Effects of Truncations of the Cytoplasmic Tail of the Luteinizing Hormone/Chorionic Gonadotropin Receptor on Receptor-Mediated Hormone Internalization

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The LH/CG receptor is a member of the family of G protein-coupled receptors and consists of a large N-terminal extracellular domain (which is responsible for binding hormone) attached to a region that spans the plasma membrane seven times, ending with an intracellularly located C-terminus. Binding of LH or human CG (hCG) to the LH/CG receptor causes a stimulation of adenylyl cyclase, presumably via activation of Gs. The binding of hormone also leads to its subsequent internalization by receptor-mediated endocytosis. In order to investigate the role of the cytoplasmic tail of this receptor in these events, we prepared a series of mutants in which progressively larger portions of the cytoplasmic tail were deleted.

Deletion of 58 amino acids from the C-terminus, in which only 11 cytoplasmic residues remain, resulted in a receptor that was not expressed on the plasma membrane. Receptors rat LHR (rLHR)-t653 and rLHR-t631, in which 21 or 43 amino acids were removed, respectively, were properly expressed. These results suggest that a region(s) between residues 616 and 631 of the rLH/CG receptor are required for proper insertion and/or targeting of the receptor into the plasma membrane. Cells expressing rLHR-t653 or rLHR-t631 bound hCG with the same high affinity as cells expressing the full-length receptor, and basal levels of cAMP were the same among the cells. However, cells expressing the truncated receptors responded to hCG with approximately 2-fold greater levels of maximal cAMP accumulation than cells expressing the full-length receptor.

Deletion of up to 43 amino acids from the C-terminus of the rLH/CG receptor had no deleterious effect on hCG internalization. In fact, mutants lacking 21 and 43 amino acids exhibited progressively faster rates of hCG internalization as compared to the full-length receptor. Once internalized, hCG was also degraded at a faster rate in cells expressing the truncated LH/CG receptors.

Since hCG-stimulated cAMP stimulation and hCG internalization are retained by rLHR-t631, it can be concluded that the residues, not necessarily the same, required for these functions reside within the 26 amino acids of the cytoplasmic tail closest to the seventh transmembrane helix and/or residues within the intracellular loops. Our data show, however, that both hCG-stimulated cAMP production and hCG internalization are enhanced by the removal of the distal portion of the cytoplasmic tail. (Molecular Endocrinology 6: 327–336, 1992)

INTRODUCTION

The LH/CG receptor is a cell surface receptor, present in gonadal tissues, which binds either LH or the highly homologous hormone CG. LH and CG are glycoprotein hormones which are related to each other as well as to TSH and FSH (1, 2). In humans LH is produced by the pituitary of adult males and females, whereas human CG (hCG) is only produced by the placenta of pregnant females. In both males and females, stimulation of the LH/CG receptor leads to the synthesis of steroids critical to normal reproductive function. The primary, if not sole, second messenger mediating the actions of LH and hCG in target cells has been shown to be cAMP (3–5), and the LH/CG-induced increase in adenylyl cyclase activity is presumably mediated by a Gs protein (4).

Cloning of the cDNA for the LH/CG receptor has shown that it is a single polypeptide composed of two

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general domains of roughly equivalent size (6–8). The amino-terminal domain resides extracellularly (9), is composed of a repeating leucine-rich repeat motif (6), and contains six potential sites for N-linked glycosylation. It has been shown that this portion of the molecule is entirely sufficient for binding hormone with high affinity (10). The carboxy-terminal domain contains seven regions of hydrophobic amino acids and is related to other G protein-coupled receptors (6). As such, it is thought to span the plasma membrane seven times, with the carboxy-terminal tail extending intracellularly. Based upon studies with other receptors (11–14), it is presumed that amino acids within the intracellular loops and proximal portion of the cytoplasmic tail of the LH/CG receptor are involved in its coupling to and activation of Gs. Other notable features of the cytoplasmic domain of the rat LH/CG receptor include the presence of numerous serines, threonines, and tyrosines that may serve as potential sites for phosphorylation and the presence of two potential sites for tryptic cleavage. Although the mature receptor does not appear to be cleaved at either of those sites (9), it is not yet known if such a cleavage occurs under certain physiological conditions.

