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Zero-Length Cross-Linking Reveals that Tight Interactions between the Extracellular and Transmembrane Domains of the Luteinizing Hormone Receptor Persist during Receptor Activation

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Several molecular models of glycoprotein hormone receptor activation have been proposed. It has been suggested that ligand binding to the ectodomain (ECD) leads to major changes in intramolecular interactions between the ECD and the transmembrane domain. We studied these intramolecular modifications by generating a recombinant LH/CG receptor (LHR) bearing an intramolecular cleavage site. We did this by inserting a furin site at position 316 in the hinge region of the ECD (LHR_Fur316). Affinity for human chorionic gonadotropin (hCG) and cAMP production upon hCG stimulation was identical to those of wild-type LHR. Western blot analysis showed that the LHR_Fur316 receptor was cleaved into two subunits linked by disulfide bridges. Chemical shedding of the ECD from the transmembrane domain did not increase basal adenylate cyclase activity, indicating that the first 294 residues did not act as an inverse agonist. The truncated LHR_316 was still activated by hCG but with an EC_{50} higher than that for the wild-type receptor. Zero length cross-linking was used to study intramolecular interactions between the two domains of LHR_Fur316. Cross-linking efficiency was similar for the basal and activated states, which indicated that the two domains interacted closely in the basal state, and this tight interaction persisted during activation. Our data suggest that activation of the LHR results from subtle modifications of intramolecular interactions between the two domains and low-affinity binding of hCG to the extracellular loops or residues preceding the first transmembrane segment. (*Molecular Endocrinology* 19: 2086–2098, 2005)

GLYCOPROTEIN HORMONE RECEPTORS (GPHRs) belong to the G protein-coupled receptor superfamily. They form a subgroup characterized by a large extracellular domain, composed of a leucine-rich repeat (LRR) region and a hinge region located between the LRR region and the first transmembrane helix. The activation mechanisms of GPHRs resemble those of G protein-coupled receptors (GPCRs) homologous to rhodopsin in several aspects. Common mechanisms have been described for the transmembrane domain (TMD) or serpentine region, which is the region most similar to rhodopsin (for review see Refs. 1–3). Specific mechanisms result from ligand binding with high affinity to the LRR region of the ectodomain (ECD) of GPHR but also from the role of the hinge region in the normal folding of the receptors (4) and in signal transduction (4–6). The molecular mechanisms by which the serpentine region adopts an active conformation after ligand binding remain largely unknown. It has been suggested that the ligand interacts directly with the TMD by low-affinity interactions, which may stabilize the active conformation of the serpentine region (7, 8). The description of constitutive mutations affecting the hinge region of TSH and LH receptors (TSHRs and LHRs) suggested functional interactions between the ECD and the serpentine region (9, 10). Expression of TSHR without the extracellular domain is associated with an increase in constitutive activity, suggesting that this region acts as an inverse agonist of the serpentine region (11, 12). According to this model, it was proposed that ligand binding disrupts interactions between ECD and TMD (11, 12). The higher constitutive activity of the N-terminally truncated TSHR bearing an

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Abbreviations: ab, Antibody; CG, chorionic gonadotropin; DTT, dithiothreitol; ECD, ectodomain; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; FACS, fluorescence-activated cell sorting; GPCR, G protein-coupled receptor; GPHR, glycoprotein hormone receptor; HEK, human embryonic kidney; LHR, LH receptor; LRR, leucine-rich repeat; NHSS, *N*-hydroxy-sulfo-succinimide; TMD, transmembrane domain; TSHR,TSH receptor; WT, wild type.

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activating mutation within TMD6 and the lack of increase in activity for activating mutations affecting the first extracellular loop led Costagliola and associates (13) to suggest that the ECD of the TSHR may also act as an agonist of the TMD. However, Sangkuhl *et al.* (14) found no evidence of inverse agonist activity for the ECD of the LHR in a study using a similar experimental approach. Ligand binding to the LHR may thus reveal agonist structures within the ECD, leading to the induction or stabilization of more constrained interactions between the ECD and the serpentine region. The higher resistance to protease treatment of constitutively activated LHRs compared with that of the wild-type (WT) receptor is consistent with this hypothesis (15).

The differences between the TSHR and LHR activation models was unexpected for such very homologous receptors, the ECDs of which are readily exchanged (16) and in which ligand specificity is controlled by only a few residues (17). These differences may be due to the experimental model used in these studies, as deletion of the extracellular domain resulted in low levels of receptor expression at the cell surface (11, 12, 14). Furthermore, the expression of ECD-truncated receptors provided insight into the functional interactions between ECD and the serpentine region, but the intramolecular interactions between the two domains were not studied.

In this study, we used a new experimental approach to study functional interactions between ECD and TMD. This model also provides evidence of tight intramolecular interactions between the two domains in basal and activated states. A recombinant LHR bearing an intracellular proteolytic site, inserted into the hinge region of the extracellular domain, was produced. This recombinant receptor was functional and was fully cleaved into two subunits linked by disulfide bridges. Chemical shedding of the N-terminal part of the recombinant receptor from the serpentine region did not increase intracellular cAMP concentration, but human chorionic gonadotropin (hCG) activation was observed. We demonstrated, by using a zero-length cross-linking strategy, tight interactions between the two domains in both basal and activated states. Our results confirm that the ECD of the LHR does not act as an inverse agonist and that modifications of intramolecular interactions between the ECD and TMD during the LHR activation are limited to few residues.

RESULTS

Cleavage of the Recombinant LHR with a Proteolytic Site

We investigated intramolecular interactions between the ECD and TMD of GPHRs by inserting a cleavage site into the ECD of the LHR. This cleavage site needed to be recognized by a highly specific protease. We initially inserted a thrombin cleavage site. However, only incomplete *in vitro* cleavage was observed on Triton-solubilized LHR. We therefore inserted a cleavage site recognized by a proprotein convertase active in the posttranslational processing of secreted proteins (18). This made it possible to cleave the expressed recombinant receptor during its processing. The RARRRR (furin) site was chosen because this site is the most efficient of this type and is recognized by several proconvertases (18). The furin site was inserted at residues 277, 316, and 356 of the ECD of the pig LHR, corresponding to the beginning of the hinge region, the middle of this region, and just before the first TMD (Fig. 1A). Recombinant receptors bearing the furin site at positions 277 and 356 were not expressed at the surface of transfected human embryonic kidney (HEK)293T cells (data not shown). Western blots performed with LHR775ab on membrane extracts of transiently transfected cells expressing these receptors showed only the uncleaved immature form of the LHR_Fur272 and LHR_Fur356 receptors. Flow cytometry on HEK293 cells transiently transfected with the LHR_Fur316 construct showed normal cell surface expression, as shown by comparison with the WT LHR (data not shown). This recombinant receptor was named LHR_Fur316.

