normalization were also presented (Table II). Of interest, the chimeric LDR showed high constitutive activity, which was increased by hCG treatment with further increases in cAMP production (Fig. 1). Due to variations in receptor expression levels, all cAMP data for this and subsequent experiments were normalized based on cell surface M1 antibody binding (Table I). For determination of cAMP data without normalization were also presented (Table II). Of interest, the chimeric LDR showed high constitutive activity, which was increased by hCG treatment with further increases in cAMP production (Fig. 1).
Expression of different receptors was estimated based on the cell surface binding of the labeled M1 antibody against the FLAG epitope appended to the N terminus of individual receptors. All results were compared with the expression level of the wild type LH receptor that is set at 100%. Iodinated hCG was used as a tracer in the radioligand receptor assay to determine the $K_d$ and maximal binding of the wild type LH receptor and various chimeric receptors. For fly LGR2, LDR(EL-3), and LDR(EL-2,3), negligible binding was detected.

| Cell surface expression | Ligand binding | $K_d$ (nm) | Maximal binding (pmol/mg protein) |
|-------------------------|----------------|----------|---------------------------------|
| LHR WT                  | 100            | 103.7 ± 12.1 | 237,142 ± 34,997 |
| fly LGR2                | 43             | 0.08 ± 0.06  | 390.92 ± 23.27 |
| LDR                     | 102 ± 0        | 33.9 ± 0.5   | 253,098 ± 10,858 |
| LDR (EL-1)              | 102 ± 3        | 52.5 ± 5.5   | 402,299 ± 57,856 |
| LDR (EL-2)              | 102 ± 7        | 25.2 ± 3.3   | 345,821 ± 22,877 |
| LDR (EL-3)              | 116 ± 2        | N.D.         | N.D. |
| LDR (EL-1,2)            | 101 ± 2        | 49.7 ± 6.8   | 626,788 ± 40,310 |
| LDR (EL-1,3)            | 111 ± 4        | 32.0 ± 2.4   | 158,130 ± 32,945 |
| LDR (EL-2,3)            | 87 ± 1         | N.D.         | N.D. |

* N.D., not detectable.

We further tested whether a chimeric receptor FDR with the ectodomain from the human FSH receptor and the TM region from the fly LGR2 could also confer constitutive activity. As shown in Fig. 2A, the chimeric FDR also showed major increases in basal cAMP production. In addition, treatment with FSH further increased cAMP production. This chimeric receptor exhibited high affinity to labeled FSH, comparable with that of the wild type FSH receptor based on Scatchard plot analysis (Fig. 2B). The cell surface expression of FDR, estimated using M1 antibody binding, was 59 ± 1% of that of the wild type FSH receptor.

Substitution of Exoloop 2 from the LH Receptor in the Chimeric LDR Reduced Basal Activity but Not Agonist Activation—Based on the hypothesis that the ectodomain of the LH receptor could contact one or more of the exoloops in the TM region to constrain the receptors to an inactive conformation, we further substituted individual exoloops of the chimeric LDR with the LH receptor sequence. As shown in Fig. 3, the length of the three exoloops is conserved between the human LH receptor and fly LGR2. Among these residues, 36 (9/25), 33 (7/21), and 33 (4/12)% are conserved in exoloops 1, 2, and 3, respectively. Although all three mutants with individual exoloop substitution were expressed on the cell surface (Table I and Fig. 4A), two mutants, LDR(EL-1) and LDR(EL-2), showed high affinity binding to labeled hCG based on Scatchard plot analysis (Table I and Fig. 4C), but the LDR(EL-3) mutant showed negligible ligand binding, likely the result of an abnormal conformation.

Table I

| Cell surface expression | Ligand binding | $K_d$ (nm) | Maximal binding (pmol/mg protein) |
|-------------------------|----------------|----------|---------------------------------|
| LHR WT                  | 100            | 103.7 ± 12.1 | 237,142 ± 34,997 |
| fly LGR2                | 43             | 0.08 ± 0.06  | 390.92 ± 23.27 |
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| LDR (EL-1,3)            | 111 ± 4        | 32.0 ± 2.4   | 158,130 ± 32,945 |
| LDR (EL-2,3)            | 87 ± 1         | N.D.         | N.D. |

* N.D., not detectable.

