Deletions of Portions of the Extracellular Loops of the Lutropin/Choriogonadotropin Receptor Decrease the Binding Affinity for Ovine Luteinizing Hormone, but Not Human Choriogonadotropin, by Preventing the Formation of Mature Cell Surface Receptor*

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The rat lutropin/choriogonadotropin receptor (rLHR) is a G protein-coupled receptor which binds either human choriogonadotropin (hCG) or lutropin (luteinizing hormone, LH) and, therefore, plays a central role in reproductive physiology. In addition to the seven transmembrane helices, three extracellular loops, three intracellular loops, and a cytoplasmic tail characteristic of all G protein-coupled receptors, the rLHR also contains a relatively large N-terminal extracellular domain. Since high affinity hormone binding occurs to this N-terminal extracellular domain and since G proteins are activated by intracellular regions of the receptor, it has been hypothesized that upon hormone binding a portion of the hormone or the receptor's extracellular domain might interact with the receptor's extracellular loops and/or transmembrane helices, thus evoking an intracellular conformational change. To explore this possibility, we prepared and characterized several mutants of the rLHR in which portions of the extracellular loops were deleted.

Ultimately, it was not possible to examine the signal transduction properties of the mutants because all but one mutant were retained intracellularly. Although the intracellularly retained mutants must be somewhat misfolded, all were found to bind hCG with high affinity if the cells were first solubilized in detergent. However, the binding of oLH to the detergent solubilized mutants was altered. Thus, whereas the wild-type rLHR bound oLH with two apparent affinities, the solubilized deletion mutants bound oLH with only one apparent affinity. Although these data could be interpreted to suggest that an ovine LH (oLH) binding site on the extracellular loops of the rLHR was deleted, data shown argue against this hypothesis. Rather, the results presented suggest that the two apparent affinities of the wild-type rLHR for oLH represent the binding affinities of two populations of rLHR where the mature, cell surface form binds oLH with a higher affinity than the immature, intracellular form. Furthermore, we show that mutations of the rLHR which cause intracellular retention of the receptor result in a decrease from two to one apparent binding sites for oLH due to the absence of the high affinity oLH binding component contributed by the mature cell surface receptor. Therefore, whereas hCG cannot discriminate between the mature cell surface wild-type receptor and an intracellularly retained rLHR mutant, oLH can make this discrimination, thus suggesting a conformational difference between the two forms of the receptor.

The abbreviations used are: rLHR, rat lutropin/choriogonadotropin receptor; hCG, human choriogonadotropin; LH, luteinizing hormone (lutropin); oLH, ovine LH; FSH, follicle-stimulating hormone (follicotropin); FSHR, FSH receptor; rFSHR, rat FSHR; BSA, bovine serum albumin; wt, wild type; GTPγS, guanosine 5′-O-(thiotriphosphate).

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with the extracellular loops of the rLHR.

To further examine whether the extracellular loops of the rLHR play a role in the binding of glycoprotein hormones, we created a series of rLHR extracellular loop deletion mutants, the hypothesis being that if hormone interacted with a loop or a portion thereof, deletion of a portion of that loop would disturb hormone binding. The data presented demonstrated that, while mutations within all three extracellular loops of the rLHR do not affect hCG binding to the rLHR, they reduce the affinity of the rLHR for hCG from two apparent affinities to one apparent affinity. However, we further show that the reduction in hCG binding to one apparent affinity is not due to the deletion of a binding site, but instead due to the absence of the mature population of the rLHR.

**MATERIALS AND METHODS**

Supplies—Highly purified hCG (CR-125 and CR-127) and oLH (NIADDK-oLH-26) were kindly provided by the National Hormone and Pituitary Agency of the NIADDK (Baltimore, MD). hCG was iodinated as described previously (13). Crude hCG, Nonidet P-40, fibronectin, and most of the other reagents were obtained from Sigma. Tissue culture plates and transfection reagents were obtained from Corning (Corning, NY) and Life Technologies, Inc., respectively. Modular incubator chambers were obtained from Billups-Rothenberg, Inc. (Del Mar, CA). Poly(vinylidene fluoride) membranes and electrophoresis reagents were purchased from Bio-Rad. Enhanced chemiluminescence blotting reagents were obtained from Amersham Corp.

