# Transfected Cells Express Mostly the Intracellular Precursor of the Lutropin/Choriogonadotropin Receptor but This Precursor Binds Choriogonadotropin with High Affinity†

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ABSTRACT: Previous studies from several laboratories have shown that the cell surface rLHR is a 85−92 kDa protein synthesized from a 68−73 kDa intracellular precursor. While all investigators agree that the cell surface rLHR binds hCG with high affinity, it is not clear if the intracellular precursor can also bind hCG. In order to directly determine if the intracellular rLHR present in cells transfected with the wild-type rLHR binds hCG with high affinity, we devised a method that selectively degrades the cell surface rLHR while preserving the intracellular rLHR. The binding of hCG to intact cells was completely lost following mild proteolysis of the cells, but binding to detergent extracts prepared from proteolyzed cells was largely preserved. Measurements of the hCG binding affinity to intact cells or to detergent extracts prepared before and after proteolysis display very similar or identical binding affinities. Since binding to nonproteolyzed intact cells, detergent extracts prepared from nonproteolyzed cells, or detergent extracts prepared from proteolyzed cells occurs only to the 85−92 kDa rLHR, the 85−92 and 68−73 kDa rLHR, and the 68−73 kDa rLHR, respectively, we conclude that the cell surface rLHR and the intracellular rLHR bind hCG with the same affinity. Quantitation of the relative abundance of the cell surface and intracellular rLHR by immunological methods indicates that transfected cells express mostly the intracellular precursor. A comparison of the binding capacity of control and proteolyzed cells with that of their detergent extracts indicates that hCG binding assays greatly underestimate the relative abundance of the intracellular rLHR.

Studies from several laboratories (*1*−*8*) have identified at least three different species of the LHR[1] expressed in transfected cells and in gonadal cells expressing the endogenous LHR. These three species have molecular masses of 68−73 kDa, 85−92 kDa, and 165 kDa, and can be detected by Western or ligand blots, as well as by immunoprecipitation and/or affinity purification procedures from metabolically labeled cells.

The 85−92 kDa species is the bona fide LHR present at the cell surface. This glycoprotein has mature carbohydrate side chains as judged by its resistance to EndoH digestion and its sensitivity to neuraminidase and PGNAse F digestion (*2*, *5*−*8*). The cell surface location of this form of the LHR was deduced by its sensitivity to proteolytic degradation following exposure of intact cells to crude preparations of "collagenase" (*2*). This 85−92 kDa LHR binds hCG with high affinity (100−500 pM) as measured in equilibrium binding experiments using intact cells (*2*−*6*) and can be readily detected using immunological procedures such as Western blots or immunoprecipitations as well as methods that depend on hCG binding such as ligand blots and affinity purification on immobilized hCG (*1*−*4*, *8*).

The 68−73 kDa species is an intracellular glycoprotein that contains the high-mannose type of carbohydrate side chains characteristic of glycoproteins that reside in the endoplasmic reticulum (*9*). This form of the LHR can be readily detected on Western blots or immunoprecipitates, and is sensitive to degradation with EndoH or PGNase F, but is insensitive to neuraminidase (*2*, *3*, *7*, *8*). The 68−73 kDa rLHR is resistant to proteolytic degradation following exposure of intact cells to crude preparations of "collagenase", but is readily degraded by proteases following lysis of the cells with nonionic detergents (*2*). The presence of an intact polypeptide chain in the 68−73 kDa species was documented by the finding that this protein can be readily visualized on Western blots using antibodies to the extreme N- or C-terminus (*2*). Lastly, pulse−chase experiments have clearly shown that the 68−73 kDa LHR is a precursor of the 85−97 kDa LHR (*2*, *4*, *5*, *7*, *8*).

Direct measurements of the hormone binding affinity of the 68−73 kDa intracellular LHR have not been possible.

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[1] Abbreviations: LHR, lutropin/choriogonadotropin receptor; LH, lutropin; CG, choriogonadotropin; EndoH, endoglycosidase H; PGNAse F, peptide *N*-glycosidase F; wt, wild-type; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; G418, geneticin; r, rat; h, human; ELISA, enzyme-linked immunoassays.

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Equilibrium binding experiments performed in detergent extracts of cells, a condition that allows access of the ligand to the 68−73 kDa as well as the 85−92 kDa cell surface LHR, yield a single hCG binding affinity comparable to that detected in intact cells where hCG has access only to the 85−92 kDa cell surface LHR (*4*−*6*, *10*−*12*). If the maximal binding capacity of detergent extracts were higher than those of intact cells, the finding that the extracts and intact cells bind hCG with the same affinity could be readily interpreted to mean that hCG binds with the same affinity to the 85−92 and 68−73 kDa LHR. Since maximal binding capacities are not always higher in the detergent extracts than in intact cells, however (*4*−*6*, *10*−*12*), a more likely interpretation of the hormone binding results is that the 68−73 kDa LHR does not bind hCG at all. Nonquantitative assessments of the hormone binding properties of the 68−73 kDa LHR have also produced conflicting data. Thus, visualization of receptor species using $^{125}$I-hCG binding to electrophoretic blots readily detects the 85−92 kDa cell surface receptor but fails to detect the 68−73 kDa species (*2*, *13*, *14*). While affinity purification procedures utilizing immobilized hCG readily show the 85−92 kDa cell surface receptor, they have been reported to detect the 68−73 kDa LHR in some studies (*2*, *4*, *8*), but not in others (*1*). Lastly, it has been argued that the hormone binding properties of the 68−73 kDa detected in cells expressing the LHR-wt may be deduced by extrapolating results obtained with a number of LHR mutants that are trapped intracellularly as EndoH-sensitive 68−73 kDa proteins (*5*, *6*, *11*, *12*). While these studies have shown that detergent extracts prepared from cells expressing these mutant forms of the LHR bind hCG with the high affinity expected of the 85−92 kDa cell surface LHR, it cannot be concluded that the 68−73 kDa protein present in cells expressing LHR mutants is functionally equivalent to that present in cells expressing the LHR-wt.