Recombinant LHR with a furin site at position 316 was characterized by immunoblotting with various antibodies against the ECD or TMD, with and without reduction by β-mercaptoethanol. On Western blots of Triton-solubilized LHR_Fur316 membrane extracts, with LHR775 antibody (ab), in the presence of β-mercaptoethanol, we detected a strong band with an apparent molecular mass of 56 kDa and a weaker band at 68 kDa (Fig. 1B). We detected two bands (96 kDa and 68 kDa) with a similar intensity pattern for the WT receptor (Fig. 1B). The cleavage of LHR_Fur316 was complete, as no band corresponding to the predicted size of the uncleaved receptor was detected. Deglycosylation of LHR_Fur316 with *N*-endoglycosidase F converted the 56-kDa band into a single band with an apparent molecular mass of 32 kDa, corresponding to the predicted size of the unglycosylated peptide bearing the first 294 amino acids of the ECD (22–316) (Fig. 1C). This result is consistent with all the glycosylation sites being located between the N-terminal end of the LHR and the furin site. The 56-kDa subunit was named ECD-316.

Western blots with an ab recognizing the c-Myc tag located at the C terminus of the recombinant LHRs showed a band with an apparent molecular mass of 98 kDa, corresponding to the uncleaved, mature WT LHR. For LHR_Fur316, the c-Myc ab detected a band with an apparent molecular mass of 42 kDa (Fig. 1C), corresponding to the residues located between residue 316 and the c-Myc tag, including the C-terminal end of the ECD, the TMD, and the intracellular domain. This part of the receptor was named the 316-TMD. The molecular mass of the TMD of LHR_Fur316 was not affected by deglycosylation with endoglycosidase F (Fig. 1C), confirming that this part of the receptor was

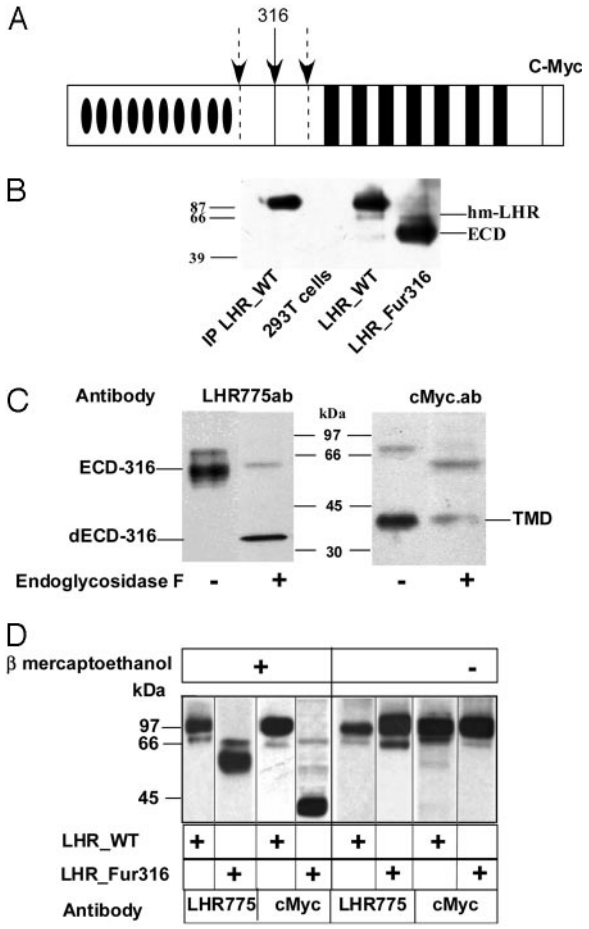

Fig. 1. Western Blot Characterization of the Recombinant Furin-LHR Chimera

A, Schematic representation of the LH recombinant receptor. *Arrows* indicate the position of the furin sites. *Closed ellipses* indicate leucine repeats, and *closed rectangles* indicate hydrophobic segments within the TMD. B, Membrane extracts were purified from HEK293T cells transiently transfected with expression plasmids encoding the WT receptor (LHR_WT), the LHR bearing the furin site at residue 316 (LHR_Fur316) and proteins were solubilized with 1% Triton. The protein samples were resolved by 8% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed for an ECD epitope (LHR775ab). A WT receptor immunopurified (ip LHR_WT) from a stably transfected HEK293 cell line expressing LHR, as described elsewhere (19), was used as a control. HEK293T cells, Nontransfected cells. ECD-316, Extracellular domain of LHR_Fur316. The high-mannose form of the LHR (hm-LHR) is indicated. C, Deglycosylation of LHR_Fur316 with endoglycosidase F: Triton extracts were incubated with endoglycosidase F and Western blotted, as described in panel B. The samples were probed for an ECD epitope (LHR775ab) or a c-Myc epitope (c-Myc.ab) at the C-terminal end of the receptor. dECD-316, Deglycosylated ECD-316. D, Western blot of LHR_WT and LHR_Fur316-solubilized membrane extracts in the presence and absence of β-mercaptoethanol. Cellular membrane extracts were purified from transfected cells expressing the WT or the LHR_Fur316 receptor. The samples were resolved by 8% SDS-PAGE in the presence and absence of 0.7 M β-mercaptoethanol.

not N glycosylated. In all lanes, c-Myc ab detected a band that decreased in size from 68 kDa to 60 kDa after endoglycosidase treatment, confirming that the 68-kDa band corresponded to the immature form of the LHR_ Fur316 receptor.

Western blots performed with LHR775ab or c-Myc abs in the absence of β-mercaptoethanol showed that LHR_Fur316 and LHR_WT both had an apparent molecular mass of 98 kDa (Fig. 1D). The separation of LHR_Fur316 into the ECD and TMD in the presence, but not in the absence, of β-mercaptoethanol indicates the existence of disulfide bridges between ECD-316 and 316-TMD.

Intracellular cleavage of the recombinant LHR_Fur316 is therefore efficient and precise in HEK293 cells. The LHR_Fur316 precursor has a molecular mass similar to that of the WT receptor in the presence of β-mercaptoethanol. This result is consistent with proconvertase cleavage in the trans-Golgi compartment. Separation of the two domains of LHR_Fur316 in the presence, but not in the absence, of β-mercaptoethanol demonstrates that the ECD-316 domain and the 316-TM domain of the LHR_Fur316 are linked by disulfide bonds.

Functional Characterization of Recombinant LHR_Fur316

We investigated the functional characteristics of LHR_Fur316 by transiently transfecting HEK293T cells. We evaluated the function of the recombinant LHR_F316 by measuring intracellular cAMP accumulation in the absence of hCG and after stimulation by hCG. Levels of hCG-induced cAMP production were identical for the recombinant LHR_Fur316 and the WT LHR (Fig. 2A). Basal levels of cAMP production were identical for both receptors (Fig. 2A, *inset*). Flow cytometry showed that cell surface expression levels were similar for LHR_WT and LHR_Fur316. Homologous competitive binding experiments showed that LHR_Fur316 bound hCG with an affinity similar to that of the WT receptor (Fig. 2B). These results indicate that insertion of the furin site at residue 316 did not disturb activation of the LHR.

Stable HEK293 cell lines expressing WT receptor or LHR_Fur316 were established by neomycin selection. The cell surface expression of LHRs was confirmed by immunocytochemistry with an ab directed against the extracellular domain (LHR 38ab) (19). Two cell lines expressing high levels of LHRs were selected. Normal basal and hCG-induced adenylate cyclase activation was observed in these cell lines.