**Preparation of rLHR and Mutants**—For preparation of the wild-type rLHR, the full coding sequence of the rLHR cDNA (1) was subcloned into pcDNA I/neo (Invitrogen, San Diego, CA). The rLHR extracellular loop deletion mutants were created by polymerase chain reaction using the overlap extension method (14, 15) and were subcloned into pcDNA I/neo. The entire region that was synthesized by a polymerase chain reaction was sequenced to verify the removal of the correct base pairs and the integrity of the remaining sequence. rLHR-t616 was constructed and initially characterized as described previously (16).

**Cells and Transfection—Human embryonic kidney 293 cells (ATCC CRL 1573; American Type Culture Collection, Rockville, MD) were maintained in a high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum (growth medium) and kept in a sterile, humidified, 5% CO2 environment. Using the calcium phosphate precipitation method (17), cells were transiently transfected with pcDNA I/neo containing either the wild-type rLHR cDNA or one of the extracellular loop deletion mutant cDNAs, or with pc15 containing rLHR-t616 cDNA. Sixteen to twenty hours after transfection, cells were washed twice with warm Waymouth’s MB752/1 medium containing 1 mg/ml gentamicin (assay medium). Dishes were incubated overnight at 4°C with a saturating concentration (100 ng/ml, final concentration) of 125I-hCG in the presence or absence of an excess of unlabeled hCG (50 IU/ml). After 24 h, the reaction was terminated by scraping the cells into Hank’s balanced salt solution supplemented with 1 mg/ml BSA and 50 μg/ml gentamicin. Cells were pelleted by centrifugation (1500 × g, 10 min), washed with the same buffer, and counted in a gamma counter. All determinations were performed in duplicate.

For the soluble binding assays, cells were plated on ice for 15 min and washed with cold buffer A (150 mM NaCl and 20 mM HEPES, pH 7.4). The cells were then scraped into cold buffer A containing protease inhibitors (19). Cells were collected by centrifugation at 4°C (1500 × g, 5 min), and the pellet was resuspended in cold buffer A containing 1% Nonidet P-40 and 20% glycerol. The suspension was incubated for 15 min on ice and subsequently centrifuged (12,000 × g, 15 min, 4°C). The supernatant, which contains the solubilized receptor, was diluted 10-fold with cold buffer A containing 20% glycerol and protease inhibitors to bring the detergent concentration to 1% Nonidet P-40. A saturating concentration of 75I-hCG (100 ng/ml, final concentration) was added to aliquots of the detergent extracts in the presence or absence of excess unlabeled hCG (50 IU/ml). The binding assay was terminated by filtration through polyethyleneimine-treated filters (20), and the filters were subsequently counted in a gamma counter. All determinations were performed in duplicate.

**Determination of Equilibrium Binding Constants**—To determine the equilibrium binding constants for hCG and oLH, competition experiments were performed using detergent solubilized cell extracts in buffer A containing 20% glycerol, 0.1% Nonidet P-40, and protease inhibitors prepared as described above. Aliquots of extracts were incubated overnight at 4°C with a saturating concentration (2 ng/ml, final concentration) of 125I-hCG and increasing concentrations of unlabeled hCG (0–4.3 μg/ml) or unlabeled oLH (0.03–105 μg/ml). Assays were terminated by filtration as described above. All determinations were performed in duplicate. The data from each experiment were analyzed for both one site and two site models of ligand binding using the computer program LIGAND (21), and the best statistical fit for the data was determined.

**Temperature Shift Experiments**—For temperature shift experiments, clones at the highest expressing levels of rLHR were transfected with GTP-γ-S, detergent-solubilized extracts (0.1% Nonidet P-40, final concentration) of a nonclonal rLHR-wt expressing cell line were prepared as described above and incubated with a trace concentration of 125I-hCG and increasing concentrations of unlabeled hCG or oLH as described above in the absence or presence of 100–200 μM GTP-γ-S. Reactions were incubated at 4°C and terminated 24 h later by filtration as described above. Filters were counted on a gamma counter and determinations were performed in duplicate.