TABLE II

Basal and ligand-stimulated cAMP production by wild type and different mutant constructs

Production of cAMP is expressed as pmol/ml without normalization of cell surface receptor expression.

|               | Basal | Ligand-stimulated |
|---------------|-------|-------------------|
|               | pmol/ml |                  |
| LHR WT       | 5.55 ± 0.30 | 390.92 ± 23.27 |
| fly LGR2     | 21.90 ± 1.00 | 24.61 ± 15.65 |
| LDR          | 172.81 ± 1.76 | 274.57 ± 22.96 |
| LDR (EL-1)   | 187.00 ± 27.24 | 239.37 ± 34.7 |
| LDR (EL-2)   | 53.20 ± 7.31 | 281.64 ± 21.08 |
| LDR (EL-3)   | 11.93 ± 0.53 | 23.31 ± 1.89 |
| LDR (EL-1,2) | 46.29 ± 2.26 | 410.92 ± 21.48 |
| LDR (EL-1,3) | 140.78 ± 15.13 | 204.74 ± 6.20 |
| LDR (EL-2,3) | 0.53 ± 0.11 | 2.15 ± 0.08 |
| FSHR WT      | 0.39> | 394.67 ± 19.50 |
| FDR          | 142.07 ± 3.52 | 322.38 ± 23.87 |
| fly LGR2 (TPPS) | 77.64 ± 3.09 | N.D. |
| fly LGR2 (TPPS) (EL-2) | 27.69 ± 2.26 | N.D. |

* N.D., not detectable.

Of interest, the constitutively active LDR could be silenced when exoloop 2, but not exoloop 1, was replaced with the LH receptor sequence (Fig. 4B). In addition, treatment with hCG restored cAMP production mediated by LDR(EL-2) to levels comparable with those of LDR-expressing cells. These data suggest that an attraction between exoloop 2 of the LH receptor and its homologous ectodomain in the chimeric LDR(EL-2) mutant constrains the receptor in an inactive conformation. Ligand binding to the ectodomain disrupts this interaction, resulting in receptor activation. In addition, treatment of LDR(EL-2) with increasing doses of hCG indicated a dose-dependent stimulation of this chimeric receptor with an ED50 of 1.7 ng/ml (Fig. 4D), a value higher than that for the wild type LH receptor (ED50: 0.25 ng/ml). Although LDR(EL-3) also showed low basal cAMP production, its inability to respond to hCG stimulation is consistent with the observed defective ligand binding of this chimeric receptor (Table I).

To further confirm the importance of exoloop 2, but not exoloops 1 and 3, in mediating the interactions between the ectodomain and TM region of the chimeric LDR receptor, we substituted two individual exoloops of the chimeric receptor (Fig. 4A). As shown in Table I and Fig. 4C, LDR(EL-1,2) and LDR(EL-1,3) were expressed on the cell surface and exhibited high affinity ligand binding. In contrast, LDR(EL-2,3) showed negligible ligand binding despite adequate cell surface expression. Furthermore, LDR(EL-1,2) showed lower basal cAMP production as compared with the chimeric LGR, whereas LDR(EL-1,3) still exhibited constitutive activity (Fig. 4B), confirming the important role of exoloop 2 in receptor constraint. Of interest, treatment with hCG stimulated cAMP production by LDR(EL-1,2) to levels comparable with those induced in the wild type LH receptor following ligand stimulation. Although LDR(EL-1,3) remained responsive to hCG treatment with a slight increase in cAMP production, the ligand binding of this receptor was partially defective. As shown in Table I, LDR(EL-1,3) exhibited lower maximal binding ability despite high cell surface expression. In contrast, the ligand signaling mechanism was defective for LDR(EL-2,3), suggesting the expression of a dysfunctional protein (Fig. 4B).