Like other cell surface receptors, the LH/CG receptor mediates the internalization of receptor-bound ligand. Thus, the binding of hCG or LH to ovarian granulosa or luteal cells or to testicular Leydig cells results in the intracellular accumulation of hormone (15–19). The internalized hormone is subsequently degraded and released from the cells. In MA-10 Leydig tumor cells, a cell line that expresses functional LH/CG receptors (20), it has been shown that receptor down-regulation occurs as a result of increased degradation of the LH/CG receptor, which results as a consequence of the receptor-mediated endoctyosis of hCG (17, 21). In these cells there is no evidence of receptor recycling. The relative contributions of receptor degradation *vs.* receptor recycling that occur during LH/CG receptor-mediated internalization of hCG in other gonadal cell types, however, have not yet been clearly determined.

The present studies were undertaken in order to ascertain what roles, if any, the cytoplasmic tail of the LH/CG receptor have on the ability of the receptor to bind hormone, activate adenylyl cyclase, and internalize hCG. To address these questions, we have prepared mutants of the rat LH/CG receptor in which progressively larger portions of the cytoplasmic tail have been deleted. As shown herein, the truncated receptors have an increased ability to stimulate adenylyl cyclase when challenged with hCG. These studies further show that deletion of as much as 62% of the cytoplasmic tail has no deleterious effect on hCG internalization, suggesting that residues within the proximal 26 amino acids of the cytoplasmic tail and/or residues within the intracellular loops are required for receptor-mediated internalization. In fact, deletion of up to 43 amino acids from the C-terminus of the cytoplasmic tail led to progressively greater rates of hormone internalization.

RESULTS

The carboxy-terminal tail of the rat LH/CG receptor consists of 69 amino acids if one assumes that the seventh membrane-spanning region terminates with T^{605}. In order to test the role of the cytoplasmic tail in the biological functions of the LH/CG receptor, three mutants were constructed in which progressively larger portions of the cytoplasmic tail were removed. Thus, as shown schematically in Fig. 1, receptor mutants rLHR-t653, rLHR-t631, and rLHR-t616 (in which each receptor was truncated after the designated residue) lack 30%, 62%, and 84% of the cytoplasmic tail, respectively.

Effects on hCG Binding and cAMP Accumulation

293 human kidney cells were transiently transfected with the cDNAs encoding for the full-length and truncated receptors and were tested for [^{125}I]hCG binding activity. Surprisingly, little or no cell surface [^{125}I]hCG binding could be detected in cells expressing rLHR-T616. When these cells were solubilized in detergent, however, high affinity binding activity was observed in the solubilized extracts (data not shown). These data suggest that this form of the LH/CG receptor, in which only 11 residues of the proximal region of the cytoplasmic tail remain, cannot be properly transported to and/or incorporated into the plasma membrane. Due to this limitation, it was not possible to more fully characterize the properties of rLHR-t616.

Table 1 summarizes the ability of cells expressing the full-length receptor, rLHR-t653, or rLHR-t631 to bind hCG and respond with increased cAMP production. These experiments were performed in which predetermined amounts of plasmid DNA were used to transfect the cells so that comparable amounts of receptors per cells would be expressed by the wild type and mutant receptors. It can be seen that truncations of the cytoplasmic tail have no apparent effect on the affinity of hCG binding to the receptor. Nor do these truncations have an effect on the EC_{50} of cAMP production in response to hCG binding. However, the maximal amounts of cAMP produced (R_{max}s) in cells expressing either rLHR-t653 or rLHR-t631 are approximately twice those of cells expressing the full-length receptor. This reflects a greater fold-stimulation of cAMP production in response to hCG by the cells expressing the truncated receptors, since the basal levels of cAMP were comparable among the different cells.