Shedding of the ECD from the Cell Surface

Stable cell lines expressing the WT or the cleaved receptor were detached by EDTA treatment, washed twice with PBS, and incubated with 150 mM dithiothreitol (DTT) in HEPES buffer, pH 8. Western blots were carried out on Triton-solubilized membrane extracts, with the c-Myc ab, in the absence of

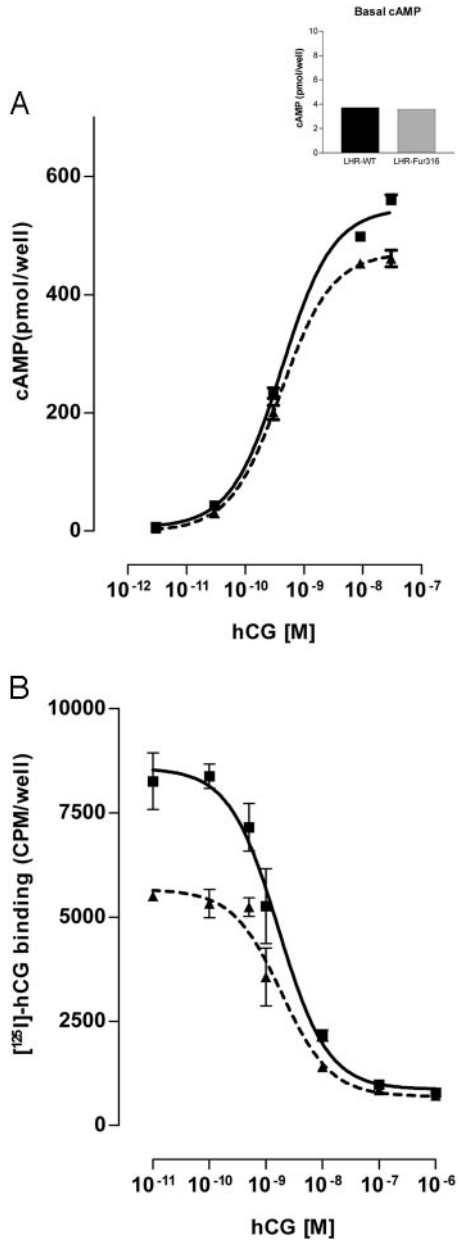

Fig. 2. Functional Characterization of the LHR_Fur316 Receptor

A, Adenylate cyclase activity of LHR_WT (■) and LHR_Fur316 (▲), measured by assessing intracellular cAMP production after stimulation with various concentrations of hCG. Basal cAMP accumulations for both receptors are indicated in the *inset*. The assays were performed in triplicate. B, Competitive binding of ^{125}I hCG in the presence of various concentrations of unlabeled hCG for LHR_WT (■) and LHR_Fur316 (▲).

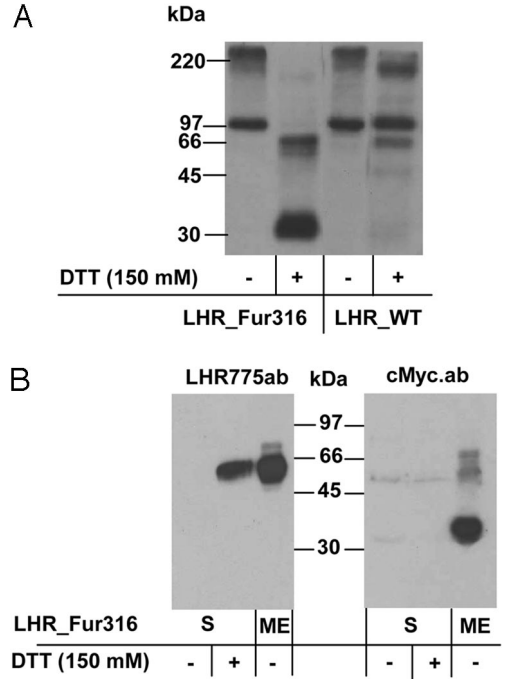

Fig. 3. Shedding of ECD from the Cell Surface by Disulfide Bridge Reduction

Stably transformed cell lines expressing WT and furin-recombinant LHRs were treated with 150 mM DTT for 30 min at 4 C. A, Cells were washed twice with HEPES buffer; solubilized cell membrane extracts were produced and Western blotted with c-Myc ab in the absence of β-mercaptoethanol. B, Western blot performed on concentrated supernatant (S) in the presence of β-mercaptoethanol, using LHR775ab (*left*) and c-Myc.ab (*right*). LHR_Fur316 receptor solubilized from membrane extracts (ME) and not treated with DTT was used as a control.

β-mercaptoethanol. We detected a band at 98 kDa for the WT LHR and LHR_Fur316 in the absence of DTT (Fig. 3A). DTT treatment led to the detection of a band at 42 kDa for LHR_Fur316 receptor but did not affect the size of the WT LHR (Fig. 3A). This result indicates complete reduction by DTT incubation of the disulfide bridges between ECD and TMD.

Western blot was performed with LHR775ab on DTT-concentrated supernatant to determine whether disulfide bridge reduction led to ECD shedding from the cell surface. We detected a band in the supernatant of LHR_Fur316 cells that was similar in size to the control LHR_Fur316 receptor solubilized from membrane extract (Fig. 3B). No band was detected in the supernatant of DTT-treated LHR_WT cells (data not shown). We stripped the membrane and incubated it with c-Myc ab. No band was detected in the supernatant, although a band corresponding to the TMD was observed in the membrane extract lane. Thus, the reduction of disulfide bridges between ECD and TMD is sufficient to induce the shedding of ECD from TMD in living cells.

We assessed the accumulation of intracellular cAMP in DTT-treated cells and control cells. DTT decreased cAMP accumulation in both LHR_Fur316 cells and LHR_ (Fig. 4A). These lower levels of basal cAMP result from weaker adenylate cyclase activity in the presence of DTT as forskolin adenylate cyclase stimulation was also decreased by DTT treatment (Fig. 4B).