For the determination of hCG and oLH binding affinities to intact cells expressing rLHR-wt, competition experiments were performed. Cells were washed twice with cold assay medium, and then incubated overnight at 4°C with a trace concentration of 125I-hCG and increasing concentrations of unlabeled hCG or oLH. After 24 h, cells were scraped into cold buffer A (1% Nonidet P-40, final concentration) of a nonclonal rLHR-wt or rLHR-E3M expressing cell line were prepared as described above and incubated with a shake mixture of 1 mg/ml BSA and 50 μg/ml gentamicin (wash medium). Cells were centrifuged (1500 x g, 10 min, 4°C), washed with washed medium, vortexed, centrifuged again, and counted in a gamma counter. All determinations were performed in duplicate.

**Temperature Shift Experiments**—For temperature shift experiments, cells were incubated at 26°C in the presence of unlabeled hCG or oLH as described above and then incubated at 37°C in the absence or presence of unlabeled hCG or oLH. After 24 h, dishes were transferred to modular incubator chambers filled with a humidified 95% O2, 5% CO2 mixture and then placed in either a 26 or 37°C incubator. Forty-eight hours later, cells were harvested. Total 125I-hCG binding to intact cells and detergent solubilized extracts thereof were performed to determine cell surface expression as described above. Competition binding assays utilizing detergent solubilized extracts containing 5.1 ng of hCG bound per lane for extracts containing rLHR-E3M.
The following experiments were performed to determine equilibrium binding constants as described above.

**RESULTS**

To examine whether the extracellular loops of the rLHR are involved in the binding of gonadotropin hormones, a series of deletion mutants were created in which small portions, three to six amino acids at a time, were removed from regions of the extracellular loops. The deduced amino acid sequence of the rLHR and the specific amino acids deleted in each mutant are shown in Fig. 1. The E in the designations of the mutants is to indicate that these are deletions in the extracellular loops. The number defines whether it is the first, second, or third loop relative to the N terminus, and N, M, and C refer to the N-terminal, middle, and C-terminal portions of each extracellular loop, respectively. For example, rLHR-E1M corresponds to the full-length rLHR in which the middle portion of the first extracellular loop of the rLHR was deleted.

Initially, it was important to determine if the rLHR extracellular loop deletion mutants were expressed. As shown in Fig. 2, Western blotting of solubilized extracts from transiently transfected 293 cells revealed that all nine mutants were stably expressed. The wild-type rLHR appears as a mature 85-kDa protein and an immature 68-kDa precursor protein (18, 22). In addition to the specific rLHR bands at 85 and 68 kDa, a prominent band appears between the 85- and 68-kDa bands in the wild-type rLHR and in all the extracellular loop deletion mutants (Fig. 2). However, this band represents a nonspecific interaction of the antibody with an unidentified protein since it was also detected in the untransfected 293 cells (Fig. 2). Further examination of the Western blot of the rLHR extracellular loop deletion mutants reveal that E2N migrates as two proteins with the same molecular masses as the wild-type rLHR. All the other mutants, however, appear only as the smaller 68-kDa protein (Fig. 2).

The following experiments were performed to determine if the receptor mutants could bind hCG and to determine if they were properly localized to the cell surface or not. To address these questions, intact 293 cells transiently expressing the extracellular loop deletion mutants and solubilized extracts thereof were tested for their ability to bind a saturating concentration of 125I-hCG. The results of these experiments are shown summarized in Table I. Intact cells expressing wild-type, E2N, and E3M receptors bound hCG, but the intact cells expressing the other mutants failed to bind hormone. However, solubilized extracts from cells expressing the wild-type or any of the mutant rLHRs were able to bind hCG. The absolute amounts of hormone bound depended on the efficiency of transfection and varied from experiment to experiment. However, by comparing the amount of 125I-hCG bound to intact cells with the total binding detected in solubilized extracts within a given experiment, the percentage of rLHR expressed at the cell surface can be calculated. As seen in Table I, approximately 75% of the rLHR-wt and rLHR-E2N are expressed on the cell surface. In contrast, only 20% of rLHR-E3M and 1.5% or less of all the other extracellular loop deletion mutants are expressed on the cell surface. The lack of cell surface expression of most of these extracellular loop mutants does not exclude the possibility that these receptors may bind hormone with high affinity as it has been previously demonstrated that many rLHR mutants that fail to reach the cell surface can still bind hCG with high affinity (5, 23–25).

