Different activation mechanisms of glycoprotein hormone receptors, which are members of the G protein-coupled receptor superfamily, have been proposed. For example, the large ectodomain of glycoprotein hormone receptors may function as an inverse agonist keeping the transmembrane domain in an inactive conformation. To provide support for this hypothesis, we have generated different lutropin/choriogonadotropin receptor (LHR) constructs lacking the ectodomain. Although some ectodomain-deficient LHR constructs were targeted to the cell surface, cAMP levels remained unchanged under basal conditions and agonist application but could be increased by a mutation within the transmembrane domain 6 (D578H). Taking advantage of a constitutive activating mutation (S277N) located in the extracellular domain, we showed that the intact leucine-rich repeat-containing ectodomain is essential for constitutive activation of the LHR by mutation of the hinge region. Our findings support an activation scenario in which agonist binding or mutational alterations expose a structure within the ectodomain, which then activates the transmembrane core.

The biological actions of lutropin/choriogonadotropin are mediated by their interaction with a specific cell membrane receptor that belongs to the leucine-rich repeat (LRR)1-containing G protein-coupled receptors (GPCRs), a group of at least seven structurally related mammalian receptors (1). Upon agonist activation, the lutropin/choriogonadotropin receptor (LHR) activates the Gα/adenyl cyclase and phospholipase C pathways. The structure of glycoprotein hormone receptors is predicted to consist of a large extracellular hormone binding domain connected to a transmembrane core that shares a common molecular architecture with other GPCRs of family 1 (2). The ectodomain of the LHR is composed of nine LRRs that are thought to form a horseshoe-like structure (3). The transmembrane core assembles from seven mostly α-helical transmembrane domains (TMDs 1–7) that are connected by three extracellular and three intracellular loops. In the LHR, a tightly packed hydrophobic cluster and a specific H-bonding network formed between the TMDs is thought to maintain the inactive receptor conformation (4). To date, a large number of activating mutations within the TMDs of glycoprotein hormone receptors has been identified, and it has been proposed that receptor activation is associated with the disruption of key inter- and intrahelical side-chain interactions (5). However, the molecular mechanism of glycoprotein hormone receptor activation by their agonists is still unknown.

Several mechanisms of glycoprotein hormone receptor activation have been proposed. First, the glycoprotein hormone binds to the extracellular domain, and distinct portions of the hormone act as agonist on the transmembrane receptor core. This mechanism is supported by studies showing that human choriogonadotropin (hCG) and peptides derived from the hCG α-chain can directly activate an LHR mutant that lacks the ectodomain (6, 7). Second, the ectodomain of glycoprotein hormone receptors functions as an inverse agonist keeping the TMD region in an inactive conformation. This model is supported by recent findings indicating that deletion of the ectodomain of the thyrotropin receptor (TSHR) increases its constitutive activity (8). Hormone binding and activating mutations within the ectodomain (9–11) may induce the disruption of the ectodomain-TMD core interaction. The latter findings are consistent with the activation mechanism proposed by Szkudlinski and co-workers (8) but also implicate a third scenario of receptor activation in which an agonistic structure within the ectodomain is exposed to the TMD core following hormone binding or mutational alteration.

To provide support for one of these hypotheses, we experimentally addressed all three mechanisms by site-directed mutagenesis approaches. Although some LHR constructs lacking the ectodomain were targeted to the cell surface, cAMP levels remained unchanged under basal conditions and high concentrations of the agonist. Our data provide no support for an activation scenario in which the non-liganded ectodomain of the LHR stabilizes an inactive receptor conformation. The fact that a mutation within the ectodomain of the LHR (S277N) constitutively activates the receptor only in the presence of the N-terminally located nine LRRs implicates an activation model in which distinct determinants of the ectodomain participate directly at least in the mutually induced receptor activation. Systematic deletion of all extracellular LRRs within the constitutive active receptor (S277N) was utilized to identify domains in the ectodomain participating in receptor activation. The study provides evidence for a cooperative model of the single LRR and other N-terminal portions in forming the global ectodomain structure that is required for proper cell surface targeting and probably for mutational receptor activation.