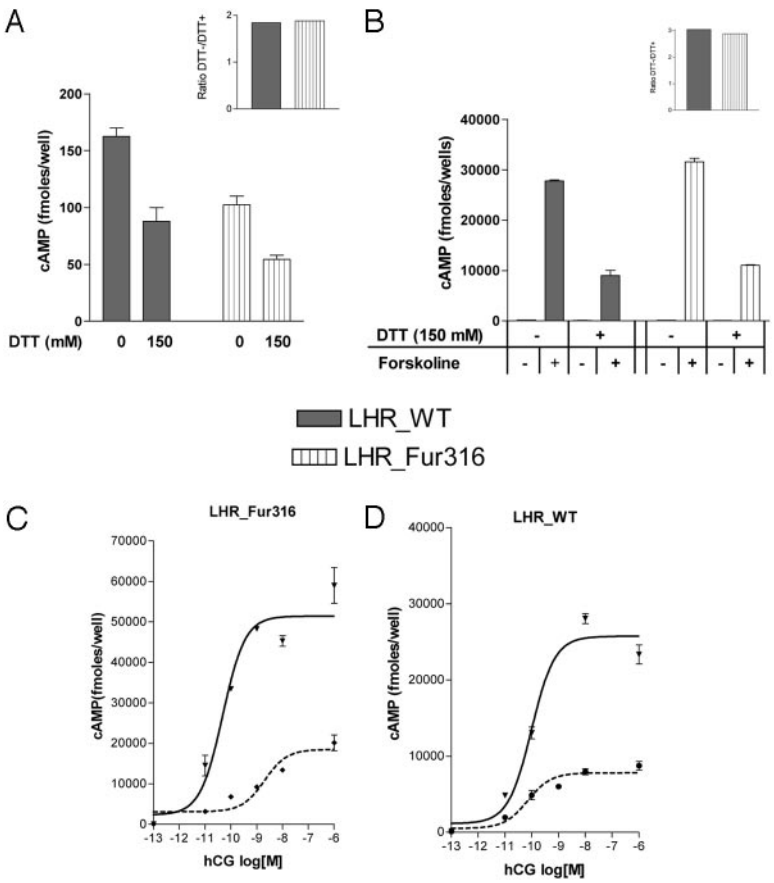

Fig. 4. Intracellular cAMP Accumulation after ECD Shedding in Response to DTT

Stably transfected cell lines expressing WT or furin recombinant receptors were treated with DTT as described in the legend to Fig. 3, except that cells were washed twice with 0.1% BSA in DMEM after DTT treatment. cAMP assays were performed as described in *Materials and Methods*. A, Basal intracellular cAMP levels in DTT-treated cells expressing LHR_WT or LHR_Fur316. B, Effect of DTT treatment on forskolin-stimulated intracellular cAMP levels. *Inset*, Ratios of cAMP levels in cells not treated and treated with DTT. C, hCG stimulation after ECD shedding in response to DTT treatment.

The ratio of basal cAMP levels in DTT-treated cells to basal cAMP levels in control cells was identical for LHR_Fur316 cells and LHR_WT cells. We therefore conclude that shedding of the ECD of the LHR from the cell surface did not increase basal LHR activity, indicating that the ECD-316 domain does not act as an inverse agonist of the TMD.

We then investigated the activation of LHR_Fur316 by hCG, after chemical shedding of the ECD-316 domain. Hormone stimulation was performed for 30 min on DTT-treated LHR_WT and LHR_Fur316 cell lines, and adenylate cyclase activation was compared with that of untreated cells. The 316-TMD receptor was activated by hCG, with a decrease in maximal stimulation and shifting of the dose-response curve to the *right* (Fig. 4C). The EC_{50} was 4.7×10^{-11} M for the full-length receptor LHR_Fur316 and increased to 2×10^{-9} M after shedding of the ECD-316 domain (Fig. 4C). A similar decrease in maximal stimulation was observed for the WT receptor, but EC_{50} values calculated with and without DTT treatment were similar (9.7 and 7.1×10^{-11} M for the untreated and DTT-treated LHR_WT receptors, respectively). Thus, hCG interacts with the 316_TDM and stabilizes it in an active conformation.

Intramolecular Cross-Linking between ECD and TMD

It has been shown recently that the ECD adopts a new conformation in activated LHR, probably modifying intramolecular interactions between the two domains. We therefore studied ECD-TMD interactions, using cross-linkers with a very small spacer, making it possible to reveal differences in intramolecular interactions during activation. Zero-length cross-linkers are defined as reagents joining two intrinsic groups without introducing extrinsic material (20). Such cross-linkers may be specific for particular functional groups, but this is not necessarily the case (20). We needed a hydrophilic reagent that would react extracellularly to limit our study only to the intramolecular interaction between ECD and TMD. We therefore used 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), which is

a hydrophilic, zero-length hetero-bifunctional cross-linker targeting carboxyl and amino groups (20).

Cross-linking was carried out in intact cells, after repeated washing with PBS to remove all soluble molecules containing amines. ECD-TMD cross-linking of the LHR_Fur316 was efficient, as demonstrated by the observed decrease in intensity of the 56-kDa band and simultaneous increase in intensity of the 98-kDa band corresponding to uncleaved mature LHR_WT (Fig. 5). We began by investigating various concentrations of EDC (10–40 mM), with the aim of optimizing cross-linking reactions but detected only a weak cross-linked complex (data not shown). The addition of *N*-hydroxy-sulfo-succinimide (NHSS) to the incubation buffer stabilized the intermediate metabolite of EDC and increased the intensity of cross-linking (21, 22). Increasing the concentration of NHSS increased the intensity of the band with an apparent molecular mass of 98 kDa, corresponding to uncleaved LHR_WT (data not shown). We therefore defined incubation with 40 mM EDC plus 5 mM NHSS at 4 C for 30 min as the optimal conditions for cross-linking ECD to TMD in the LHR. EDC cross-linking in whole cells showed that the 294 N-terminal residues of the LHR closely interacted with residues 317–360 or the extracellular loops of the LHR.

Effect of hCG Stimulation on the Efficiency of the Cross-Linking between ECD and TMD

To analyze interactions between the ECD and TMD of the LHR during receptor activation, stably transfected cells expressing LHR_Fur316 were incubated with 10^{-8} M hCG at 37 C for 15 min. Cells were washed three times with PBS at 4 C, and cross-linking was then carried out by incubating the cells in 40 mM EDC/5 mM NHSS at 4 C. In the absence of the cross-linker, two bands at 56 kDa and 68 kDa were detected with the ab LHR775ab. After incubation with EDC-NHSS, a band with an apparent molecular mass of 98 kDa was detected (Fig. 5). The intensity of cross-linking, as reflected by the intensity of the 98 kDa band, was highest after incubation for 30 min (Fig. 5). No band with a molecular mass exceeding 98 kDa was detected on any of the blots. This suggests that these soluble membrane extracts did not contain molecular complexes bearing hCG cross-linked to LHR. Tight interaction between the ECD and the TMD of the LHR was maintained after ligand binding to the ECD.

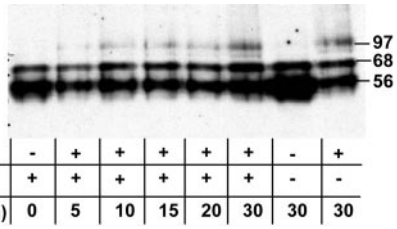

Fig. 5. LHR Intramolecular Cross-Linking by EDC

Western blots were performed with LHR775ab, on solubilized membrane extracts of stable cell lines expressing LHR_Fur316, after stimulation by incubation with hCG (10^{-8} M) for 15 min at 37 C and incubation with 40 mM EDC and 5 mM NHSS.

Comparison of EDC Cross-Linking Intensity in Constitutively Activated LHRs

The visualization of cross-linked complexes after hCG binding depends on the number of active LHR molecules at the cell surface. After 15 min at 37 C, these ligand-receptor complexes may be no longer available for cross-linking with a hydrophilic reagent that cannot pass through the membrane. We thus carried out cross-linking on constitutively activated receptors because such receptors are continuously available in the activated conformation at the cell surface before internalization. The replacement of serine 277, within the hinge region, by any of the other 19 natural amino acids leads to a constitutive increase in rat LHR levels (9). These activating mutations should mimic the conformational change in the ECD observed during stimulation due to the binding of hCG to the LRR. We compared the efficiency of cross-linking between Ser277 mutants and the Asp578His mutant. The molecular mechanism underlying Asp578His-mutated LHR activation is thought to involve modification of the hydrogen bonds between hydrophobic segments (23, 24).