The next set of experiments tested the crucial question concerning the potential effects of the deletion of small portions of the rLHR extracellular loops on the affinity of the receptor for hormone. Previously, it was shown that hCG binds to the rLHR with picomolar affinity (1). It was also demonstrated that the extracellular domain alone binds hCG with an affinity comparable to that of the full-length rLHR (8). Because of these prior results, the possibility exists that even if hCG interacts with the extracellular loops with low affinity, a decrease in the affinity for hCG in the solubilized extracts of cells expressing the extracellular loop deletion mutants might not be detected.
Panels A

fected 293 cells using unlabeled hCG or oLH to displace $^{125}$I-

performed using solubilized extracts from transiently trans-
deletion mutants for hCG and oLH, competition assays were 
as for hCG, because oLH has a much lower affinity for the 
terminal 340 amino acids of the LHR. Consequently, we chose 
because of the high affinity interaction of hCG with the N-
terminal 340 amino acids of the rLHR. Hence, we chose to 
measure the affinity of the receptor mutants for oLH, as well 
as for hCG, because oLH has a much lower affinity for the 
rLHR (25).

To measure the binding affinities of the wild-type rLHR and 
deletion mutants for hCG and oLH, competition assays were 
performing using solubilized extracts from transiently trans-
fected 293 cells using unlabeled hCG or oLH to displace $^{125}$I-
cG. Consistent with previous reports, computer analyses of 
the data utilizing the LIGAND program (21) indicated that a 
single class of high affinity (360 pM) hCG binding sites was 
present in extracts from cells expressing the wild-type rLHR 
(Table II and Fig. 3) (23, 25). The results for the binding of hCG 
to all the mutants are compiled in Table II. Significantly, the 
deletion of any portion of any of the three extracellular loops 
had no effect on the affinity of the receptor mutants for hCG.

When competition assays were performed with unlabeled oLH 
as the displacing ligand, analyses of data from extracts of cells 
expressing rLHR-wt revealed two apparent classes of oLH 
binding sites, one of 7.1 nM and a lower affinity site of 64 nM 
(Table III). Fig. 4, Panels A and B, shows the binding of oLH to 
the wild-type rLHR, where the curvilinear nature of the Scatchard plot is readily apparent. oLH binding affinities for the 
wild-type rLHR and all rLHR extracellular loop deletion mu-
tants are summarized in Table III. Solubilized extracts of cells 
expressing rLHR-E2N bound oLH with two apparent affinities 
similar to the wild-type rLHR. In contrast to the rLHR-wt and 
rLHR-E2N, however, deletion of any of the other portions of 
any of the extracellular loops resulted in oLH binding with only 
a single apparent binding affinity of 33–46 nM (see Table III).

As an example, Fig. 4, Panels C and D, illustrates the binding 
of oLH to rLHR-E1M. A comparison of Panels B and D of Fig.

4 clearly shows a shift in oLH binding from two apparent 
affinities for rLHR-wt to a single affinity for rLHR-E1M.

Thus far, the shift from two apparent affinities to one appar-
ent affinity observed for all the extracellular loop deletion 
mutants, with the exception of rLHR-E2N, could be explained 
by the deletion of a binding site for oLH on the extracellular 
loops. However, reevaluation of the Western blot in Fig. 2, 
cell surface expression data in Table I, and the oLH binding affinity 
data in Table III shows that rLHR-E2N is the only extracellu-
lar loop deletion mutant that is expressed as both the mature 85-
kDa and the immature 68-kDa forms of the receptor, that is 
expressed at the cell surface at levels comparable to rLHR-wt, 
and that binds oLH with two apparent affinities. These observa-
tions suggest an alternative hypothesis to explain the shift 
from two apparent affinities to one affinity observed for all the 
extracellular loop deletion mutants other than rLHR-E2N. Thus, 
the introduction of mutations into the extracellular loops 
of the rLHR may result in the improper folding and retention 
of the mutant receptors intracellularly. This retention may result 
in a lack of processing of the mutants to the mature 85-kDa cell 
surface receptor which may be reflected by the absence of high 
affinity oLH binding. To examine this further, the following 
experiments were performed.