The 165 kDa form of the LHR is poorly characterized. There are no experiments addressing the possibility that this receptor species binds hCG. This form of the LHR appears to be an aggregate or oligomer of the 68−73 kDa species described above. Thus, pulse−chase experiments show that the 165 kDa band labels and decays with the same kinetics as the 68−73 kDa band (*2*, *5*). Moreover, this 165 kDa species is sensitive to EndoH digestion (W. R. Hipkin and M. Ascoli unpublished observations) and can be detected in cells expressing mutant forms of the LHR that remain trapped as a 68−73 kDa intracellular receptor instead of maturing into the 85−92 kDa cell surface receptor (*5*).

The studies presented herein were designed to directly determine if the 68−73 kDa intracellular LHR present in cells transfected with the wild-type LHR bind hCG with high affinity and to better ascertain the relative levels of the 68−73 kDa intracellular LHR and the 85−82 kDa cell surface LHR.

## MATERIALS AND METHODS

*Plasmids and Cells.* The plasmid encoding for rLHR-myc was prepared by inserting a small oligonucleotide (5′-GAACAAAAGCTTATTTCTGAAGAAGACTTG-3′) encoding for the myc epitope (EQKLISEEDL, see ref *15*) between the C terminus of the signal peptide (S-1) and the N-terminus (R1) of the mature rLHR (*16*) in pcDNA1neo. The insertion of the oligonucleotide was done using standard PCR procedures (*17*, *18*). The methods used for transfection and selection of clonal cell lines have been described elsewhere (*5*, *10*, *19*). The 293L(wt-17) and 293L(wt12) cells are previously described clonal lines of 293 cells stably transfected with the rLHR-wt (*20*−*22*).

Transfected cells were maintained in DMEM supplemented with 10 mM Hepes, 10% newborn calf serum, 50 $\mu$g/mL gentamicin, and 700 $\mu$g/mL G418. Prior to experiments, the cells were cultured for 2−3 days in medium without G418, however.

*Proteolysis of Intact Cells.* Cells (plated in 100 mm dishes) were placed on ice and washed twice with 4 mL portions of cold Hank's balanced salt solution (medium A). The cells were then incubated on ice with in a total volume of 4.2 mL of medium A alone or supplemented with 250 $\mu$g/mL protease type XIV. At the desired time, protease activity was quenched by the addition of 4 mL of Waymouth MB752/1 medium supplemented with 20 mM Hepes, 15% horse serum, 1 mM phenylmethanesulfonyl fluoride, 2 mM EDTA, and 5 mM *N*-ethylmaleimide (medium B). The cells were then scraped from the plate (protease digestion resulted in the release of the cells from the dishes to an extent that depended on the duration of protease treatment) and collected by centrifugation. The pellet was washed once more with 4 mL of medium B and either resuspended in medium B, in order to measure $^{125}$I-hCG binding to intact cells, or used to prepare detergent extracts (see below).

*Hormone Binding Experiments.* Binding of $^{125}$I-hCG to intact cells was performed during an overnight incubation at 4 °C as described elsewhere, except that the cells were suspended in medium B (see above). Detergent extracts used to measure $^{125}$I-hCG binding were obtained by solubilizing the cells in 0.5% NP-40, 20 mM Hepes, 100 mM NaCl, 20% glycerol, and 1 mM EDTA, pH 7.4, using a constant ratio of 100 $\mu$L of detergent solution/million cells as described elsewhere (*10*, *23*). The detergent concentration was diluted to 0.1% prior to measuring $^{125}$I-hCG binding as described before (*10*, *23*). Equilibrium binding parameters were calculated using the computer program LIGAND (*24*).

*Immunoprecipitation of the rLHR from Metabolically Labeled Cells.* Cells were metabolically labeled during a 12−24 h incubation at 37 °C in methionine- and cysteine-free DMEM supplement with 1% newborn calf serum, 20 mM Hepes, 50 $\mu$g/mL gentamicin, and 100−200 $\mu$Ci/mL [$^{35}$S]Translabel. Cell lysates were prepared in 0.5% NP-40, 20 mM Hepes, 100 mM NaCl, and 1 mM EDTA, pH 7.4, using a constant ratio of 100 $\mu$L of detergent solution/million cells, purified on wheat germ aglutinnin bound to agarose, and immunoprecipitated with the appropriate antibodies (*2*, *5*, *21*, *22*, *25*). Most of the immunoprecipitations presented here were done using 150 $\mu$g of a rabbit polyclonal antibody raised against the 85 kDa rLHR purified from rat corpora lutea (*26*) or with a mixture (75 $\mu$g each) of two polyclonal antibodies raised against synthetic peptides derived from rLHR. One of these antibodies is designated R02, and it was raised against a synthetic peptide corresponding to residues 194−207 (*27*). The other one is a new polyclonal antibody raised against a synthetic peptide corresponding to residues 1−14 (designated antiL). The myc-tagged rLHR was immunoprecipitated using 50 $\mu$L of a 10-fold dilution of ascites fluid prepared from mice injected with the 9E10

hybridoma cell line. Immunoprecipitates were resolved on SDS gels, and fluorograms of the dried gels were obtained using Kodak BioMax MS film. All fluorograms were scanned using a BioRad Molecular Imaging System and captured in a digital format for presentation.