Flow cytometry showed similar cell surface expression profiles for all mutated receptors except LHR_Fur_S277L, which displayed lower levels of surface expression (Fig. 6A). Western blots of membrane extracts with LHR775ab displayed strong signals at 56 kDa for all receptors except LHR_Fur_S277L (Fig. 6B). For this receptor, a strong band at 68 kDa, corresponding to the immature form, was observed. A similar low level of expression was observed for the S277L mutated receptor without a furin site (data not shown). These results suggest that the low level of cell surface expression of LHR_Fur results from abnormal processing or increase degradation rather than the inserted furin site.

Basal intracellular cAMP levels were high in all mutated LHR_Fur316 receptors, confirming that all mutations constitutively activated the porcine LHR (Fig. 7A). Constitutive activation was weakest for the S277N mutation. The basal level of cAMP accumulation observed for LHR_Fur_S277L was similar to that for LHR_Fur_S277V, which was expressed normally at the surface of HEK293T cells. All receptors responded to hCG stimulation similarly to the WT receptor (Fig. 7B). The similar response to hCG of the S277L receptor and LHR_WT indicates that the overall conformation of the S277L mutated receptor was highly sensitive to ligand-mediated activation.

For cells transiently expressing WT or mutated receptors, cross-linking was carried out at 4 C for 30 min. An intense band at 98 kDa, corresponding to the

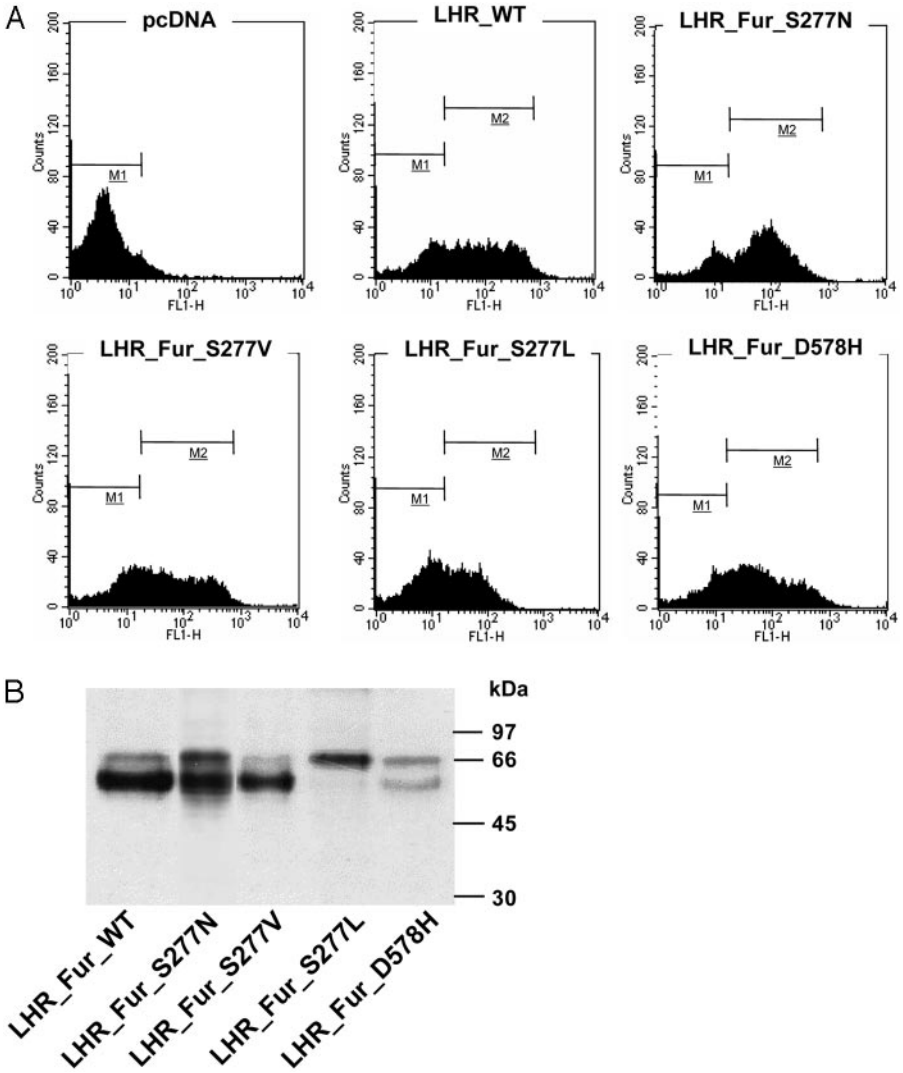

Fig. 6. Cell Surface Expression of Constitutively Activated LHRs

A, Cell surface expression was measured with a FACS, using LHR775ab on HEK293T cells transiently transfected with plasmids encoding the WT and constitutively activated mutated LHR_Fur316 receptors. B, HEK293T cells were transiently transfected with plasmids encoding WT and mutated receptors, as described in *Materials and Methods*. Western blots were performed as described in the legend to Fig. 1A, with LHR775ab. pcDNA, Plasmid; M1, nonspecific fluorescence; M2, specific fluorescence; FL1-H, fluorescence intensity.

cross-linked complexes, was observed for the WT, LHR_Fur_S277V, and LHR_Fur_D578H receptors (Fig. 8). A very faint band was detected at 98 kDa for the LHR_Fur_S277L receptor correlating with the low level of expression of this mutated receptor at the cell surface. Weak cross-linking was observed for LHR_Fur_S277N despite its normal expression at the cell surface. The low cross-linking efficiency of this receptor probably resulted from the conformational modification induced by the S277N substitution, as this mutated receptor displayed a smaller increase in basal activity than the three other mutations tested. Our results indicate that the tight ECD-TMD interactions revealed by EDC cross-linking persist during the constitutive activation of LHRs.

DISCUSSION

Functional Integrity of the LHR Was Not Disturbed by Furin Cleavage within the ECD

In this study, we used a new experimental model to study intramolecular interactions between the ECD and the TMD of the LHR. We introduced a cleavage site recognized by the proprotein convertase furin in the extracellular domain facilitating chemical separation of the ECD from the TMD. Previous studies on the activation of GPHR suggested that the ECD of the TSHR acts as an inverse agonist of the serpentine region whereas the ECD of the LHR acts only as an agonist (11, 12, 14). These models were based primar-