If the loss of high affinity oLH binding to the intracellularly 
retained rLHR mutants is due to the absence of the fully 
processed cell surface rLHR, one would predict that expression 
of the extracellular loop deletion mutants at the cell surface 
should restore high affinity oLH binding. Incubation of some 
malfunctioning proteins such as the ΔF508 CFTR mutant (26, 27), 
some vesicular stomatitis virus G proteins with mutations in 
their glycosylation sites (28, 29), and some rLHR mutants2 at 
reduced temperatures restored proper localization of these mut-
transporter proteins to the cell surface. Therefore, $^{125}$I-oLH binding 
assays were performed on nondonal 293 cells stably trans-
fected with the different intracellularly trapped rLHR extracellu-
lar loop deletion mutants after a 48-h preincubation at 26 
versus 37 °C. With the exception of rLHR-E3M, little or no cell 
surface binding was detected for all of the intracellularly 
trapped extracellular loop deletion mutants incubated at either 
temperature (data not shown), indicating that these mutants 
continued to be mislocalized intracellularly even when incu-
bated at reduced temperature. However, rLHR-E3M, which 
was expressed at <20% at the cell surface at 37 °C, was ex-
pressed at the cell surface with levels similar to the wild-type

\[ \text{Table I} \]

| Receptor       | Exp. no. | Intact cells (A) | Detergent extracts (B) | Percentage on cell surface (A/(B × 100)) |
|---------------|---------|------------------|------------------------|----------------------------------------|
| rLHR-wt       | 1       | 5.2              | 6.1                    | 85                                     |
|               | 2       | 4.1              | 5.4                    | 76                                     |
| rLHR-E1N      | 1       | 0                | 1.2                    | 0                                      |
|               | 2       | 0                | 2.6                    | 0                                      |
| rLHR-E1M      | 1       | 0                | 2.0                    | 0                                      |
|               | 2       | 0.01             | 2.0                    | 0.3                                    |
| rLHR-E1C      | 1       | 0                | 0.40                   | 0                                      |
|               | 2       | 0.02             | 2.5                    | 0.8                                    |
| rLHR-E2N      | 1       | 2.4              | 3.5                    | 69                                     |
|               | 2       | 5.1              | 6.3                    | 80                                     |
| rLHR-E2M      | 1       | 0.03             | 1.6                    | 1.5                                    |
|               | 2       | 0.01             | 2.2                    | 0.5                                    |
| rLHR-E2C      | 1       | 0.03             | 4.2                    | 0.7                                    |
|               | 2       | 0                | 0.58                   | 0                                      |
| rLHR-E3N      | 1       | 0.01             | 2.6                    | 0.4                                    |
|               | 2       | 0                | 3.2                    | 0                                      |
| rLHR-E3M      | 1       | 0.26             | 1.1                    | 23                                     |
|               | 2       | 0.31             | 1.6                    | 19                                     |
| rLHR-E3C      | 1       | 0.01             | 2.7                    | 0.4                                    |
|               | 2       | 0                | 5.0                    | 0                                      |

\[ \text{Table II} \]

| Receptor       | Affinity of rLHR extracellular loop deletion mutants for hCG | K_d (nM) |
|---------------|-------------------------------------------------------------|---------|
| rLHR-wt       | 0.36 ± 0.06 (n = 11)                                         |         |
| rLHR-E1N      | 0.41 ± 0.12 (n = 5)                                          |         |
| rLHR-E1M      | 0.25 ± 0.04 (n = 4)                                          |         |
| rLHR-E1C      | 0.32 ± 0.02 (n = 3)                                          |         |
| rLHR-E2N      | 0.34 ± 0.05 (n = 5)                                          |         |
| rLHR-E2M      | 0.39 ± 0.12 (n = 4)                                          |         |
| rLHR-E2C      | 0.23 ± 0.03 (n = 4)                                          |         |
| rLHR-E3N      | 0.59 ± 0.25 (n = 2)                                          |         |
| rLHR-E3M      | 0.43 ± 0.08 (n = 4)                                          |         |
| rLHR-E3C      | 0.53 ± 0.15 (n = 3)                                          |         |

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publication.
B

![Graph showing binding of hCG to wild-type rLHR](http://www.jbc.org/content/journals/)

**Fig. 3. Binding of hCG to the wild-type rLHR.** Detergent-solubilized extracts from 293 cells transiently expressing the wild-type rLHR were incubated with a trace concentration of \(^{125}\text{I}\)-hCG and increasing concentrations of unlabeled hCG as described under “Materials and Methods.” The results are representative of 11 independent experiments. Each point represents the mean of duplicate determinations. The computer program LIGAND (21) was used to analyze the data. The program determined that a single class of binding sites was the best statistical fit through the data points (p < 0.05).