*Enzyme-Linked Immunoassays.* Detergent extracts of control and proteolyzed cells were prepared and purified on wheat germ agglutinin−agarose as summarized above. Throughout these procedures, all volumes were normalized to cell number by keeping the purified extracts at a constant ratio of 100 $\mu$L of solution/million cells. Fifty microliter aliquots of serial dilutions of the partially purified extracts were applied to 96 well plates and incubated for 2 h at room temperature in a humidified atmosphere. This solution was aspirated, and the wells were filled with blocking solution (10 mM sodium phosphate, 100 mM NaCl, 10% milk powder, 0.2% Tween-20, and 1% goat serum, pH 7.4) and incubated for 16 h (when 9E10 was used as the primary antibody) or for 4 h (when R02 was used as the primary antibody) at room temperature. The blocking solution was aspirated, and the wells received 50 $\mu$L of primary antibody (9E10 was used as a 100−200-fold dilution of the ascites fluid, while R02 was used as a 20−50 $\mu$g/mL solution of purified IgGs). The wells were then incubated with primary antibody for 1 h (9E10) or 16 h (R02) at room temperature and washed 5 times (5 min each time) with 250 $\mu$L of the blocking solution. Each well was then incubated with 50 $\mu$L of a 2000 fold-dilution of goat anti-mouse IgG labeled with horseradish peroxidase (when using 9E10 as the primary antibody) or with goat anti-rabbit IgG labeled with horseradish peroxidase (when using R02 as the primary antibody) for 1 h at room temperature. Following aspiration of this solution, the wells were washed 5 times (5 min each time) with 250 $\mu$L of 10 mM sodium phosphate, 100 mM NaCl, pH 7.4. Finally, the wells were incubated with 100 $\mu$L of ImmunoPure TMB Substrate for 10 min at room temperature, and the reaction was stopped by adding 100 $\mu$L of 2 M sulfuric acid to each well. Quantitation of the product was performed by reading the $A_{450}$ in a microplate reader.

*Other Methods.* Concentration−response curves for the hCG-induced increases in cAMP accumulation were obtained by measuring total cAMP levels in cells that had been incubated with at least five different concentrations of hCG for 30 min at 37 °C in the presence of a phosphodiesterase inhibitor. The different parameters that describe the concentration−response curves were calculated as described elsewhere (*21*, *22*).

*Hormones and Supplies.* Human CG (CR-127) was generously provided by the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases. This hormone was radiolabeled with $^{125}$I as previously described (*28*). Protease type XIV (bacterial, EC 3.4.24.31), cycloheximide, protease inhibitors, and goat anti-rabbit IgG coupled to horseradish peroxidase were from Sigma. Cell culture plasticware and other cell culture supplies were from Corning and Gibco, respectively. The 9E10 cell line was obtained from the American Type Culture Collection. All electrophoresis supplies and goat anti-mouse IgG coupled to horseradish peroxidase were obtained from BioRad. The ImmunoPure TMB Substrate was purchased from Pierce. The sources of all other materials have been described (*2*, *5*, *21*, *22*, *25*).

## RESULTS

*Proteolysis and Inactivation of the 85 kDa rLHR.* In this initial series of experiments, we sought to establish a method to directly determine if the 68 kDa intracellular form of the rLHR can bind hCG with high affinity. This can be done by measuring hCG binding (to intact cells and detergent extracts) after selective inactivation of the 85 kDa cell surface rLHR. Selective inactivation of cell surface receptors has traditionally been accomplished by protease treatment of intact cells at reduced temperature to prevent uptake of the protease. We have indeed shown that the cell surface LHR of target or transfected cells can be selectively degraded by proteases that contaminate crude preparations of "collagenase" (*2*, *29*, *30*). This enzyme preparation, however, is not suitable for the experiments planned here because it merely "nicks" the cell surface LHR and does not affect its hCG binding affinity (*29*, *30*). Thus, we conducted a series of preliminary experiments with a variety of proteases and found that the hCG binding activity of a clonal strain of 293 cells expressing the rLHR [designated 293L(wt-17)] was rather resistant to proteolytic destruction under mild (i.e., 4 °C) incubation conditions. Eventually we found a "crude" protease preparation (henceforth referred to as protease XIV) obtained from *Staphyloccocus aureus* (protease type XIV from Sigma) that can readily and selectively degrade the cell surface rLHR and abolish hCG binding to intact cells.

The data presented in Figure 1A,B show that incubation of intact 293L(wt-17) cells with protease XIV at 4 °C results in a rapid and quantitative loss of the cell surface 85 kDa rLHR, while preserving the intracellular 68 kDa rLHR. The data presented in Figure 1C show that protease treatment induces a pronounced loss of hCG binding to intact cells, with little or no change in hCG binding to detergent extracts prepared from the same cells. Since only the 68 kDa rLHR remains in protease-treated cells, our ability to detect hCG binding to detergent extracts of protease-treated cells clearly shows that the 68 kDa rLHR is capable of binding hCG. The data presented in Figure 1C also show that in the absence of protease treatment the binding capacity of detergent extracts (about 25 ng of $^{125}$I-hCG/$10^6$ cells) is only slightly higher than that of intact cells (about 20 ng of $^{125}$I-hCG/$10^6$ cells) and that the binding capacity of proteolyzed cells is virtually undetectable. Thus, one would predict that the binding capacity of detergent extracts prepared from proteolyzed cells would drop to about 5 ng of $^{125}$I-hCG/$10^6$ cells. As shown in Figure 1C, however, the binding capacity of detergent extracts prepared from proteolyzed cells drops only to about 20 ng of $^{125}$I-hCG/$10^6$ cells.