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EXPERIMENTAL PROCEDURES

Generation of Mutant LHRs—LHR mutations (see Fig. 1 and Table I) were introduced into LHR-pcDps (12), a mammalian expression vector containing the entire coding sequence of the human LHR, using a PCR-based site-directed mutagenesis and restriction fragment replacement strategy (15). For generation of S277N and LHR deletion mutants, PCR fragments containing the mutations were digested with Bgl II and Bsu36I and used to replace the corresponding fragments in LHR-pcDps. The D578H mutation was introduced into LHR-pcDps via BstBI and SpeI.

The HA-tagged V₂ vasopressin receptor (V₂R) and M₃ muscarinic receptor (M₃R)/LHR chimeras were constructed using the V₂R and M₃R cDNAs as template (14), and a fragment replacement via BglII (for V₂R) or HindIII (for M₃R) and Bsu36I was performed with the LHR-pcDps vector. An overlapping PCR-strategy and the restriction sites Bsu36I and DraIII were utilized to generate ÆTM1–2 and ÆTM1–4. The N terminus of V₂R was ligated into the latter constructs using BglII and Bsu36I. To generate the TMD1-LHRterm and TMD1-V₂term constructs, a silent mutation (codon position 386; ACA to ACT) that produces a new SpeI site was introduced into the wild-type (wt) LHR plasmid. Then, PCR-derived fragments were inserted using the new SpeI site and the SpeI site in the 3' polylinker region.

To allow for immunological detection, wt and mutant LHRs were tagged with an N-terminal nine-amino acid residue epitope (YPYDVPDYA) derived from the influenza virus hemagglutinin protein (HA tag) after the signal peptide. In addition, constructs were tagged with a C-terminal eight-amino acid residue epitope (DYA) derived from the influenza virus hemagglutinin protein (HA tag) after the signal peptide. For functional assays, COS-7 cells were transiently transfected using LipofectAMINE (Invitrogen). cAMP accumulation assays were performed in 12-well plates (2 × 10⁵ cells/well), and transfectected cells were washed once in serum-free Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 7% CO₂ incubator. For radioligand binding studies, cells were harvested 72 h after transfection (10 μg of plasmid DNA/100-mm dish), and displacement and saturation binding assays were performed using membrane homogenates. ¹²⁵I-hCG saturation binding studies (¹²⁵I-hCG 1800 Ci/mmol; PerkinElmer Life Sciences) were carried out for 2 h at 22 °C as described (12). Nonspecific binding was defined as binding in the presence of 5 μM hCG. The Kᵢ value for the human LHR transiently expressed in COS-7 cells was 2.4 nm.

Immunological and Immunofluorescence Studies—To estimate cell surface expression of receptors carrying an N-terminal HA tag, we used an indirect cellular ELISA (17), further referred to as surface ELISA. Briefly, COS-7 cells were seeded into 48-well plates, transfected (0.25 μg of DNA and 0.6 μl of LipofectAMINE/well), formaldehyde-fixed without disrupting the cell membrane, and incubated with a biotin-labeled anti-HA monoclonal antibody (12CA5; Roche Molecular Biochemicals). Bound anti-HA antibody was then detected with the help of a peroxidase-labeled streptavidin conjugate (Sigma). After removal of excess unbound conjugate, H₂O₂ and O-phenylenediamine (2.5 mM each in 0.1 M phosphate-citrate buffer, pH 5.0) were added to serve as substrate and chromogen, respectively. After 15 min the enzyme reaction was stopped by the addition of 1 × Na₂SO₄ containing 0.05 M Na₂SO₄, and color development was measured photometrically at 492 and 620 nm using an ELISA reader (Titertek Multiskan MCC/340; Flow Laboratories, McLean, VA).

To further assess the amounts of full-length HA- and FLAG-tagged receptor constructs, a previously developed sandwich ELISA was used (17). In brief, transfected cells (5 μg of DNA and 10 μl of LipofectAMINE/60-mm dish) were harvested, and membrane preparations were solubilized under continuous rotation in lysis buffer overnight. Microwell plates were coated with an anti-FLAG monoclonal antibody
Various LHR mutants and V₆R LHR chimeras were constructed by site-directed mutagenesis as described under “Experimental Procedures.”

The construct designation and the exact amino acid positions that were deleted or present in the receptor constructs are outlined (see also Fig. 1).