Studies Using Mutant Fly LGR2 Revealed Interactions between the Exoloop 2 and the Hinge Region of the LH Receptor—Earlier studies suggested the important role of several residues in the hinge region (Fig. 5A, asterisks) of the human LH receptor for the conformational constraint of this protein (18). Single point mutations of serine 277 and proline 276 of the LH receptor were associated with ligand-independent activation of the receptor. In addition, photoaffinity labeling and affinity cross-linking of peptide fragments of the LH receptor suggested a potential interaction between the hinge region and the exoloop 2 of this protein (20). Taking advantage of similar structural arrangements of fly LGR2 and the human LH receptor, we replaced key residues in the hinge region of fly LGR2 with those of the LH receptor (from SYAY to TYPY, Fig. 5A) and expressed the mutant receptor in 293T cells. After correcting for cell surface expression levels based on M1 antibody binding of tagged sequences (Fig. 5B, parentheses), basal cAMP production was determined for wild type and mutant receptors. As shown in Fig. 5B, the mutant fly LGR2 (TPPS) showed constitutive activity suggesting a loss of receptor constraint. We hypothesized that the observed receptor constraint could be
restored with the interacting LH receptor sequence and further replaced the exoloop 2 of this mutant receptor with that of the human LH receptor. The double mutant fly LGR2 (TYPS)(EL-2) was expressed in the cell surface but its constitutive activity was suppressed by 85% as compared with basal cAMP production mediated by LGR2 (TYPS). These data suggested that in the mutant fly LGR2, the exoloop 2 of the human LH receptor likely interacts with key residues in the hinge region of the same receptor to constraint the double mutant fly protein.

**DISCUSSION**

The present findings suggest the importance of exoloop 2 of the LH receptor in interacting with the hinge region of the ectodomain, thus constraining the TM region from activating the Gs protein. The chimeric receptor approach takes advantage of the similar domain arrangement of related GPCRs. In contrast to biochemical studies in which the interactions of different receptor fragments were tested in cell-free conditions, the chimeric receptors were expressed in intact cells to reveal
the gain- or loss-of-function of different mutants. The present results are consistent with cell-free studies in which synthetic peptides corresponding to exoloop 2 were found to compete for the binding between labeled hCG and a stretch of amino acids corresponding to the hinge region (20).

There are at least three different steps involved in the ligand signaling of the LH and related glycoprotein hormone receptors, each probably requiring unique but overlapping domains of the receptor. First, the heterodimeric ligands (LH and hCG) interact with the leucine-rich repeats in the ectodomain of the LH receptor. Based on structural modeling, the seven to nine leucine-rich repeats of the LH receptor are believed to form a 1/3 donut structure important for interaction with its large ligands. Recent studies indicated that leucine-rich repeats 2 and 4 are crucial for hormone binding (27, 28). Second, ligand binding leads to the disruption of the constraint on the TM region exerted by the interactions between the ectodomain (likely the hinge region) and exoloop 2. Indeed, point mutations of key residues in the hinge region of all three glycoprotein hormone receptors led to constitutive activation of these receptors (15–18). Furthermore, an earlier affinity labeling study (20) and the present findings provide evidence for direct interactions between the hinge region and exoloop 2. Third, the relaxed TM region, as the result of ligand binding, interacts with the Gs protein to activate the adenyl cyclase enzyme.

Analysis of chimeric human/fly receptors provided a unique opportunity to separate the first and second steps in LH receptor ligand signaling. Although the chimeric LDR showed optimal ligand binding and constitutive activity, this receptor was constrained following introduction of exoloop 2, but not exoloop 1, of the LH receptor. Of interest, the constrained LDR(EL-2) could be activated following ligand stimulation, indicating that the ligand signaling mechanism remains intact in this mutant receptor containing minimal LH receptor sequence in the TM region. A recent study further indicated that the N-terminal region of leucine-rich repeat 4 in the ectodomain is responsible for interaction with hCG, whereas the C-terminal region of this repeat is important for signal generation by binding the ex-
oloop 2 (29). The exact relationship among exoloop 2, leucine-rich repeat 4, and the hinge region awaits structural analysis following future crystallization of the receptor. Although alanine-scanning mutagenesis of exoloop 2 in the LH receptor indicated that ligand binding affinity was enhanced by some alanine substitution (30), similar mutagenesis of individual residues in this region did not lead to constitutive activation of the receptor (31). Based on the screening of a comprehensive series of overlapping synthetic peptides, several synthetic peptides corresponding to sequences surrounding the hinge region competed for the binding of radiolabeled hCG to rat ovarian LH receptors (32). However, loop 3, but not loop 2, showed activity in this assay, probably due to the requirement of extremely high concentrations of these peptides in the radioligand receptor assay. Likewise, our attempts to activate the wild type LH receptors following incubation with synthetic peptides corresponding to either exoloop 2 or the hinge region of the LH receptor were unsuccessful (data not shown).