Equilibrium binding assays were next performed using control or protease-treated cells, and detergent extracts prepared from these cells. For these experiments, we utilized two different cell lines [designated 293L(wt-12) and 293L-(wt-17)] expressing different densities of cell surface rLHR. The results of a representative experiment are shown in Figure 2, and a summary of several experiments is presented in Table 1. These data clearly show that the $^{125}$I-hCG binding affinities of intact cells or of detergent extracts (before and after proteolysis) are very similar or identical. Since $^{125}$I-hCG binding to non-proteolyzed intact cells, detergent extracts prepared from nonproteolyzed cells, or detergent extracts prepared from proteolyzed cells occurs only to the

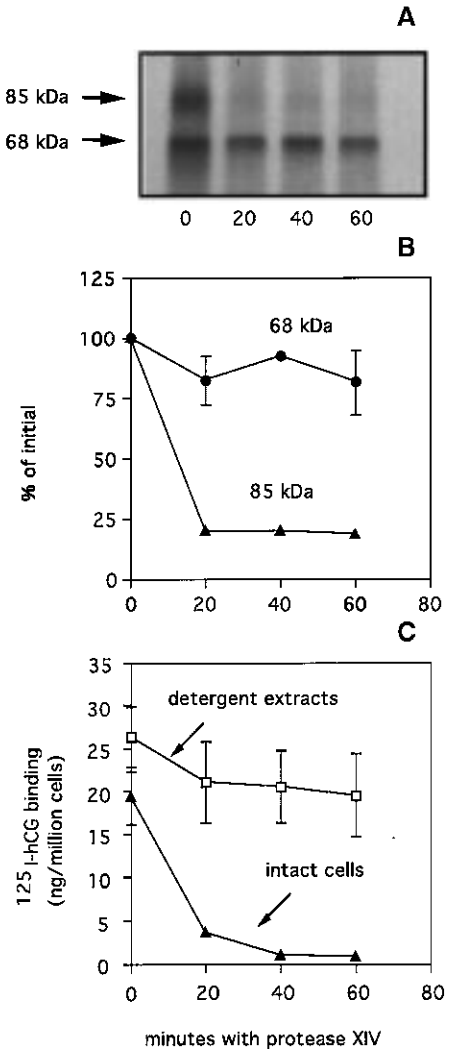


FIGURE 1: Effect of proteolysis on the 85 and 68 kDa rLHR. 293L-(wt-17) cells were metabolically labeled with 200 $\mu$Ci/mL [$^{35}$S]-Translabel (panels A and B) or used without labeling (panel C). After washing (to stop the labeling), they were incubated at 4 °C with 250 $\mu$g/mL protease XIV for the times indicated and then used for binding experiments or to prepare cell lysates. Cell lysates from metabolically labeled cells were then used to immunoprecipitate and quantitate the rLHR as described under Materials and Methods while those prepared from the nonlabeled cells were used to measure their $^{125}$I-hCG binding capacity. Binding of $^{125}$I-hCG to intact cells or detergent extracts was measured during an overnight incubation with a saturating concentration (100 ng/mL) of $^{125}$I-hCG as described under Materials and Methods. A polyclonal antibody raised against the purified 85 kDa rLHR isolated from rat corpora lutea (see Materials and Methods) was used in the immunoprecipitations. Panel A = densitometric scan of a representative fluorogram. Panel B = densitometric quantitation of the 85 and 68 kDa forms of the rLHR obtained from two experiments, such as that presented in panel A. Each point represents the average of these two experiments, and the bars extend to the individual values obtained in each experiment. Panel C = binding of $^{125}$I-hCG to intact cells and detergent extracts. Each point represents the average ± SEM of three independent experiments.

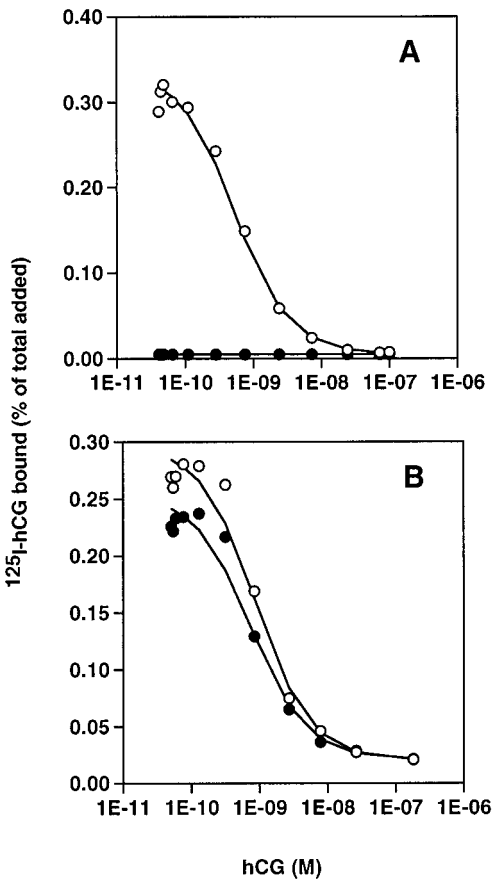


FIGURE 2: Competition binding assays for $^{125}$I-hCG in control and protease-treated cells and their detergent extracts. 293L(wt-17) cells were incubated for 30 min at 4 °C with (closed symbols) or without (open symbols) 250 $\mu$g/mL protease XIV as indicated. The cells were then used directly for binding assays (panel A), or they were used to prepare lysates (see Materials and Methods) which were subsequently used for binding assays (panel B). Equilibrium binding parameters were calculated as described under Materials and Methods using a competition binding protocol in which cells or detergent extracts are incubated with a trace concentration of $^{125}$I-hCG and increasing concentrations of hCG as shown. The results of a representative experiment are shown.

85 kDa rLHR, the 85 and 68 kDa rLHR, and the 68 kDa rLHR, respectively, we conclude that the cell surface 85 kDa rLHR and the 68 kDa intracellular rLHR bind $^{125}$I-hCG with the same affinity. The results presented in Table 1 as well as those shown in Figure 1C underscore a problem with the quantitation of binding capacity. For both cell lines, the maximal binding capacity measured in intact nonproteolyzed cells is basically the same as the maximal binding capacity measured in detergent extracts prepared from the same cells. Thus, one would predict that following proteolysis, a procedure that reduces binding to intact cells to undetectable levels should also reduce binding in detergent extracts to undetectable levels. This is clearly not the case, as the maximal binding capacity of detergent extracts prepared from proteolyzed cells is only slightly lower than that of detergent extracts prepared from control cells (Table 1).

Altogether the data presented in this section show that the 68 and 85 kDa rLHR bind hCG with the same affinity but indicate that the hCG binding capacity of intact cells and detergent extracts cannot be used to quantitate the relative abundance of the 85 and 68 kDa rLHR.