The amino acid residue positions are referred to the human LHR and to the human V₆R amino acid sequences (without the epitope tags). ‡, epitope tag present; —, no epitope tag.

| Construct       | Deleted amino acid residues in the LHR | LHR amino acid residues in the construct | V₆R amino acid residues in the construct | HA tag | Flag tag | Other modifications |
|-----------------|----------------------------------------|------------------------------------------|------------------------------------------|--------|----------|---------------------|
| LHR (wt)        | —                                      | 1–699                                    | —                                        | +      | +        |                     |
| S277N           | —                                      | 1–699                                    | —                                        | +      | +        | S277N               |
| D578H           | —                                      | 1–699                                    | —                                        | +      | +        | D578H               |
| M₆LHR           | 1–360                                  | 361–699                                  | 1–66‡                                    | +      | +        |                     |
| V₆₆LHR          | 1–360                                  | 361–699                                  | 1–40                                     | +      | +        |                     |
| V₆₆LHRD578H     | 1–360                                  | 361–699                                  | 1–40                                     | +      | +        | D578H               |
| Δecto D578H     | 29–358                                 | 1–27; 359–699                            | —                                        | —      | —        | D578H               |
| HA-ecto         | 29–358                                 | 1–27; 359–699                            | —                                        | —      | —        |                     |
| HA-ecto D578H   | 29–358                                 | 1–27; 359–699                            | —                                        | +      | +        | D578H               |
| ΔG–10S277N      | 29–275                                 | 1–28; 276–699                            | —                                        | —      | —        | S277N; D578H        |
| ΔG–10S277N/D578H| 29–275                                 | 1–28; 276–699                            | —                                        | —      | —        | S277N; D578H        |
| ΔG–9S277N       | 29–267                                 | 1–28; 268–699                            | —                                        | —      | —        | S277N; D578H        |
| ΔG–9S277N/D578H | 29–267                                 | 1–28; 268–699                            | —                                        | —      | —        | S277N; D578H        |
| ΔG–7S277N       | 29–223                                 | 1–28; 224–699                            | —                                        | —      | —        | S277N; D578H        |
| ΔG–7S277N/D578H | 29–223                                 | 1–28; 224–699                            | —                                        | —      | —        | S277N; D578H        |
| ΔTMD1–2         | 364–439                                | 1–363; 440–699                           | —                                        | +      | +        |                     |
| ΔTMD1–4         | 364–524                                | 1–363; 525–699                           | —                                        | +      | +        |                     |
| S277N/ΔTMD1–2   | 364–439                                | 1–363; 440–699                           | —                                        | +      | +        | S277N               |
| S277N/ΔTMD1–4   | 364–524                                | 1–363; 525–699                           | —                                        | +      | +        | S277N               |
| ΔTMD1–2D578H    | 364–524                                | 1–363; 525–699                           | —                                        | +      | +        | D578H               |
| ΔTMD1–4D578H    | 364–524                                | 1–363; 525–699                           | —                                        | +      | +        | D578H               |
| V₆₆TMD1–2       | 1–439                                  | 440–699                                  | 1–40                                     | +      | +        |                     |
| V₆₆TMD1–4       | 1–524                                  | 525–699                                  | 1–40                                     | +      | +        |                     |
| V₆₆TMD1–2D578H  | 1–439                                  | 440–699                                  | 1–40                                     | +      | +        | D578H               |
| V₆₆TMD1–4D578H  | 1–524                                  | 525–699                                  | 1–40                                     | +      | +        | D578H               |
| TMD1–V₂Cterm    | 388–699                                | 1–387                                    | 327–371                                  | +      | +        |                     |
| TMD1–LHRCTerm   | 388–627                                | 1–387; 628–699                           | —                                        | +      | +        | S277N               |
| S277N/TMD1–V₂Cterm | 388–699                           | 1–387                                    | 327–371                                  | +      | +        | S277N               |
| S277N/TMD1–LHRCTerm | 388–627                           | 1–387; 628–699                           | —                                        | +      | +        | S277N               |
| ΔS277N          | 51–74                                  | 1–50; 75–699                             | —                                        | +      | +        | S277N               |
| ΔS277N          | 75–99                                  | 1–74; 109–699                            | —                                        | +      | +        | S277N               |
| ΔS277N          | 100–125                                | 1–99; 126–699                            | —                                        | —      | +        | S277N               |
| ΔS277N          | 126–149                                | 1–125; 150–699                           | —                                        | —      | +        | S277N               |
| ΔS277N          | 150–175                                | 1–149; 176–699                           | —                                        | —      | +        | S277N               |
| ΔS277N          | 176–198                                | 1–175; 199–699                           | —                                        | —      | +        | S277N               |
| ΔS277N          | 198–223                                | 1–198; 224–699                           | —                                        | —      | +        | S277N               |
| ΔS277N          | 224–245                                | 1–223; 246–699                           | —                                        | —      | +        | S277N               |
| ΔS277N          | 246–267                                | 1–245; 268–699                           | —                                        | —      | +        | S277N               |
| ΔS277N          | 268–275                                | 1–267; 276–699                           | —                                        | —      | +        | S277N               |

* Amino acid residues 1–66 of the rat M₂ muscarinic receptor.