Effects on Receptor-Mediated Endocytosis of hCG

Because it is well documented that the LH/CG receptor is internalized as a consequence of hormone binding, and because it has been shown for numerous other cell surface receptors that residues located intracellularly appear to mediate receptor internalization, we chose to

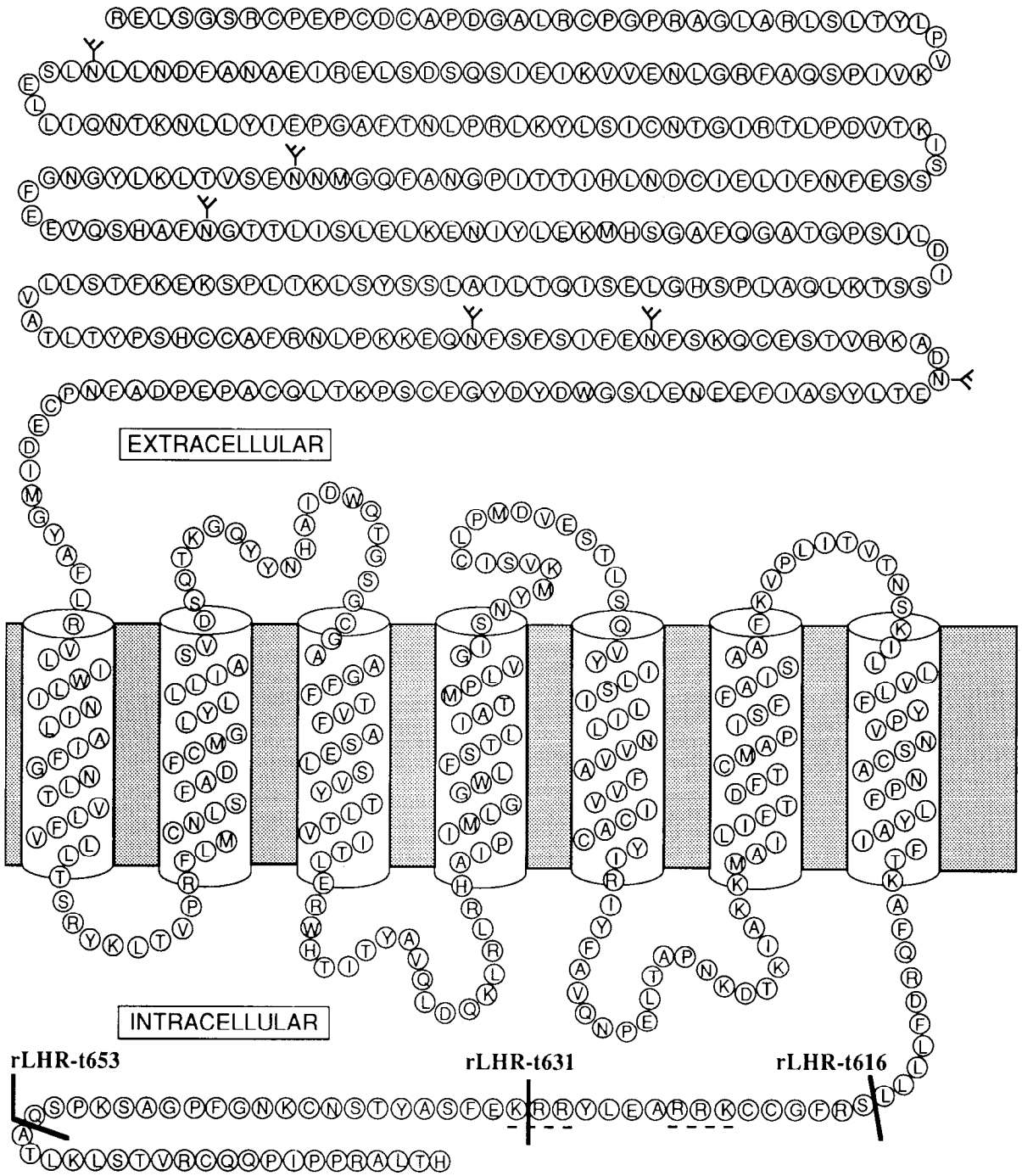

Fig. 1. Schematic Representation of the Full-Length Rat Luteal LH/CG Receptor and Truncated Mutants Thereof

Shown is the deduced amino acid sequence of the rat luteal LH/CG receptor (6). Potential sites for N-linked glycosylation are denoted by the *branch-like structures*. Amino acids underlined by the *dotted lines* represent potential tryptic cleavage sites within the cytoplasmic tail.

examine the ability of cells expressing rLHR-t631 and rLHR-t653 to mediate [^{125}I]hCG internalization. In the first set of experiments, the fate of receptor-bound [^{125}I] hCG was examined under conditions allowing multiple rounds of endocytosis to occur.