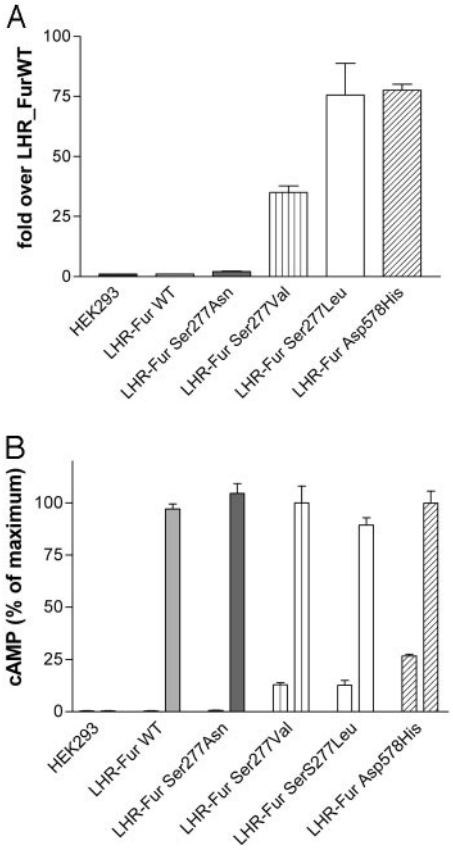

Fig. 7. Basal and hCG-Stimulated cAMP Production for WT, S277 LHR_Fur316, and D578 LHR_Fur316 Mutated Receptors

We assessed cAMP production 48 h after the transfection of HEK293T cells with plasmids encoding WT or mutated LHR_Fur316 receptors. A, Basal levels. cAMP levels are expressed as fold over the basal level observed for LHR_FurWT after normalization to the levels of the receptor expression measured by FACS analysis. B, hCG (10^{-7} M)-stimulated cAMP accumulation. cAMP levels are expressed as the percentage of the level observed for LHR_FurWT.

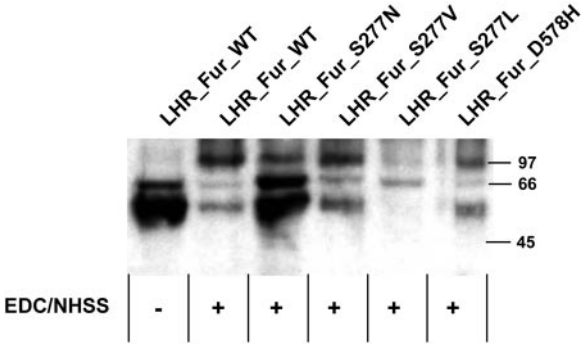

Fig. 8. Intramolecular Cross-Linking of Constitutively Activated LHRs

Cross-linking with EDC/NHSS was carried out in EDTA-detached HEK293T cells expressing recombinant LHR_Fur316 receptors, as described in *Materials and Methods*. Western blotting was performed with LHR775ab.

ily on truncated receptors that are poorly expressed at the cell surface. In contrast, we investigated whether the ECD-truncated TMD of a cleaved LHR expressed at the cell surface at similar levels to the WT receptor was able to activate adenylate cyclase. With this approach, we aimed to demonstrate functional and specific intramolecular interactions between the two domains. We inserted the cleavage site at position 316, as this point resembles the most N-terminal proteolytic site reported for the TSHR (25, 26). Residue 316 is encoded by exon 10 of the LHR gene. The residues encoded by exon 10, which is spliced out during mRNA processing in New-World monkeys (27), seem to play a role in stimulation of the receptor by LH but not by hCG (28). The recombinant cleaved LHR_Fur316 responded normally to hCG stimulation, demonstrating its functional integrity. In contrast, insertion of the proteolytic site at two other positions, LHR_Fur277 and LHR_Fur356, impeded cell surface expression. These cleavage sites are located in highly conserved regions, and insertion of the cleavage site may therefore interfere with receptor folding and trafficking (2, 4).

Western blot characterization of the LHR_Fur316 receptor showed that the first 294 residues of the mature LHR are linked to 316-TMD by disulfide bridges. This result is consistent with the presence of a functional LHR when part of the ECD (1–297) was coexpressed with the C-terminal 402 amino acids of the pig LHR (29). These two domains are probably linked by disulfide bridges (30). Coexpression of the full-length ECD (residues 1–364) with the seven transmembrane segments and the intracellular domain (residues 365–698) did not result in the expression of a functional receptor at the cell surface (14, 31). However, an ECD fused to the first TMD coexpressed with the serpentine region is functional (31). These data indicate that the first 294 residues (22–316) of the mature LHR interact with residues preceding the first TMD, and that this is important for the normal folding and trafficking of the intact receptor. Fralish *et al.* (32) compared the secondary structure of the domain bearing the first 294 residues of the LHR with that of the whole ECD (341 residues without the signal peptide) by circular dichroism. They showed that the 47 C-terminal residues of ECD (294–341, rat numbering) corresponding to the extracellular end of the 316_TMD of the recombinant LHR_Fur316 stabilize the secondary structure of the LRR (32). Sangkuhl *et al.* (14) obtained similar results showing that an intact overall conformation of the ECD is also required for constitutive activation of the LHR by mutations within the hinge region. This stabilization probably involves disulfide bridges.

The cysteines of the ECD are very well conserved between glycoprotein receptors (2). Two clusters surrounding the LRR have been described, and a third cluster is located just before the first TMD. Cysteines at the N terminus of the ECD are involved in establishing disulfide bridges leading to the formation of an

N-terminal loop (33). The cysteines located within extracellular loops 1 and 2 are conserved in all G protein-coupled receptors homologous to rhodopsin and form a disulfide bridge. Disulfide bridges between the C terminus of ECD-316 and the N terminus of 316-TMD should involve cysteines 279 and 280 with the cysteine cluster (C343 or C353) located before the first TMD. Another disulfide bridge should involve Cys304 with Cys336 (Fig. 9). Normal folding of the intact LHR would therefore require close interaction between the residues surrounding these cysteines 279, 280, and 304 and residues preceding the first TMD. Such interactions were recently proposed for the TSHR (34).

The molecular form of the mature LHR_Fur316 expressed at the cell surface is very similar to the mature TSHR as these two receptors are cleaved at similar positions. In contrast to the single cleavage observed in our model, spontaneous cleavage of the TSHR occurs sequentially from positions 316 to 366–378 (25, 26). Studies with LHR-TSHR chimera have shown that TSHR cleavage is not essential for the stimulation of adenylate cyclase (35). We show here that a single cleavage at residue 316 during posttranslational processing of the LHR is insufficient to induce constitutive activation. In contrast to what was observed for LHR_Fur316, we observed a faint but clear band corresponding to the TMD of TSHR in Western blots in the absence of β-mercapthoethanol (data not shown). This confirms the observed spontaneous shedding of TSHR-ECD into cell culture media (36). We observed no such spontaneous shedding of the extracellular domain of LHR_Fur316. Disulfide isomerase is thought to be involved in the shedding of the TSHR-ECD (32), but this mechanism is not sufficient to account for shedding of the LHR.