(1 μg/ml in borate buffer, pH 8.0; Sigma). After incubation with the membrane solubilisates, bound HA-tagged receptors were detected with the combination of a biotin-labeled anti-HA monoclonal antibody (12CA5; Roche Molecular Biochemicals) and a peroxidase-labeled streptavidin conjugate. Color reaction and measurements were performed as described for the surface ELISA.

Immunofluorescence studies were carried out to examine the subcellular distribution of the wt and mutant LHRs. COS-7 cells were transfected into 6-well plates containing sterilized glass cover slips and transfected. Approximately 48 h later, cells were fixed and probed with an anti-HA monoclonal antibody (10 μg 12CA5/ml in phosphate-buffered saline). To detect intracellularly retained receptors, cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline. Fluorescence images were obtained with a confocal laser-scanning microscope (LSM 510; Zeiss).

RESULTS

**Functional Relevance of the LHR Ectodomain and the Transmembrane Receptor Core**—It is well established that the ectodomain of glycoprotein hormone receptors binds the hormone, and the TMD region mediates signal transduction. Recent studies suggest that the ectodomain of some glycoprotein hormone receptors acts as inverse agonist (8, 18). In initial studies, we expressed an LHR construct (Δecto) that completely lacks the ectodomain (see Fig. 1 and Table I). Functional analysis of Δecto in transiently transfected COS-7 cells revealed no basal or agonist-induced activity even at 10 μM hCG (Table II). To test whether Δecto can be activated mutationally we introduced a missense mutation (D578H) into TMD6 (Δecto D578H), which is known to induce strong constitutive activity of the wt LHR (19). However, no increase in basal activity was observed (see Table II). We next asked whether Δecto is properly delivered to the cell surface. By introducing the N-terminal epitope tag we demonstrated in a cell surface ELISA that none of the constructs (Δecto, Δecto D578H) were efficiently transported to the plasma membrane (see Table II).

To circumvent the problem of poor plasma membrane expression, we replaced the LHR ectodomain with the N terminus of the V₆R to assure cell surface targeting (see Fig. 1). For quantification of the receptor expression, all receptor constructs contained an artificial N-terminal HA epitope. Additionally, the C terminus of the LHR constructs was tagged with a FLAG epitope. COS-7 cells were transfected with the V₆R/LHR chimera, and cell surface expression was compared with the HA-tagged version of the wt LHR (see “Experimental Procedures”). As
shown in cell surface ELISA (see Table II) and in immunofluorescence studies (see Fig. 3). V2RLHR was expressed at high levels at the plasma membrane and displayed an even higher total cellular expression than the wt LHR (see Table II). However, functional analysis of V2RLHR revealed neither elevated basal nor agonist-stimulated cAMP levels (Fig. 2) even when different preparations of hCG (Calbiochem, Schering, Sigma) and concentrations up to 10 μM hCG were applied.

Introduction of D578H into the V2RLHR chimera resulted in a pronounced constitutive activity in the cAMP (see Fig. 2) and the IP signal transduction pathways, indicating a properly folded transmembrane core (see Table II). Interestingly, the D578H mutation drastically increased cell surface expression levels of both the wt LHR and the V2RLHR chimera (see Table II).

To exclude the possibility that the V2R N terminus somehow interferes with hCG binding to the TMD core we replaced the LHR ectodomain with the N terminus of the rat M3 muscarinic receptor (M3LHR). Similar to the V2RLHR chimera, M3LHR was transported to the cell surface but displayed no basal or agonist induced activity in the cAMP assay (see Table II).

Taken together, these data suggest that the ectodomain of the LHR does not act as an inverse agonist on the TMD core. The functional folding and stabilization of the TMD core occurs independently from the extracellular domain. Further, we found no evidence for a direct agonistic action of the hormone at the TMD core.