Although the chimeric receptor approach provides support for the role of the exoloop 2 in ligand signaling mediated by the LH receptor, the role of exoloop 3 cannot be ruled out. Earlier mutagenesis studies (33, 34) have indeed suggested an important role of this region in LH receptor activation. In the present study, the mutant LDR(EL-3) and LDR(EL-2,3) showed low basal cAMP production and normal or adequate cell surface expression. However, ligand binding and activation of these receptors were defective, suggesting mis-folding of these mutants. Because a mutant LH receptor with only transmembrane loop 1 is capable of ligand binding (35), the observed decreases in hCG binding in LGR(EL-3) and LDR(EL-2,3) suggested the involvement of exoloop 3 in the ligand binding step.

Although our earlier data suggested that fly LGR1, but not fly LGR2, exhibited constitutive activity when overexpressed in mammalian cells (7), the present results indicate that fly LGR2 is also likely to signal through the Gs protein, and the G protein-signaling mechanism could be established between the two fly receptors and Gα proteins expressed in human cells used for transfection. The observed ligand signaling of different chimeric receptor mutants consisting of human LH receptor and fly LGR2 also suggested that fly LGR2 likely belongs to the same subgroup of LGRs together with glycoprotein hormone receptors and fly LGR1 (8). Despite the lack of knowledge on the endogenous ligand(s) for the fly LGR2, understanding of the signaling pathway for this orphan receptor could facilitate future identification of its ligands using bioinformatic and biochemical approaches (36).

In conclusion, the present chimeric receptor analyses, together with earlier mutagenesis and cross-linking studies, provide a unified model of ligand signaling for the LH receptor. In this model, the hinge region and exoloop 2 of the receptor interact to constrain the unliganded transmembrane protein in an inactive conformation. Following ligand (hCG) occupancy of the leucine-rich repeats (2 and 4) in the ectodomain of the receptor (28), the α subunit of hCG displaces the hinge region from exoloop 2 (20), leading to the relaxation of the TM region. With or without the additional stabilizing effects of the agonist on the TM region, ligand-induced conformation changes in this region allows subsequent activation of the Gs protein. Thus, the

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**FIG. 5.** Replacement of key residues in the hinge region of fly LGR2 by the human LH receptor sequence led to constitutive receptor activation: decreases in basal cAMP production following replacement of both the hinge region and exoloop 2 of the fly LGR2 with the human LH receptor sequences. A. sequence comparison between the hinge region of fly LGR2 and the human LH receptor. The asterisks indicate two key residues previously found to be essential for constraining the human LH receptor. Conserved residues are shaded. B. human 293T cells were transfected with expression plasmids encoding the wild type human LH receptor (LHR WT), or fly LGR2 with or without substitution of the hinge region by the LH receptor sequence (SYAY to TYPS). The double fly mutant, fly LGR2 (TYPS)(EL-2), has both hinge and exoloop 2 from the LH receptor. Basal and hCG-stimulated cAMP production mediated by different receptors is shown. All data were normalized based on cell surface M1 antibody binding as indicated in parentheses with the LHR WT arbitrarily set as 100%. Lower panel, diagrammatic representation of different receptors.
β subunits of different glycoprotein hormones provide specificity in their binding to the receptor-specific leucine-rich repeats whereas the common α subunit is important in the displacement of the hinge-exoloop 2 interactions. These findings provide the basis for future structural approaches to understand the detailed mechanism of ligand signaling mediated by glycoprotein hormone receptors and other GPCRs with a large ectodomain.

Acknowledgments—We thank Dr. C. J. Grimmelikhuijzen, University of Copenhagen, Denmark, for providing the full-length fly LGR2 cDNA; and Caren Spencer for editorial assistance. We also thank the National Pituitary and Hormone Distribution Program for the cAMP antiserum and hCG preparations.

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J. Biol. Chem. 2002, 277:3958-3964. doi: 10.1074/jbc.M109617200 originally published online November 26, 2001

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