In this experimental approach the cells were incubated at 37 C in the continuous presence of [^{125}I]hCG and at various time points were assayed for surface-bound and internalized [^{125}I]hCG. The results of these experiments are shown in Fig. 2. In examining cells expressing the full-length receptor (Fig. 2A), it can be seen that by 6 h approximately 30% of the hormone bound to the cells is located intracellularly. In spite of the internalization of [^{125}I]hCG, however, the levels of total and surface-bound hormone remain relatively unchanged between 1–6 h after hormone addition, sug-

Table 1. Effects of Truncations of the Cytoplasmic Tail of the Rat LH/CG Receptor on hCG Binding and hCG-Stimulated cAMP Production

Exp	Receptor	[^{125}I]hCG Binding		hCG-Stimulated cAMP Production	
		K_d (pM)	B_{max} (ng/10^6 cells)	EC_{50} (pM)	R_{max} (pmol/10^6 cells)
1	rLHR	454	3.6	131	107
	rLHR-t653	454	3.4	131	235
	rLHR-t631	625	3.8	131	226
2	rLHR	312	3.9	144	111
	rLHR-t653	435	3.7	144	221
	rLHR-t631	526	3.5	144	223

Cells expressing the different LH/CG receptors were assayed for equilibrium [^{125}I]hCG binding and hCG-stimulated cAMP production as described in *Materials and Methods*. The binding data were analyzed by Scatchard analysis and the cAMP data using the Allfit computer program (38). Basal levels of cAMP in all groups of cells was 20 ± 4 pmol/10^6 cells.

gesting that some receptor recycling may be occurring. If one now examines the results obtained with cells expressing the truncated receptors rLHR-t653 and rLHR-t631 (Fig. 2, B and C), it can be seen that these cells too internalize [^{125}I]hCG. Quantitatively, however, the levels of [^{125}I]hCG located intracellularly throughout the 6-h time course in cells expressing rLHR-t653 are much lower than in cells expressing the full-length receptor or rLHR-t631. Another notable difference is that in cells expressing rLHR-t631, but not rLHR-t653 or the full-length receptor, the levels of total and surface-bound hormone peak and then decline somewhat with time.

In order to directly assay the rates of internalization of hCG mediated by the full-length *vs.* the truncated receptors, experiments were performed in which the cells were allowed only a single round of internalization of hormone. In this experimental paradigm, the cells are allowed to prebind [^{125}I]hCG under conditions in which little or no internalization has occurred. The cells are then washed to remove the unbound hormone, and the fate of the cell surface receptor-bound [^{125}I]hCG is examined by incubating the cells at 37 C in the absence of any added hormone. Under these conditions, one can follow the fate of the hormone during one round of endocytosis in the absence of any potential receptor recycling. The results, shown in Fig. 3, show that the surface-bound hormone in cells expressing the full-length receptor decreases such that 50% of the radioactivity has disappeared by approximately 4.5 h. Within 30 min a significant portion (about 20%) of the initial receptor-bound hormone is located intracellularly, which then remains relatively constant throughout the 6-h time course. Degraded hormone release into the media is not appreciable until 4 h, with only 15% of the initial receptor-bound hormone being degraded by 6 h.

When examining [^{125}I]hCG internalized during one round of endocytosis, the results observed with cells expressing the truncated receptors rLHR-t653 and rLHR-t631 appear quite different from those expressing the full-length receptor (*cf.* Fig. 3A with Fig. 3, B and C). Notably, the disappearance of receptor-bound [^{125}I]hCG from the surface of cells expressing the truncated receptors is much faster than from those expressing the full-length receptor. Thus, the times at which 50% of the hormone has been internalized in cells expressing rLHR-t653 *vs.* rLHR-t631 are 2.7 h and 0.5 h, respectively, as compared to 4.5 h for cells expressing the full-length receptor. In agreement with the faster and more extensive loss of surface-bound [^{125}I]hCG in rLHR-t631-expressing cells, those cells also show a more rapid accumulation of internalized and degraded hormone.