Requirement of All Transmembrane Segments for LHR Activation—Studies with the CCR5 and CXCR4 chemokine receptors have challenged the established view that an intact TMD core containing all seven TMDs is essentially required for GPCR signaling (20). We addressed this issue by fusing the LHR N terminus to the N termini of TMDs 3 and 5 (∆TM1–2, ∆TM1–4). Additionally, two activating mutations, one in the ectodomain (S277N) and one in TMD6 (D578H), were introduced in either deletion mutant (S277N ∆TM1–2, S277N ∆TM1–4, ∆TM1–2D578H, ∆TM1–4D578H). Unfortunately, all six receptor constructs were poorly delivered to the plasma membrane efficiently

Table II: Functional characterization of ectodomain and TMD deletion mutants

| Transfected construct | cAMP | IP | A492 nm |
|-----------------------|------|----|---------|
|                       | Basal cAMP levels | Basal IP levels | Surface ELISA | Sandwich ELISA |
| LHR (wt)              | 23.8 ± 4.9 | 8.3 ± 1.8 | 100 | 100 |
| S277N                 | 21.6 ± 8.8 | 5.0 ± 0.2 | 132 ± 17 | 99 ± 9 |
| D578N                 | 15.4 ± 2.7 | 10.8 ± 3.5 | 406 ± 62 | 83 ± 23 |
| V2LHR                 | 0.9 ± 0.2 | 0.8 ± 0.1 | 67 ± 14 | 149 ± 36 |
| V2LHRD578N            | 10.4 ± 1.7 | 1.9 ± 0.3 | 159 ± 24 | 117 ± 57 |
| M3LHR                 | 0.9 ± 0.2 | 0.8 ± 0.1 | 90 ± 12 | 118 ± 8 |
| Δecto                 | 0.7 ± 0.1 | 0.8 ± 0.1 | 32 ± 6  | ND |
| Δecto D578N           | 0.7 ± 0.1 | 0.8 ± 0.1 | 77 ± 15 | ND |
| HA-ecto               | 0.8 ± 0.1 | 0.9 ± 0.2 | 200 ± 11 | ND |
| HA-ecto D578N         | 0.8 ± 0.1 | 0.9 ± 0.2 | 39 ± 16  | ND |
| ΔTMD1–2              | 0.8 ± 0.1 | 0.8 ± 0.1 | 79 ± 11  | ND |
| ΔTMD1–4              | 0.1 ± 0.1 | 0.7 ± 0.1 | 40 ± 10  | ND |
| S277NΔTMD1–2         | 0.7 ± 0.1 | 0.8 ± 0.1 | 87 ± 13  | ND |
| S277NΔTMD1–4         | 0.7 ± 0.1 | 0.8 ± 0.1 | 25 ± 14  | 92 ± 7 |
| ΔTMD1–2D578H         | 0.7 ± 0.1 | 0.8 ± 0.1 | 42 ± 21  | 104 ± 43 |
| ΔTMD1–4D578H         | 0.7 ± 0.1 | 0.8 ± 0.1 | 34 ± 24  | 115 ± 4 |
| V2ΔTMD1–2            | 0.7 ± 0.1 | 0.8 ± 0.1 | 16 ± 12  | 91 ± 13 |
| V2ΔTMD1–4            | 0.7 ± 0.1 | 0.8 ± 0.1 | 22 ± 14  | 109 ± 12 |
| V2ΔTMD1–2D578H       | 0.7 ± 0.1 | 0.8 ± 0.1 | 19 ± 17  | 108 ± 19 |
| V2ΔTMD1–4D578H       | 0.8 ± 0.1 | 0.8 ± 0.1 | 20 ± 13  | 199 ± 43 |
|                       | 201 ± 23 | 215 ± 56 |