*Recovery of the Cell Surface rLHR following Proteolysis.* To complete the characterization of the effects of proteolysis on the cell surface rLHR, we characterized the recovery of $^{125}$I-hCG binding to the cell surface following proteolysis of intact cells. These results are presented in Figure 2 and show that following proteolysis of 293L(wt-17) cells $^{125}$I-hCG binding to the cell surface returns with a half-life of

Table 1: Effect of Proteolysis on $^{125}$I-hCG Binding to Intact Cells and Detergent Soluble Extracts[a]

| | | $^{125}$I-hCG binding | | | |
|---|---|---|---|---|---|
| | | intact cells | | detergent extracts | |
| cell line | proteolysis | $K_d$ (pM) | (binding capacity (molecules/cell) | $K_d$ (pM) | binding capacity (molecules/cell) |
| 293L(wt-17) | no | 261 ± 44 | 174000 ± 39000 | 477 ± 106 | 149000 ± 43000 |
| | yes | ND | ND | 531 ± 161 | 126000 ± 39000 |
| 293L(wt-12) | no | 230 ± 31 | 105000 ± 13000 | 259 ± 42 | 94000 ± 19000 |
| | yes | ND | ND | 325 ± 47 | 84000 ± 11000 |

[a] Cells were incubated with or without 250 $\mu$g/mL protease XIV for 30 min at 4 °C. The equilibrium binding parameters for $^{125}$I-hCG were measured during an overnight incubation at 4 °C using intact cells or detergent extracts as described under Materials and Methods. Each number represents the mean ± SEM of 4 independent experiments. ND = not detectable.

Table 2: Equilibrium Binding Parameters for $^{125}$I-hCG following Recovery of Proteolyzed Cell Surface Receptors[a]

| | $K_d$ (pM) | maximal binding (% of nonproteolyzed cells) |
|---|---|---|
| no treatment | 310 ± 67 | 100 |
| +protease XIV followed by 4 h recovery without cycloheximide | 400 ± 7 | 20 ± 4 |
| +protease XIV followed by 4 h recovery with cycloheximide | 329 ± 52 | 10 ± 2 |

[a]Cells were incubated with or without 250 $\mu$g/mL protease XIV for 30 min at 4 °C, and the proteolyzed cells were allowed to recover in the presence or absence of 5 $\mu$g/mL cycloheximide for 4 h at 37 °C as indicated. The equilibrium binding parameters for $^{125}$I-hCG were then measured as described under Materials and Methods.

7−8 h. A parallel experiment was conducted in the presence of cycloheximide to assess the relative contribution of de novo synthesis of receptors and simple translocation of the preformed 68 kDa intracellular rLHR to the cell surface. These data are also shown in Figure 2 and indicate that processing of the preformed intracellular rLHR to the cell surface rLHR is complete within 4 h of recovery, and it accounts for only 10−20% of the cell surface binding recoverable after protease digestion. These data are consistent with previous pulse−chase experiments done in metabolically labeled cells showing that the conversion of the 68 kDa intracellular rLHR to the 85 kDa cell surface rLHR is completed in 4−6 h (*2*, *5*).

The data summarized in Table 2 show that the $^{125}$I-hCG binding affinity measured in intact cells allowed to recover from proteolysis in the presence or absence of cycloheximide is similar to that of nonproteolyzed cells. Thus, the binding affinity of the cell surface rLHR is the same regardless of whether it originates only from maturation of the preformed intracellular rLHR or when synthesis of additional precursor is involved.

*Quantitation of the rLHR Using Immunological Methods.* In order to overcome the problem of quantitation of the relative abundance of the 85 and 68 kDa rLHR by measuring $^{125}$I-hCG binding capacity, we sought to establish an ELISA to quantitate the rLHR.

There are a number of polyclonal antibodies that have been raised against the rLHR that would be useful for this assay. Since there is no guarantee that a given antibody would recognize the 85 and 68 kDa rLHR to the same extent, we reasoned that it would be best to compare data obtained with as many different antibodies as possible. We initially tried to establish ELISAs using Bugs, a polyclonal antibody raised against a purified preparation of the rat luteal LHR (*26*); R02, a polyclonal antibody raised against a synthetic peptide corresponding to residues 194−207 of the rLHR (*27*); and antiL, a new polyclonal antibody raised against a synthetic peptide corresponding to residues 1−14 of the rLHR. Since only R02 turned out to be useful in this assay (see below), we prepared a myc epitope-tagged rLHR and used a well-characterized antibody against this epitope (9E10, see ref *15*) to set up an additional ELISA (see below).

The myc-tagged rLHR (designated rLHR-myc) was constructed by introducing a 10 amino acid residue sequence (EQKLISEEDL) at the N-terminus of the mature rLHR. This epitope is derived from human c-myc and is recognized by the 9E10 antibody (*15*). Several stably transfected 293 cell lines expressing rLHR-myc were established, and one of them, designated 293L(wt-myc11), was chosen for further studies. Intact 293L(wt-myc11) cells bind hCG with high affinity (209 ± 15 pM, $n = 11$) and have a maximal binding capacity (98000 ± 10000 molecules of $^{125}$I-hCG/cell, $n = 11$) comparable to that of 293L(wt-12) cells (cf Table 1).