As shown in Fig. 3, cells expressing the truncated receptors release more degraded hormone into the media than cells expressing the full-length receptor. Although this may be accounted for by the faster accumulation of hCG into those cells, the following experiment was performed in order to determine if, once internalized, the hormone is also being degraded more rapidly. In this experiment cells were preincubated with [^{125}I]hCG for 1 h at 37 C to allow hormone internalization. After removing both the free and the receptor-bound [^{125}I]hCG, the cells were then further incubated at 37 C in the absence of added hormone. As such, the fate of the initial hormone that was internalized could be followed. The results of this experiment, shown in Fig. 4, indicate that [^{125}I]hCG internalized by cells expressing the truncated LH/CG receptors is indeed degraded at a faster rate than the hormone internalized by cells expressing the full-length receptor. The data from Fig. 4 were used to calculate the half-lives of [^{125}I]hCG degradation in the different cells, and this is shown in Table 2. Cells expressing rLHR-t653 and cells expressing rLHR-t631 degrade internalized hCG 2.8- and 5.5-fold faster, respectively, than cells expressing the full-length LH/CG receptor. Therefore, the greater accumulation of hCG degradation products in the media of cells expressing the truncated receptor (see Fig. 3) is due to both an increase in the rate of internalization of surface-bound hCG and to an increase in the rate of degradation of hCG once internalized.

Effects on hCG-Induced Down-Regulation of Receptors

It was shown above (see Fig. 2) that after 6 h at 37 C in the continuous presence of hCG, there was little or

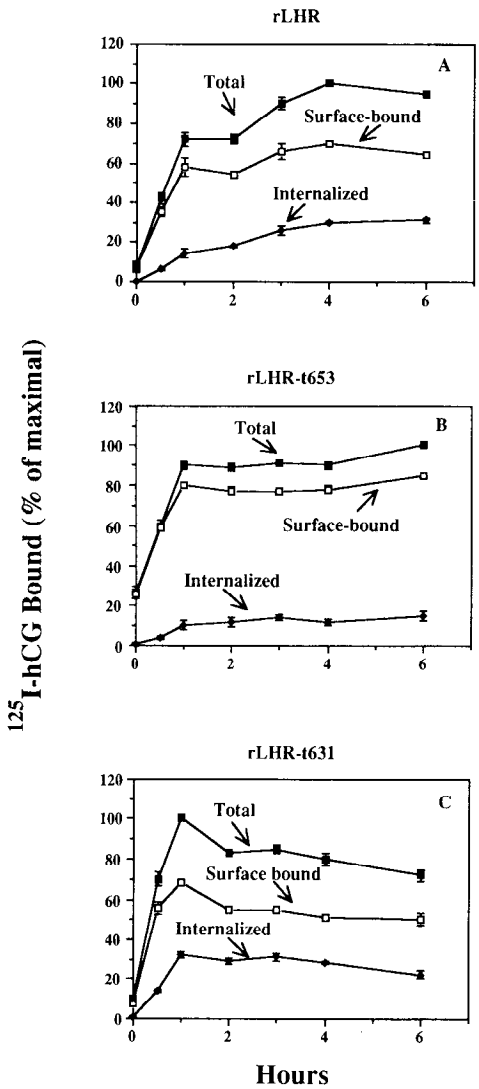

Fig. 2. Several Rounds of Endocytosis

Cells expressing the noted LH/CG receptors were incubated at 37 C with a saturating concentration of [^{125}I]hCG for up to 6 h. At the times indicated, the cells were place on ice and washed to remove free hormone. Amounts of radioactivity associated with surface-bound and internalized hormone were determined as described in *Materials and Methods*. Each *point* represents the mean ± SEM of three independent experiments, with duplicate determinations for each point.