Fig. 2: Structural requirements for LHR activation. COS-7 cells transfected with the wild-type or different mutant LHRs were studied in cAMP assays as described under “Experimental Procedures.” Basal and agonist-induced cAMP formation are expressed as fold-over basal cAMP levels of the wt LHR (see Table II). Data are presented as means ± S.E. of three to four independent experiments, each carried out in triplicate.
To examine whether a covalent interaction between the ectodomain and the TMD receptor portion is necessary to transduce the conformational changes of an activating mutation located in the extracellular domain (S277N) to the TMD core, various membrane-anchored LHR ectodomain constructs were coexpressed with the V2LHR chimera in COS-7 cells. For control purposes, the LHR and the S277N were transfected with the empty expression vector (1:1). cAMP responses (-fold over wt basal) to 100 nM hCG from five to six experiments performed in triplicate are shown. Cell surface expression of the constructs were measured by ELISA (see “Experimental Procedures”). Receptor densities of the mutant LHR are expressed as % of the wt LHR (surface ELISA, A492 nm 1.282 ± 0.364) minus the non-specific optical density readings of GFP (surface ELISA, A492 nm 0.614 ± 0.150). Values of basal and agonist-induced cAMP formation of the coexpression experiments were tested for significant changes using the paired two-sample Student’s t test.

| Transfected constructs | cAMP | Surface ELISA |
|------------------------|------|--------------|
|                        | Basal cAMP levels | Increase in cAMP levels | A492 nm | -fold over wt basal | % wt |
| LHR (wt) + vector      | 1    | 17.5 ± 5.0   | 100    |
| S277N + vector         | 1.7 ± 0.3 | 16.6 ± 5.6   | 120 ± 5 |
| TMD1-LHRCterm + V2LHR  | 1.1 ± 0.2 | 1.4 ± 0.2 (n = 5, p = 0.008) | 18 ± 6  |
| S277N/TMD1-LHRCterm + V2LHR | 0.9 ± 0.1 | 1.2 ± 0.1 (n = 5, p = 0.035) | 27 ± 11 |
| TMD1-V2Cterm + V2LHR   | 0.8 ± 0.1 | 1.3 ± 0.2 (n = 8, p = 0.001) | 27 ± 8  |
| S277N/TMD1-V2Cterm + V2LHR | 1.0 ± 0.1 | 1.2 ± 0.1 (n = 4, p = 0.11) | 27 ± 8  |

To study whether the LRR-containing region of the LHR ectodomain is required for mutual induction of agonist-independent receptor activation, an S277N construct (Δ0–10S277N) was generated in which all LRRs were deleted. In Δ0–10S277N the signal peptide followed by the HA tag was linked directly to amino acid position 276. As measured with the cell surface ELISA, Δ0–10S277N was expressed at high levels (71% of wt LHR expression) at the plasma membrane of transiently transfected COS-7 cells (see Table II). Next, cAMP levels of Δ0–10S277N-transfected COS-7 cells were determined. No constitutive basal or agonist-induced receptor activation was observed (see Fig. 2 and Table II). To verify the signal transmission abilities of Δ0–10S277N, an additional constitutively activating mutation (D578H) was introduced. Δ0–10S277ND578H-transfected cells displayed a 6-fold elevation of basal cAMP levels (see Fig. 2 and Table II). These data indicate that the amino acid sequence between position 29 and 275 is required for receptor activation by S277N and that deletion of all LRRs does not interfere with proper trafficking and functional folding of the TMD core. Interestingly, further N-terminal extension of Δ0–10S277N as made in Δ0–9S277N and Δ0–7S277N reduces cell surface expression levels and, therefore, the mutational activation by D578H (see Table II).

LHR Reconstitution from Transmembrane and Extracellular Domains—Next, we asked whether a covalent interaction between the ectodomain and the TMD receptor portion is necessary to transduce the conformational changes of an activating mutation located in the extracellular domain (S277N) to the TMD core for G-protein coupling. It has been demonstrated that the LHR ectodomain fused to a transmembrane anchor forms a functional complex with the TMD receptor portion when coexpressed (22). Initial coexpression studies with an essentially similar ectodomain-lacking construct or the V2LHR chimera, together with an N-terminal construct containing the ectodomain (with or without S277N) and TMD1 (see Fig. 1), showed only insignificant elevations of hCG-induced intracellular cAMP levels, probably because of a low cell surface expression of the ectodomain/TMD1 construct (data not shown).