rLHR and Binding Affinity for oLH

**Table III**

Affinity of rLHR extracellular loop deletion mutants for oLH

| Receptor     | \(K_d\) (nM) |
|--------------|--------------|
| rLHR-wt      | 7.1 ± 2.1    |
| rLHR-E1N     | 4.3 ± 1.5    |
| rLHR-E1M     | 46 ± 4.7     |
| rLHR-E1C     | 44 ± 2.1     |
| rLHR-E2M     | 42 ± 5.6     |
| rLHR-E2N     | 40 ± 4.2     |
| rLHR-E3N     | 33 ± 0.8     |
| rLHR-E3M     | 45 ± 3.9     |
| rLHR-t616    | 56 ± 9.3     |

Data shown are the mean ± standard error of the mean for the indicated number of experiments. Equilibrium binding assays where a trace concentration of \(^{125}\text{I}\)-hCG was incubated with increasing concentrations of unlabeled oLH were performed on detergent-solubilized extracts from transiently transfected 293 cells as described under “Materials and Methods.”

The computer program LIGAND (21) was used to analyze the data. The results are representative of 11 independent experiments. Each point represents the mean of duplicate determinations. The computer program LIGAND (21) was used to analyze the data. The program determined that a single class of binding sites was the best statistical fit through the data points (p < 0.05).

The putative hormone binding site. One such mutant, rLHR-t616, is a truncation mutant of the cytoplasmic tail of the rLHR. The question remains, whether the two apparent affinities for oLH represent two binding sites for oLH present on the mature, cell surface rLHR.

Thus far, the data suggest that a mature, cell surface rLHR is required for high affinity oLH binding. The question remains, whether the two apparent affinities for oLH represent two binding sites for oLH present on the mature, cell surface rLHR.

Fig. 3, Binding of hCG to the wild-type rLHR. Detergent-solubilized extracts from 293 cells transiently expressing the wild-type rLHR were incubated with a trace concentration of \(^{125}\text{I}\)-hCG and increasing concentrations of unlabeled hCG as described under “Materials and Methods.” The results are representative of 11 independent experiments. Each point represents the mean of duplicate determinations. The computer program LIGAND (21) was used to analyze the data. The program determined that a single class of binding sites was the best statistical fit through the data points (p < 0.05).
rLHR and Binding Affinity for oLH

Fig. 4. Binding of oLH to rLHR-wt and rLHR-E1M. Detergent-solubilized extracts from 293 cells transiently transfected with the cDNA for rLHR-wt or rLHR-E1M were incubated with a trace concentration of 125I-hCG and increasing concentrations of unlabeled oLH as described under "Materials and Methods." The computer program LIGAND (21) was used to analyze the data to determine the best fit through the data points. A and B are a representative displacement curve and Scatchard plot, respectively, for rLHR-wt from 11 independent experiments. For the binding of oLH to rLHR-wt, a model of two classes of binding sites was found to be the best statistical fit for the data points (p < 0.05). C and D are a representative displacement curve and Scatchard plot, respectively, for rLHR-E1M from four independent experiments. For the binding of oLH to rLHR-E1M, a model of a single class of binding sites was determined to be the best statistical fit (p < 0.05).

Table IV

Affinities of temperature shifted rLHR-wt and rLHR-E3M for oLH

Data shown are the mean ± range or standard error of the mean for the indicated number of experiments. 125I-hCG bindings to intact cells and detergent-solubilized extracts thereof were performed as described under "Materials and Methods." The percentage of receptor expressed on the cell surface was calculated by dividing the amount of 125I-hCG binding to intact cells by the amount bound to solubilized extracts. Equilibrium binding assays where a trace concentration of 125I-hCG was incubated with increasing concentrations of unlabeled oLH were performed with detergent-solubilized extracts from a clonal stable 293 cell line expressing rLHR-wt or with extracts from a nondonal stable 293 cell line expressing rLHR-E3M as described under "Materials and Methods."