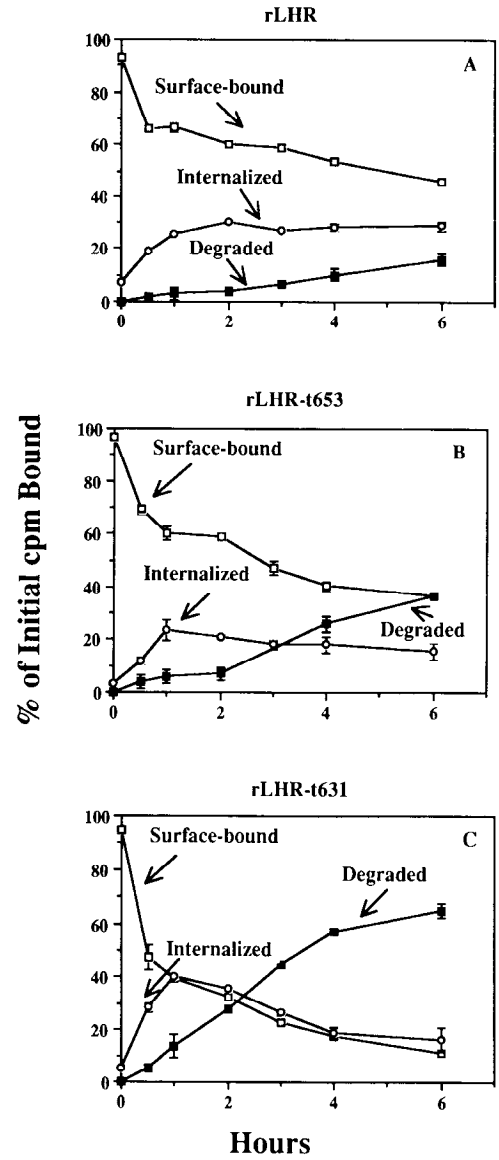

Fig. 3. One Round of Endocytosis

Cells expressing the noted LH/CG receptors were preincubated with a saturating concentration of [^{125}I]hCG for 5 min at 37 C and then washed to remove unbound hormone. Cells were either processed immediately (t = 0) or incubated at 37 C for up to 6 h in hormone-free medium. At the times indicated, amounts of radioactivity associated with surface-bound, internalized, and degraded hormone were determined as described in *Materials and Methods*. Each *point* represents the mean ± SEM of two independent experiments, with duplicate determinations for each point.

no decrease in the levels of cell surface binding in cells expressing either the full-length receptor or the truncated receptors. This would indicate that within this time frame little down-regulation of cell surface LH/CG receptors had occurred. In order to determine if receptor down-regulation would occur after a longer period of exposure to hormone, the experiment shown in Fig. 5 was performed. In this case, cells were incubated for 12 h at 37 C in the presence of a saturating concentration of unlabeled hCG. After removing both the free and the receptor-bound hCG, levels of LH/CG receptor remaining at the cell surface were assayed by [^{125}I]hCG binding. Indeed after a prolonged period of time (*i.e.* 12 h), there is down-regulation of the LH/CG receptor in cells expressing either the full-length or the truncated receptors. After 12 h with hCG, approximately 40% of the cell surface receptors of cells expressing the full-length LH/CG receptor have been lost. Similar results are observed in cells expressing rLHR-t653. However, LH/CG receptor down-regulation was more extensive in those cells expressing rLHR-t631, in which a greater portion of the cytoplasmic tail has been removed. The