The myc-tagged rLHR is functional as illustrated by the ability of 293L(wt-myc11) cells to respond to hCG with a robust increase in cAMP accumulation. This responsiveness is slightly diminished when compared to 293L(wt-12) cells, which express comparable density of the nontagged rLHR. Thus, the maximal cAMP response of 293L(wt-myc11) cells is reduced by about 25%, and their sensitivity to hCG is reduced about 4-fold (Table 3). The cholera toxin-induced maximal cAMP response is the same in both cell lines, however. Virtually identical results have been recently published (*12*) with another epitope-tagged form of the rLHR in which an 8 amino acid residue sequence corresponding to the Flag epitope (DYKDDDDK) was introduced in exactly the same location in which we introduced the myc epitope. As shown in Figure 3, the 68, 85, and 165 kDa forms of the rLHR can be readily immunoprecipitated using polyclonal antibodies raised against synthetic peptide derived from the rLHR or using 9E10, a monoclonal antibody directed against the myc epitope (15). The data presented in Figure 3 also show that the intracellular precursor of the rLHR detected in cells expressing rLHR-myc is somewhat larger than that detected in cells expressed the nontagged rLHR. This increase in apparent size is most likely due to the general acidic nature of the added epitope rather than to its mass because the apparent size of the mature rLHR (which is more acidic than the immature rLHR because of the presence of sialic acid, see ref *2*) is the same for the tagged and nontagged rLHR.

Table 3: Effect of hCG on cAMP Accumulation in 293L(wt-12) and 293L(wt-myc11) Cells[a]

| cell line | basal cAMP (pmol/$10^6$ cells) | hCG-induced cAMP response response | | cholera toxin-induced cAMP response maximal response (pmol/$10^6$ cells) |
|---|---|---|---|---|
| | | $EC_{50}$ (pM) | maximal response (pmol/$10^6$ cells) | |
| 293L(wt-12) | 10.6 ± 3.6 | 26 ± 5 | 1905 ± 459 | 1165 ± 342 |
| 293L(wt-myc11) | 7.4 ± 0.4 | 107 ± 34 | 1414 ± 241 | 906 ± 233 |

[a] Total levels of cAMP were measured in cells incubated in the presence of a phosphodiesterase inhibitor and increasing concentrations of hCG for 30 min, or in cells incubated with a single concentration (500 ng/mL) of cholera toxin for 2 h as described under Materials and Methods. Each number represents the average (±SEM) of four independent experiments.

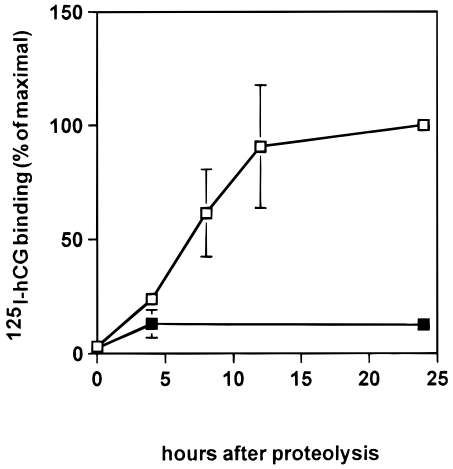


FIGURE 3: Recovery of the cell surface rLH following proteolysis. 293L(wt-17) cells were preincubated for 1 h at 37 °C with (closed symbols) or without 5 $\mu$g/mL cycloheximide (open symbols). The cells were then incubated for 30 min at 4 °C with 250 $\mu$g/mL of protease XIV, and washed as described under Materials and Methods. At this point ($t$ = 0 in the figure), the cells were resuspended in growth medium with or without cycloheximide and incubated at 37 °C for the times indicated. The binding of $^{125}$I-hCG to intact cells was then measured as described under Materials and Methods and expressed as percent of maximal. Each point represents the average ± SEM of 3 experiments.

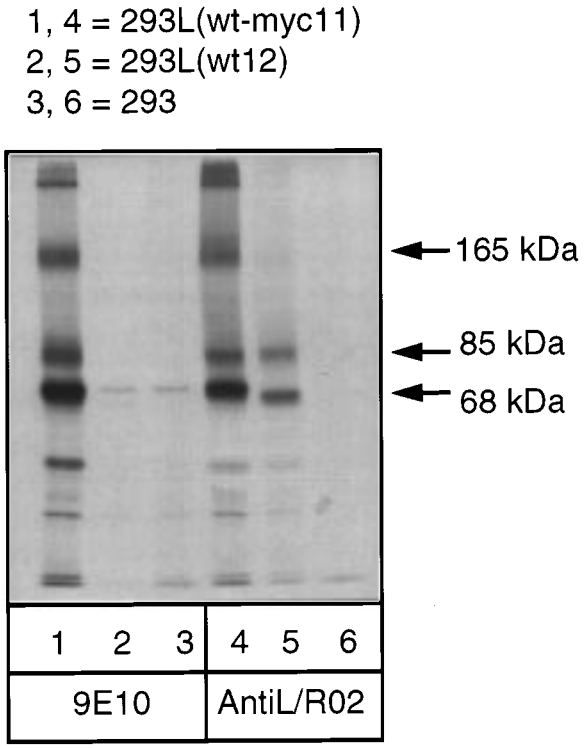


FIGURE 4: Immunoprecipitation of the rLHR and rLHR-myc. Untransfected 293 cells as well as 293L(wt-12) and 293L(wt-myc11) cells were metabolically labeled with 200 $\mu$Ci/mL [$^{35}$S]-Translabel. Cell lysates were prepared, purified, and immunoprecipitated as described under Materials and Methods using the 9E10 monoclonal antibody to the myc epitope (lanes 1−3) or a mixture of two polyclonal antibodies (R02 and antiL) to the rLHR (lanes 4−6). The results presented are from a densitometric scan of a representative fluorogram.

Results of a typical ELISA performed using the 9E10 and R02 antibodies and lectin-purified detergent extracts prepared from several cell lines are shown in Figure 5. As expected, the 9E10 antibody recognized only extracts from 293L(wt-myc11) cells, and not from 293L(wt-12) cells (Figure 5, top panel). In contrast, the R02 antibody recognized extracts from 293L(wt-myc11) and 293L(wt-12) cells, but not from untransfected 293 cells (Figure 5, bottom panel). Note that the remarkable similarity in the intensity of the signal obtained when the 293L(wt-myc11) cell line was assayed with both antibodies (compare the top and bottom panels of Figure 4) may be fortuitous, as their affinity for the rLHR as well as the affinity of the secondary antibodies for the R02 and 9E10 antibodies may be different. The difference in the magnitude of the signal obtained with the same antibody (R02) in two different cell lines [293L(wt-myc11) and 293L(wt-12)] shown in the bottom panel of Figure 5 may be a reflection of different affinities of the R02 antibody for the myc-tagged and nontagged rLHR, or of differences in the amount or receptor present in the soluble extracts from either cell line. As noted above, the density of cell surface receptors measured by $^{125}$I-hCG binding is similar among these two cell lines, but we do not know if the total receptor density is the same.