| Receptor     | Temperature | Percentage on cell surface | Kd (nM) | n  |
|--------------|-------------|-----------------------------|---------|----|
| rLHR-wt      | 37°C        | 86 ± 1.0                    | 4.0 ± 1.2| 2  |
| rLHR-wt      | 26°C        | 91 ± 6.5                    | 18.7 ± 8.1| 2  |
| rLHR-E3M     | 37°C        | 18 ± 1.0                    | 23.3 ± 2.6| 3  |
| rLHR-E3M     | 26°C        | 70 ± 5.0                    | 6.0 ± 1.8| 2  |

Taken altogether, these data clearly indicate that the shift from two apparent oLH binding affinities for rLHR-wt to one apparent affinity with most of the extracellular loop deletion...
mutants is not due to the deletion of an oLH binding site on the rLHR. Instead, deletion of portions of the extracellular loops and even part of the cytoplasmic tail most likely perturb the normal folding of the rLHR resulting in the retention of the mutant receptors intracellularly and the absence of the mature cell surface receptor. These data also suggest that the observation of two binding affinities for the binding of oLH to rLHR-wt is not due to the presence of two binding sites on the mature rLHR for oLH, but is instead most likely due to the presence of two populations of rLHR with different binding affinities for oLH where the mature, 85-kDa cell surface form of the receptor binds oLH with a higher affinity than the immature, 68-kDa intracellular form of the receptor.

**DISCUSSION**

The present study was undertaken to identify potential low affinity binding sites for hormone on the extracellular loops of the rLHR. There is much evidence for the involvement of the extracellular loops of other G protein-coupled receptors in ligand binding. For example, experiments in which portions of the neurokinin-1 receptor were substituted with the corresponding sequences of the neurokinin-2 and -3 receptors revealed that the first and second extracellular loops of the neurokinin-1 receptor, in addition to a portion of the N-terminal tail and transmembrane 3, bind substance P (30, 31) In addition, Glu-301 of the third extracellular loop of the mammalian gonadotropin-releasing hormone receptor has recently been shown to be involved in an electrostatic interaction with Arg-8 of gonadotropin-releasing hormone (32). The second and third extracellular loops of the interleukin-8 type A receptor have also been demonstrated to be part of a ligand binding site (33, 34).

Although it has been shown that hormone binds with high affinity to the N-terminal extracellular domain of the rLHR (8, 35–38), low affinity interactions of hormone with the extracellular loops and/or transmembrane helices of the rLHR have
was suggested by the finding that micromolar concentrations of a synthetic peptide corresponding to the third extracellular loop of the rLHR was able to inhibit hCG binding to the full-length rLHR (11). In addition, it was reported that a rLHR truncation mutant expressing the C-terminal portion of the receptor and either a 10- or 49-amino acid N-terminal extension was able to bind hCG with micromolar affinity and to stimulate low levels of cAMP production (12). However, without data to demonstrate that this truncation mutant was properly located to the plasma membrane, it is difficult to distinguish such a low binding affinity for hCG and low cAMP stimulation from nonspecific effects. Studies by Ascoli and co-workers found that a substitution of Asp-383 for Asn in the second transmembrane helix of the rLHR produced a receptor that had a decreased affinity for oLH in the absence of sodium (25), suggesting a binding site for oLH on the C-terminal half of the rLHR. Lastly, our laboratory has shown that, unlike the full-length rLHR which binds oLH with two apparent affinities, the rLHR extracellular domain alone binds oLH with a single affinity (18).