It has been demonstrated that fusion of the receptor C terminus to an N terminus/TMD1 construct can increase the cell surface expression of this construct dramatically (23). Therefore, the C terminus of the LHR was linked mutationally with the ectodomain/TMD1 construct (TMD1-LHRCterm; see Fig. 1) and coexpressed with the V2LHR chimera. As shown in Table III, coexpression of both constructs resulted in a small but significant increase in agonist-induced cAMP formation. This finding confirmed previous observations that the ectodomain and the TMD core behave as independent units and that a covalent interaction of both functional units is not necessary for function. Next, the V2LHR chimera was coexpressed with TMD1-LHRCterm containing the activating S277N mutation within the ectodomain. No elevated basal cAMP levels were observed. For correct interpretation of these results, HA (N terminus) and FLAG (C terminus) tags of this construct were utilized to monitor cell surface and total cellular expression levels in ELISAs (see “Experimental Procedures”). As compared with the tagged version of wt LHR, TMD1-LHRCterm displayed less than 20% cell surface expression but 85% of total cellular expression, indicating an intracellular retention. In a final attempt to increase the cell surface expression of the ectodomain/TMD1 construct, we linked the V2R C terminus downstream of TMD1 (TMD1-V2RCterm; see Fig. 1). However, the expected increase in cell surface expression levels of the TMD1-V2RCterm was not observed in ELISA studies. In accordance with this finding a low rescue efficiency was found after coexpressing TMD1-V2RCterm and V2LHR (see Table III).

Requirement of All Leucine-rich Repeats for Proper LHR Trafficking—As demonstrated above the LRR-containing region of the ectodomain is essential for mediating the constitutive receptor activation of S277N. This finding may support an activation scenario in which an intramolecular agonistic structure within the ectodomain is exposed following mutational or ligand-induced activation. To identify determinants that may be involved in the intramolecular receptor activation, single LRRs were deleted systematically in the S277N mutant...
The mechanisms of agonist-induced glycoprotein receptor activation have been studied in considerable detail. Recent studies favor an activation model in which the ectodomain acts as an inverse agonist keeping the receptor in the inactive conformation (8, 18) (Fig. 4A). To test whether such a mechanism also accounts for LHR receptor activation, we replaced the LHR ectodomain by the N terminus of the V, R to assay proper cell surface targeting of the construct. In contrast to findings with the TSHR, our data do not support an inverse agonistic function of the LHR ectodomain, because constitutive activation was not observed for the ectodomain-lacking LHR constructs. The lack of constitutive activity was not because of trafficking deficiency as shown by ELISA and immunofluorescence techniques. Additionally, introduction of an activating mutation (D578H) into the ectodomain-deficient LHR induced constitutive activity (see Table II), excluding an improperly folded TMD core. One may argue that the artificial introduction of the V, R N terminus somehow keeps the LHR TMD core in an inactive conformation. However, replacement of the LHR ectodomain by the N terminus of the rat M, muscarinic receptor gave essentially similar results. Additionally, expression of an ectodomain-lacking LHR construct that is structurally equivalent to the TSHR construct used by Zhang et al. (8) showed no constitutive and agonist-induced activity in previous (22) and present studies (Ecto constructs; see Table II). Therefore, a mechanism in which the receptor is activated by agonist- or mutation-induced disruption of an ectodomain/TMD core interaction as proposed for the TSHR is not supported experimentally for the LHR. Our and other (4, 5) data point at multiple constrains within the TMD core maintaining the inactive conformation of the LHR. In contrast to the LHR and follicle-stimulating hormone receptor, the TSHR exhibits a high basal activity (25). These obvious differences are indicative of a less “dense” network of interactions that keep the receptor in the inactive conformation. Thus, minor structural changes of the TSHR may already destabilize the ground steady state equilibrium between inactive and active conformations.

The Intact Transmembrane Core of the LHR Is Necessary for G, Activation—The exact nature of the G protein interaction sites within the LHR receptor is currently unknown. It is assumed that not only the intracellular loops but also the cytoplasmic ends of different TMDs participate in GPCR/G-protein coupling. Indeed, peptides derived from the 13 loop/TMD6 junction of the LHR can activate G proteins (26). Like-
wise, a TMD core containing TMD3–7 is sufficient for agonist-mediated signaling of the CCR5 and CXCR4 chemokine receptors (20). In analogy to the latter study we examined whether the entire TMD core is required for LHR activation. Thus, we took advantage of an agonist-independent activation of LHR constructs that lacked TMD1–2 and TMD1–4. Only constructs in which the N terminus of the LHR was replaced by the V2R N terminus were delivered properly to the cell surface, but none of the constructs containing the activating mutation D578H showed elevated basal cAMP levels. Similarly, introduction of D578G into an LHR fragment containing only TMD6–7 and the C terminus did not result in a constitutive activation of the Gs/adenylyl cyclase pathway when transfected into COS-7 cells.2 Our data indicate that, at least for mutation-induced receptor activation, Gs activation requires a global receptor assembly from all seven TMDs and the connecting loops.