Once these two assays, were established we used them to compare the amount of receptor present in detergent extracts prepared from control and protease-treated cells. Again, since protease treatment destroys only the 85 kDa cell surface rLHR (cf. Figure 1), a comparison of the signal detected in the extracts prepared from protease-treated cells with that of control cells should yield the relative amount of the 68 kDa intracellular receptor. We chose to perform these experiments with two different cell lines. 293L(wt-myc11) cells were chosen because the rLHR expressed in this cell line can be recognized by the 9E10 and R02 antibodies, thus allowing a comparison of the results obtained with two different antibodies on the same sample. 293L(wt-17) cells were also chosen to allow a comparison of the results obtained with the R02 antibody and with $^{125}$I-hCG binding (cf. Table 1).

Table 4: Immunological Quantitation of the rLHR Present in 293L(wt-17) and 293L(wt-myc11) Cells[a]

| | immunoreactive rLHR, (+protease/−protease) × 100, antibody used | |
|---|---|---|
| cell line | 9E10 | R02 |
| 293L(wt-myc11) | 76 ± 2 | 84 ± 3 |
| 293L(wt-17) | − | 87 ± 6 |

[a]Cells were incubated with or without protease XIV (250 μg/mL) for 30 min at 4 °C. Detergent extracts were prepared, purified, and assayed for rLHR using ELISAs that employed the indicated antibodies as described under Materials and Methods. The amount of immunoreactive receptor present in the extracts prepared from protease-treated cells was expressed as percentage of that detected in the control cells. Each number represents the mean ± SEM of 3−5 experiments.

The results of these experiments are presented in Table 4 and show that protease treatment of 293L(wt-myc11) cells reduces the amount of immunoreactive rLHR detected with the 9E10 or R02 antibodies by 16−24%. Likewise, the results presented in Table 4 also show that protease treatment of 293L(wt-17) cells reduces the amount of rLHR detected with the R02 antibody by about 13%. Thus, the immunological quantitation of the solubilized rLHR present in extracts prepared from control and protease-treated cells is basically in agreement with that obtained by measuring the $^{125}$I-hCG binding capacity of these extracts (Table 1). Both of these assays indicate that the 68 kDa rLHR (i.e., that which is not accessible to the protease) is much more abundant than the 85 kDa rLHR.

Lastly, it should be stressed that the immunological quantitation described here assumes that both the 68 and 85 kDa forms of the rLHR are recognized to the same extent by the antibodies. This assumption could not be tested directly, but there are three findings that argue favorably for its validity. First, all the ELISAs comparing multiple dilutions of extracts prepared from control cells (which contain both the 85 and 68 kDa rLHR) and from proteolyzed cells (which contain only the 68 kDa rLHR) gave parallel lines, a finding which is indicative of equal cross-reactivity in both samples (Figure 5). Second, a comparison of the amount of rLHR present in detergent extracts prepared from control and proteolyzed cells using ELISAs performed with two different antibodies (i.e., 9E10 and R02) directed against two distinct epitopes located in different regions of the rLHR gives basically the same results (cf. Table 4). Lastly, immunoprecipitations of the labeled rLHR from 293L(wt-myc11) cells using different antibodies (i.e., 9E10 and antiL/R02) also give the same results (compare lanes 1 and 4 in Figure 3).

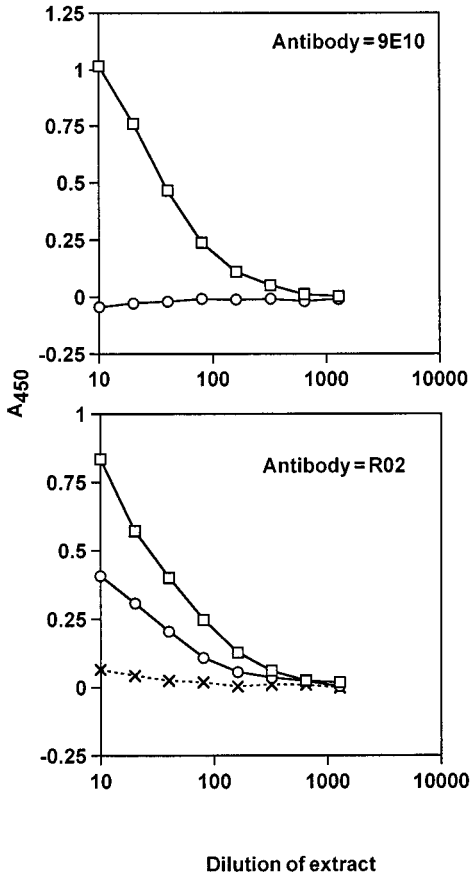


FIGURE 5: ELISA for rLHR and rLHR-myc. Detergent extracts were prepared from untransfected 293 cells (×), as well as 293L-(wt-12) (○) and 293L(wt-myc11) cells (○) and used to detect the rLHR using 9E10, the monoclonal antibody directed against the myc epitope (top panel), or R02, a polyclonal antibody directed against the rLHR (bottom panel). The results of a representative experiment are shown.