In the present studies, we examined the effects of deletions of portions of the extracellular loops of the rLHR on hCG and oLH binding affinities. Western blotting revealed that all of the mutants were expressed as stable proteins. However, unlike rLHR-wt, which is expressed as both the mature 85-kDa and immature 68-kDa forms, all the mutants, with the exception of rLHR-E2N, were expressed as only the 68-kDa precursor form of the rLHR. In addition, all the extracellular loop deletion mutants, with the exception of rLHR-E2N, were severely or completely retained intracellularly. Binding assays to detergent solubilized extracts of cells showed that although the affinities for hCG were unaffected by deletions of any portion of any of the extracellular loops, deletions of portions of all the extracellular loops with the exception of rLHR-E2N resulted in altered oLH binding. A shift from two apparent binding affinities of oLH for rLHR-wt to a single binding affinity for oLH was observed for all the mutants with the exception of rLHR-E2N. The results of experiments presented herein, therefore, at first suggested that the extracellular loops of the rLHR were contributing to the binding of oLH. However, further experiments confirmed the correlation between the presence of two binding affinities for oLH and the expression of the cell surface 85-kDa form of the receptor. Taken altogether, these data suggest that the shift from two apparent oLH binding affinities for rLHR-wt to a single binding affinity for the most of the mutants is not due to the loss of an oLH binding site on the rLHR, but is due to the absence of the fully folded, cell surface form of the receptor. We hypothesize that the mutations are causing a misfolding of the receptor that causes them to be retained intracellularly, thus preventing the further folding and processing of the mutant receptor into the mature cell surface form. Our data also show that the observation of two oLH binding affinities measured for rLHR-wt does not represent two binding sites on an individual receptor. Rather, the two oLH binding affinities for rLHR-wt represent the binding affinities of oLH for two populations of rLHR. The 85-kDa mature cell surface form of the rLHR binds oLH with a higher affinity, while the 68-kDa intracellular precursor form binds oLH with a lower affinity.

For certain other G protein-coupled receptors, in particular the adrenergic receptors, the affinity of agonist for the receptor can be affected by the receptor's association with a G protein (39). This is manifest by the ability of exogenously added GTP to decrease the binding affinity of agonist for the receptor (39). The possibility that perhaps the two apparent binding affinities of the rLHR for oLH were arising from solubilization of one pool of rLHR associated with G proteins and one pool not...
associated with G proteins was, therefore, also considered. It is generally not possible to observe a change in hCG binding affinity to membranes containing the rLHR upon addition of GTP (40–44). This is thought to be due to the high affinity interactions of hCG with the extracellular domain of the rLHR (8), a situation quite unlike the binding of catecholamines to the transmembrane helices of adrenergic receptors (45). Thus, whereas it can be envisioned how an intracellular conformational change in an adrenergic receptor accompanying G protein activation could lead to an increased dissociation of catecholamine from their binding site, a similar conformational change in the rLHR would not be as likely to result in a dissociation of hCG from the extracellular binding domain. The possibility remained, however, that the binding affinity of oLH, which is much lower than that of hCG for the rLHR, might be affected by G protein activation, especially if there were additional contact sites of oLH on the carboxyl half of the receptor. If so, then this might also account for the two populations of oLH binding affinities observed in detergent extracts of cells expressing the wild-type rLHR. To test this possibility, we examined the effects of 200-300 µM GTP·S on oLH binding to detergent soluble extracts of cells expressing the wild-type rLHR. In three separate experiments, however, the same results were observed regardless of the presence or absence of GTP·S (data not shown). Therefore, it is highly unlikely that the association or lack of association of G proteins with the rLHR contribute to alterations in oLH binding to this receptor. As such, the higher affinity binding of oLH to the mature cell surface receptor as compared to the intracellular precursor receptor suggests that the newly synthesized rLHR in the endoplasmic reticulum are retained in a conformation distinct from the cell surface mature rLHR. In contrast, newly synthesized rLHR in the endoplasmic reticulum is already folded into a conformation that can bind hCG with high affinity. It must still be in a conformation distinct from the cell surface mature receptor, though, since it does not yet have the same high affinity hCG binding and reduced oLH binding affinity as compared to the cell surface mature rLHR, intracellularly retained mutants of the rFSHR lack any detectable binding activity. Assuming that mutants of the rLHR and rFSHR are retained in the same intracellular compartment (i.e. the endoplasmic reticulum), these observations suggest that the newly synthesized rLHR in the endoplasmic reticulum is already folded into a conformation that can bind hCG with high affinity. It must still be in a conformation distinct from the cell surface mature receptor, though, since it does not yet have the same high affinity hCG binding and reduced oLH binding affinity as compared to the cell surface mature rLHR. In contrast, newly synthesized rFSHR in the endoplasmic reticulum does not appear to be folded appropriately enough to allow for any hormone binding. Taken together, the data on intracellularly retained mutants of the rLHR and rFSHR suggest that the rLHR folds more readily than the highly related rFSHR.

Clearly more studies will be required in order to better understand the complex folding and maturation of the gonadotropin receptors, as well as other G protein-coupled receptors. Toward this end, the differential binding of oLH to mature versus immature rLHR should prove useful in elucidating the folding of the rLHR.

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