What Is the Agonist of the LHR, Lutropin/Choriogonadotropin/hCG or the Ectodomain?—Direct contact of the TMD core with its designated agonist is the most common mechanism of GPCR activation. Whether glycoprotein hormones participate directly in receptor transmembrane core activation is not yet solved. Activation of an LHR lacking the ectodomain has been found to occur at micromolar concentrations of hCG (6). Moreover, a peptide derived from the C-terminal end of the hCG α-chain was able to induce cAMP formation in LHR-expressing cells, probably because of a direct interaction with the transmembrane region of the LHR (7). Both studies implicate a mechanism of LHR receptor activation in which the ectodomain binds the glycoprotein with high affinity and directs the hormone to the TMD region for activation (Fig. 4B). In contrast, Hsueh and co-workers (22) were unable to demonstrate a direct activation of a very similar ectodomain-less LHR

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2 T. Schöneberg, unpublished results.
construct. None of the two studies, however, provided proof of sufficient cell surface expression, because the high affinity ligand binding domain was chopped off in these constructs. To solve the problem of proper cell surface targeting and to allow for receptor cell surface quantification, we exchanged the LHR ectodomain with the V_{5R} that contained an N-terminal HA-epitope tag. Despite high cell surface expression levels even micromolar concentrations of hCG were insufficient to activate this construct. Based on our data, a direct interaction of hCG with the TMD core in the wt LHR cannot be excluded, but we also cannot provide supporting data for a direct activating contact between the hormone and receptor core.

Identification of activating mutations within the ectodomain of glycoprotein hormone receptors now provides an invaluable tool to investigate their activation mechanism. Most activating mutations found in the ectodomain are in proximity to the hinge region (10). Consistent with an activation model proposed by Zhang et al. (8), these mutations may open ectodomain/TMD core interactions leading to a shift of the receptor equilibrium to the active conformation. The position Ser-277 in the LHR corresponds to Ser-281 in the TSHR. Mutation of this position results in constitutive activation of either receptor (9, 10). First, we asked whether a covalent association between the ectodomain and the TMD core is required for transducing the activating properties of S277N to the TMD core. Our data confirm previous findings (22) that the ectodomain and the TMD portion of the LHR behave as independent functional units. Because of the low rescue efficiency the applied coexpression strategy of both functional domains was insufficient to answer the question above clearly. Next, we asked whether the LRR domain participates in mediating constitutive activity. Despite proper plasma membrane localization the Δ0–10S277N construct displayed no constitutive or agonist-induced activity (see Fig. 2). Here we show for the first time that the LRR-containing ectodomain is essentially required for constitutive activation of the LHR by the S277N mutation. Therefore, our data with the S277N constructs and the ectodomain-less constructs are not consistent with a scenario in which an activating mutation in the extracellular domain releases the TMD core in a more active conformation.

From the data available to date, a third model of receptor activation has to be considered in which an agonistic structure within the ectodomain is exposed to the TMD core following hormone binding or mutational activation (Fig. 4C). In a first attempt to identify possible agonistic structures within the ectodomain, all LRRs were deleted systematically within the S277N receptor background. None of the constructs showed constitutive activity; however, all LRRs deletion mutants were delivered poorly to the cell surface and mainly trapped intracellularly (Table IV). Our data indicate clearly that the functional formation of the LRR ectodomain is cooperative rather than modular. Proper cell surface targeting of the LHR essentially requires all LRRs that form a global ectodomain structure. Because of improper cell surface targeting of all LRR-deletion mutants and, therefore, the uncertain interpretation of the lack of constitutive activity, this mutagenesis attempt was unsuccessful to identify determinants that may participate in an endogenous ligand formation.

Taken together, our data exclude an inverse agonistic effect of the ectodomain of the LHR. The fact that S277N requires the LRR-containing domain may support the idea of an intramolecular agonistic structure that is exposed following structural changes by LH/CG binding or mutation of the extracellular domain. Our study provides evidence for a cooperative model of the single LRRs and other N-terminal portions in forming the global ectodomain structure, which is essential for proper cell surface targeting and receptor activation.

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Structural Requirements for Mutational Lutropin/Choriogonadotropin Receptor Activation
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