## DISCUSSION

The experiments presented herein were designed to resolve the conflicting information on the hCG binding properties of the 68 kDa intracellular precursor of the rLHR. This question was directly addressed by measuring $^{125}$I-hCG binding to detergent extracts prepared from cells that had been proteolyzed at 4 °C. These conditions were shown to selectively degrade the cell surface 85 kDa rLHR (Figure 1 and Table 1), which is known to bind $^{125}$I-hCG with high affinity, thus allowing the preparation of detergent extracts that contain only the 68 kDa rLHR. The data presented clearly show that detergent extracts containing only the 68 kDa rLHR bind hCG with essentially the same affinity as that detected in intact cells, where hCG can bind only to the 85 kDa cell surface rLHR (Table 1).

As mentioned in the introduction, studies performed in several laboratories have shown that a number of rLHR mutants expressed in mammalian cells fail to be properly transported to the cell surface and remain trapped intracellularly as EndoH-sensitive 68−73 kDa proteins that can bind hCG with high affinity (*5*, *6*, *11*, *12*). Prior to the experiments presented here, there was no way to determine if the hormone binding properties of these mutants were equivalent to those of the 68 kDa rLHR present in cells expressing the wild-type rLHR. The experiments presented here argue that they are indeed equivalent, and that the inferences made about the 68 kDa rLHR present is cells expressing the wild-type rLHR from data obtained using these mutants are valid.

While the studies on the hCG binding properties of proteolyzed cells and their detergent extracts conclusively established that the 68 kDa intracellular rLHR binds hCG with high affinity, they also uncovered a hitherto unrecognized problem with the quantitation of receptor density. Based on comparisons of the maximal $^{125}$I-hCG binding capacity of intact cells and detergent extracts, one can conclude that most of the rLHR present in the two cell lines studied here is present as the 85 kDa cell surface rLHR

(Table 1 and Figure 1). The finding that surface proteolysis has only a small effect on the maximal $^{125}$I-hCG binding capacity detected in detergent extracts supports the opposite conclusion, that most of the rLHR is present as the 68 kDa intracellular species (Table 1 and Figure 1). In order to solve this discrepancy we established ELISAs using two different antibodies and quantitated the amount of rLRH present in detergent extracts prepared from control and proteolyzed cells. The results of these assays indicate that most of the rLHR is present as the 68 kDa intracellular precursor (Table 4). Quantitation of the relative abundance of 85 and 68 kDa rLHR species can also be done by densitometric scanning of the fluorograms obtained from the gels of immunoprecipitates of cells that had been metabolically labeled with [$^{35}$S]Translabel. We have refrained from quantitating the fluorograms presented here (cf. Figures 1 and 4) because more recent experiments indicate that in many cases the labeling conditions used in this paper result in an almost complete depletion of the [$^{35}$S]Translabel. As such, the labeling of the 68 and 85 kDa rLHR species may not be at steady state, and the fluorograms presented here cannot be used to quantitate their relative abundance. Fluorograms of more recent experiments (not shown) done with 293L(wt-12) and 293L(wt-myc11) cells under conditions where the isotope is not depleted and the labeling is at steady state indicate that the 68 kDa rLHR is indeed more abundant than the 85 kDa rLHR, however. Based on these fluorograms and the results of the ELISAs presented here (Table 4), we estimate that the 68 kDa rLHR accounts for about 75% and the 85 kDa rLHR accounts for about 25% of the rLHR present in 293L(wt-12) and 293L(wt-myc11) cells. These results are also in agreement with the finding that surface proteolysis reduces the maximal $^{125}$I-hCG binding capacity detected in detergent extracts by 10−20% (Table 1).

When considered together, the results presented here suggest that a comparison of the binding capacity of detergent extracts and intact cells underestimates the relative abundance of the 68 kDa rLHR. These results may be explained by the known instability of the detergent-solubilized rLHR and by the effects of detergents on hCG binding (*31*−*33*). Detergents are known to interfere with hCG binding but need to be present for the receptor to stay in solution. The concentration of detergent (0.1% NP40) present for the binding experiments shown here is not optimal for binding, but it is a compromise in that further dilution decreases the concentration of receptor too much, thus affecting the signal/noise ratio in the binding assay to the point where the results are not reliable. Moreover, further dilution of the detergent to the point where binding is not affected may lead to the precipitation or aggregation of the solubilized receptor (*31*−*33*). Lastly it should be noted that although all solutions used for solubilization and binding contain a high percentage of glycerol, a compound which is known to stabilize the binding activity of the LHR (*23*), it is impossible to know what fraction of the binding activity detected in intact cells is preserved upon solubilization. Thus, it is possible that binding assays performed using detergent extracts greatly underestimate the total density of LHR because the binding activity of the detergent-solubilized receptor is not well preserved and because detergents interfere with hCG binding.

It should be stressed that while all studies presented here were done with transfected cells, Western blots of the testicular LHR reveal the presence of a large amount of the immature 68 kDa receptor (*1*, *3*). The presence of this precursor in gonadal cells raises the possibility that the maturation of the receptor is under physiological control. The fate of the 68 kDa rLHR also needs to be considered in this context. While it is clear that this is a precursor of the 85 kDa rLHR (*2*, *4*, *5*, *7*, *8*), it also seems now that a good portion of this intracellular receptor is never converted to the 85 kDa cell surface rLHR and may instead be degraded. Thus, pulse−chase experiments indicate that even after a 6 h chase not all of the 68 kDa rLHR is converted to the 85 kDa rLHR (*2*, *5*), and the data presented here suggest that most of the recovery of the cell surface receptor that occurs after proteolysis involves de novo synthesis of the receptor rather than the simple conversion of the 68 to the 85 kDa proteins (Figure 2 and Table 2).

Lastly, some of the data presented here show that the modification of the rLHR with the myc epitope is a valid strategy for quantitating the rLHR and for studying its processing. The addition of the myc epitope between the C-terminus of the signal peptide and the N-terminus of the mature rLHR does not affect its ability to bind hCG (see text), and has only small effects on the biological properties of the receptor (Table 3). Importantly, the myc-tagged receptor can be readily immunoprecipitated (Figure 4) and quantitated (Figure 5 and Table 4) using a well-characterized and readily available antibody (9E10) to this epitope.

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