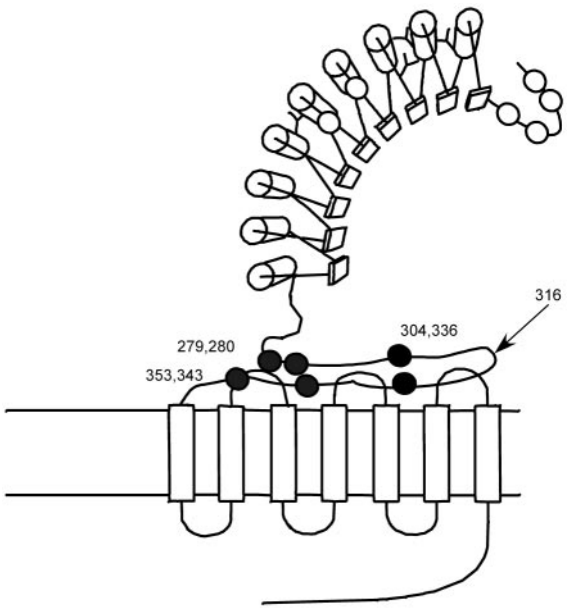

Fig. 9. Cysteines Involved in Disulfide Bridges between the Extracellular Domains and the TMDs of the LHR

Cysteines of the extracellular domain are represented by a *circle*. Putative cysteine residues involved in disulfide bridges between the extracellular domain and the transmembrane domain are indicated by *closed circles*. Residues 279 or 280 may interact with residues 343 or 353. Residue 304 may interact with residue 336. The localization of the furin site is indicated (316).

ECD-TMD Interactions Involve Lysine Interactions with Aspartate or Glutamate, which Persist during Receptor Activation

We used a zero-length cross-linker strategy to restrict our study to closely interacting residues. The cross-linker used in this study covalently links the amino group of lysine to the carboxyl groups of aspartic or glutamic acids. Our results show that such intramolecular interactions exist between the ECD-316 and the 316-TMD. Several studies have suggested that ECD interacts directly with the extracellular loops of the LH or FSH receptors (31, 37–39). Studies with a chimeric receptor bearing the ECD of human LHR and the transmembrane region of fly LRR-containing G protein-coupled receptor (LGR2) led Nishi *et al.* (38) to suggest that the hinge region surrounding serine 277 interacts directly with exoloop 2. This interaction might be released by hCG binding to the high-affinity binding site of the LRR. It has also been suggested that ECD interacts with exoloop 3 in the FSH receptor (34). Several lysine and acidic residues in the extracellular part of the receptor are involved in signal transduction. In exoloops 2 and 3, Lys488 and Lys583 are involved in LHR signaling (40, 41). The replacement of Asp397 (exoloop 1) by a basic residue such as lysine or arginine results in normal binding affinity for hCG but weaker signaling (42). Carbodiimide-specific cross-linking between residues of the ECD-316 and the TMD observed in the absence of the natural ligand, after hormone binding and in constitutively activated LHRs, indicates the close proximity of lysine and aspartic or glutamic acid residues in the native and active receptor states.

Major Modifications to the Interaction between ECD and TMD of the LHR Do Not Lead to Receptor Activation

The reduction of disulfide bridges between ECD and TMD is sufficient to dissociate the two domains of LHR_Fur316. The normal intracellular cAMP levels observed after DTT-mediated shedding of the first 294 residues show that major modifications to the intramolecular interactions between the two domains prevent TMD from adopting an activated conformation. Thus, this part of the receptor does not act as an inverse agonist of the TMD. The inverse agonist role described for the ECD of the TSHR results from the TMD of this receptor having a less constrained form than that of the LHR. However, this role seems to be less important than the agonist role of the ECD in full activation of the glycoprotein receptor.

In one model of glycoprotein receptor activation, it was suggested that hormones bind to the LRR with high affinity and to extracellular loops with low affinity. This double interaction results in activation of the TMD (43). This hypothesis was rejected because TDM is not able to stimulate adenylate cyclase in the absence of extracellular residues (14). The increase in EC_{50} observed in this study after the shedding of ECD-316 is consistent with ligands of glycoprotein receptors being involved in direct low-affinity interactions with residues preceding the first transmembrane segment or extracellular loops.

All GPCRs bear an N-terminal extracellular domain, the length of which differs from protein to protein. The crystal structure of rhodopsin has shown that the N-terminal end and the extracellular loops form a compact structure with several intramolecular interactions (44). It has been suggested that GPHR genes are derived from a fusion of a gene encoding a protein bearing a LRR domain and a GPCR ancestor gene homologous to the rhodopsin gene. A single exon (exon 11) encoding residues 317–698 and a splicing variant corresponding to the ECD-316 described in pig testis suggest that residues 317–366 of LHR_Fur316 correspond to the N-terminal extracellular end of the ancestral receptor. By analogy with the structure of rhodopsin, we suggest that interactions of residues 317–366 with extracellular loops of the LHR may be modulated by ligand binding to the LRR region and low-affinity interactions between the ligand and this region.

This work provides a new model for studying intramolecular interaction between the extracellular domains and TMDs of GPCR for glycoprotein hormones. We demonstrated that the salt bridges between the two domains persist during LHR activation, suggesting that the modifications of intramolecular interactions occurring are subtle. Combining a zero-length intramolecular cross-linking strategy with mass spectrometry of the cross-linked receptor may therefore be used to characterize these interacting residues.

MATERIALS AND METHODS

LHR Expression Constructs

Several proteolytic sites for *in vitro* cleavage were tested, but complete cleavage was not observed. We therefore decided to insert a proteolytic site for cleavage of the recombinant receptor within the cell. We chose a site recognized by furin and inserted it into the extracellular domain. The cDNA encoding the WT pig LHR was subcloned from the PSG5 vector (45) into the pcDNA 3.1-His.Myc mammalian expression vector (version B), using the *Eco*RI and *Bam*HI sites (pLHR_WT). An *Apa*I site from the pcDNA3.1 polylinker was removed by cutting the pLHR_WT vector with *Nhe*I and *Xba*I. Two *Xba*I sites are present in the polylinker of version B of pcDNA 3.1. The *Xba*I fragment bearing the LHR cDNA was ligated to the linearized *Nhe*I-*Xba*I plasmid. This removed the *Pme*I, *Apa*I, and the 5′-*Xba*I restriction enzyme sites.

Monoclonal antibodies directed against the ECD are available (19). A c-Myc epitope (EQKLISEED) was fused to the coding sequence of the LHR for the detection of TMD by Western blotting. The pLHR construct was digested with the blunt-end cutter *Hpa*I to remove the stop codon and with *Bam*HI treated by T4 DNA polymerase to blunt the ends of the *Bam*HI site and religated. This resulted in elimination of the *Bst*XI and *Bam*HI restriction sites from the pcDNA polylinker of the resulting construct. The peptide sequence SSFL appeared between the last residue of the porcine LHR and the first residue of the c-Myc epitope.

The proteolytic site recognized by proconvertases such as furin (RARRRR) was inserted into the extracellular domain by PCR. This site was named the "furin site" for reasons of clarity. A PCR fragment bearing the nucleotide sequence encoding the cleavage site was used to replace the WT sequence at the *Apa*I and *Pflm*I restriction sites. This resulting construct was named pLHR_Fur. Point mutations were generated by PCR.

All plasmid constructs were validated by dideoxy sequencing of the modified fragment and restriction enzyme mapping.

Cell Culture and *in Vitro* Transfection

We studied hCG binding and adenylate cyclase activation by the WT receptor (LHR_WT) and the chimeric LHR_Furin (LHR_Fur) receptor in HEK293T cells, grown in DMEM/10% FCS/1% penicillin-streptomycin. Cells (1.5×10^5/well) were seeded in 12-well dishes coated with fibronectin. They were transiently transfected with 1 μg of vector DNA (WT LHR or recombinant LHR_Fur) by incubation for 24 h with Superfect transfection reagent (QIAGEN, Courtaboeuf, France) or Lipofectin Plus (Invitrogen, Cergy-Pontoise, France). Negative controls included transfection with the insert-less vector pcDNA3.1. Transfections were carried out in triplicate, and each independent experiment was repeated at least once. Stably transfected HEK293 cell lines expressing high levels of LHR_WT or LHR_Fur316 were established by transfection with both plasmids by the calcium-phosphate precipitation procedure and selection of the transfected cells with neomycin. Expression of the recombinant receptors in the transfected cells was confirmed by immunocytochemistry with an ab (LHR38ab) directed against the extracellular domain (19).

cAMP Assay

LHR function was studied by assessing hormone-induced adenylate cyclase activation. Forty-eight hours after transfection or 24 h after plating or DTT treatment, the culture medium was replaced by 0.1% BSA in DMEM supplemented with 0.5 mM 3-isobutylmethylxanthine (Sigma Chemical Co., St. Louis, MO) for 30 min at 37 C. Cells were then exposed to various concentrations of hCG (10^{-12} to 10^{-6} M, Organon, Eragny sur Epte, France), with which they were incubated for 30 min at 37 C. The accumulation of cAMP was stopped by adding 300 μl of 5% perchloric acid (PCA). Intracellular cAMP content was measured by RIA (Amersham Pharmacia Biotech, Buckinghamshire, UK).

hCG Binding

We carried out hCG binding studies in cells plated in 12-well dishes transiently transfected with pLHR_WT and pLHR_Fur plasmids, as outlined above. Forty-eight hours after transfection, cells were incubated with 1.2×10^5cpm/well [^{125}I]hCG, 2850 Ci/mmol, in the presence of various concentrations (10^{-11}M to 10^{-6} M) of unlabeled hCG for 3 h at 4 C in DMEM supplemented with 1% BSA/20 mM HEPES, pH 7.4. The affinity for hCG affinity and concentration of LHR sites on the cell surface were calculated with PRISM software (version 3.0, GraphPad Software, Inc., San Diego, CA). Binding studies were performed at least twice, in triplicate.

Flow Cytometry Analysis

Cell surface expression of LHR recombinant receptors was studied by fluorescence-activated cell sorting (FACS) analysis with the LHR775ab (15). Cells were detached from cell culture dishes by incubation with 1 mM EDTA in PBS and washed twice with PBS. We incubated 500,000 cells with 1% goat serum for 20 min at 4 C and then with LHR775ab (2 μg/ml) in the same buffer for 20 min at 4 C. The cells were then washed twice with the saturating buffer and incubated with an Alexa 488 fluorescent antimouse goat ab (1/1 250) (Molecular Probes, Eugene, OR) for 20 min at 4 C. Cells were washed twice with PBS at 4 C and fixed by incubation with 2% paraformaldehyde for 1 min at 4 C. Cells were analyzed in a FACS, by collecting 10,000 events, and negative controls were carried out with untransfected cells.

Immunoblotting of LHRs

Membrane preparations were obtained from whole-cell lysates of stable cell lines or transiently transfected cells, scraped off the plates when they reached 100% confluence in PBS supplemented with protease inhibitors. Cells were centrifuged at 400 $\times$ *g* for 5 min at 4 C and the resulting cell pellet was suspended in hypotonic lysis buffer (10 mM Tris, pH 7, supplemented with protease inhibitor) at 4 C, snap frozen in liquid nitrogen, and thawed at 37 C. This procedure was repeated twice. The cells were then passed through a needle (27 G) and centrifuged at 500 $\times$ *g* for 5 min at 4 C. The supernatant was centrifuged at 40,000 $\times$ *g* for 60 min at 4 C, and the resulting pellet was solubilized in 100 μl of 50 mM Tris/150 mM NaCl/10% glycerol/2% Triton/10 mM in *N*-ethylmaleimide buffer supplemented with protease inhibitor. The resulting suspension was centrifuged at 40,000 $\times$ *g* for 30 min at 4 C, and the total protein content of the supernatant was determined by the Bradford method (Pierce Chemical Co., Rockford, IL). Receptors were deglycosylated with *N*-glycosidase F according to the manufacturer's instructions (PNGase F, New England Biolabs, Inc., Beverly, MA). The solubilized membrane extracts were subjected to electrophoresis in an 8% polyacrylamide gel containing sodium dodecyl sulfate, in reducing and nonreducing conditions, and the protein bands were electroblotted onto polyvinylidene difluoride membrane. We used the LHR775ab (19) or monoclonal purified anti-c-Myc ab (clone, 9E10) directed against the c-Myc Tag fused to the C-terminal end of the pig LHR for detection. The precise location of the LHR775ab epitope is unknown, but its specific binding, without permeabilization, to the surface of LHR-transfected HEK293 cells indicates that the epitope is extracellular. A Western blot carried out on LHR_Fur showed that the LHR775ab epitope was located between residues 22 and 316 (see *Results*).

Cross-Linking of Recombinant LHR_Fur316 Expressed in HEK 293 Cells

We studied molecular interactions between the ECD and TMD of the LHR, using the zero-length heterofunctional cross-linker EDC on attached cells and on cells detached from plates by adding EDTA (2 mM). Stably transfected HEK293 cell lines expressing WT LHR or recombinant LHR_Fur316 were grown to 100% confluence. We determined optimal conditions for cross-linking, using various concentrations of EDC (between 5 and 40 mM) and various incubation times (5–60 min) and temperatures (4 C and 37 C) in the presence and absence of NHSS. For EDC cross-linking after receptor activation by hCG, cells expressing LHR_Fur316 were washed twice with 10 ml DMEM/20 mM HEPES/0.1% BSA and exposed to hCG (10^{-8} M) for 15 min at 37 C. Cells were washed with 10 ml PBS/1 mM Ca/0.5 mM Mg at 4 C to remove unbound ligand and were then incubated with EDC at 4 C. Cross-linking was stopped by washing cells with 10 ml 0.1 mM glycine/150 mM NaCl, pH 6.8, at 4 C. Cells were scraped into hypotonic lysis buffer at 4 C, and membranes were prepared as described above.

Chemical Shedding of the Extracellular Domain

ECD was shed from the cell surface by DTT treatment. Cells stably expressing WT LHR or LHR_Fur316 were detached from the plates by treating with EDTA (2 mM) in PBS supplemented with protease inhibitors (PI). They were washed twice in HEPES buffer [20 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 1 g/liter glucose (pH 8) plus protease inhibitors] and then kept in suspension in HEPES buffer. DTT (150 mM) was added and the cells were incubated for 30 min at room temperature. The cells were then centrifuged at 400 $\times$ *g* for 10 min at 4 C. The supernatant was concentrated 10-fold on Centricon YM30 filters (Millipore Corp., Billerica,) and used for Western blots. Cell pellets were washed twice with HEPES buffer at 4 C. Cell membranes were prepared and solubilized with Triton as described above. Western blots were performed in the absence of β-mercaptoethanol. We assessed cAMP accumulation in DTT-treated cells after two washes with 0.1% BSA in DMEM, as described above.

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