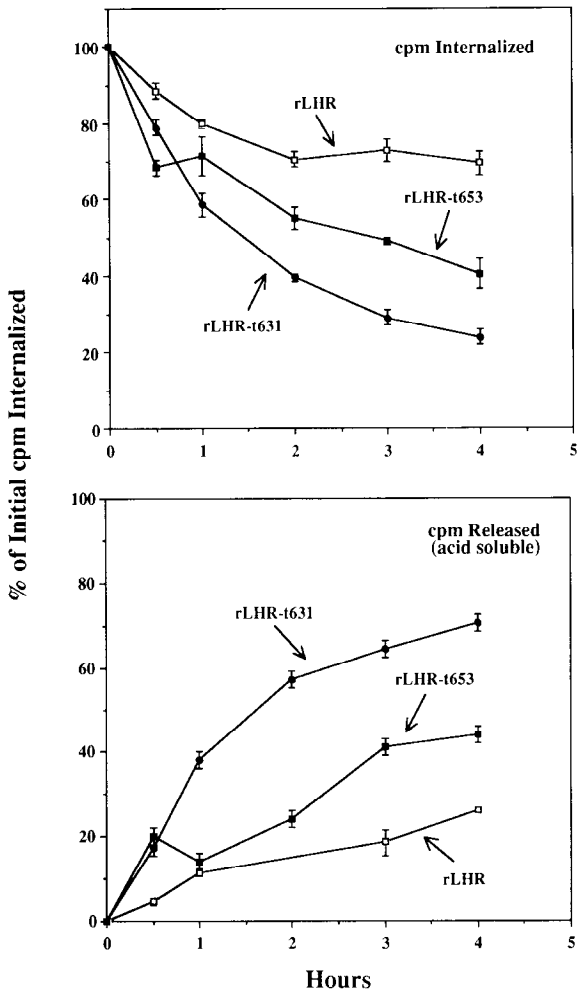

Fig. 4. Degradation of Internalized hCG

Cells expressing the noted LH/CG receptors were preincubated with a saturating concentration of [^{125}I]hCG for 1 h at 37 C. At the end of this incubation time (t = 0), the cells were placed on ice and washed to remove unbound hormone, treated with acid to remove the surface-bound hormone, and then incubated at 37 C in hormone-free medium. At the times indicated, the amounts of radioactivity that remained associated with the cells (*upper panel*) and the amounts that were released into the media (*lower panel*) were determined as described in *Materials and Methods*. The acid-insoluble counts per min released into the media was 5% or less at all time points (data not shown). Each *point* represents the mean ± SEM of three independent experiments, with duplicate determinations for each point.

Table 2. Half-Lives of Degradation of Internalized hCG in Cells Expressing the Full-Length *vs.* Truncated Forms of the LH/CG Receptor

Receptor	$t_{½}$ of hCG Degradation (h)
rLHR	11 ± 2 (n = 3)
rLHR-t653	4 ± 1 (n = 3)
rLHR-t631	2 ± 1 (n = 3)

Using the data shown in Fig. 5, the disappearance of internalized hormone was plotted as a semilog plot of the internalized hormone *vs.* time. The half-lives of degradation were calculated from the slopes, which were derived by linear regression.

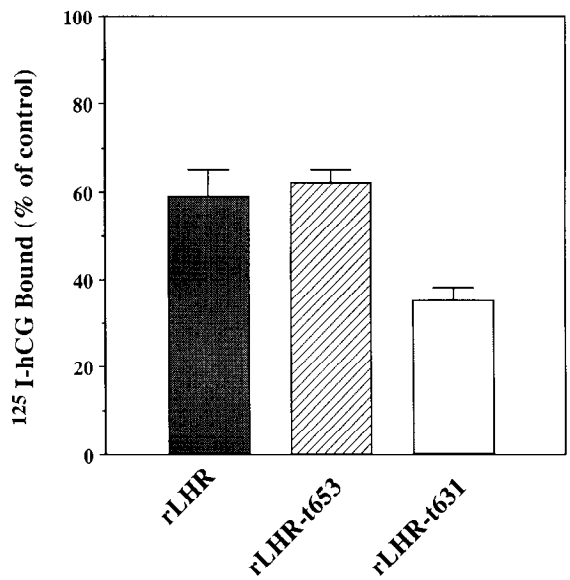

Fig. 5. Down-Regulation of Cell-Suface LH/CG Receptors

Cells expressing the noted LH/CG receptors were incubated with or without a saturating concentration of unlabeled hCG for 12 h at 37 C. At the end of this incubation, all cells were washed to remove unbound hormone and treated with acid to release surface-bound hormone. The cells were then incubated with a saturating concentration of [^{125}I]hCG overnight at 4 C. Each column represents the percentage of [^{125}I]hCG bound to the hCG-pretreated cells relative to the cells pretreated without hCG and is expressed as the mean ± SEM of two independent experiments.

greater down-regulation of receptors in cells expressing rLHR-T631 is consistent with the much higher rates of both hCG internalization and degradation observed in those cells (*cf.* Figs. 3 and 4).

DISCUSSION

The cytoplasmic regions of cell surface receptors have been shown to be involved in signal transduction, desensitization, and receptor internalization. Receptors comprising the family of G protein-coupled receptors have a complex structure involving seven transmembrane regions. As such, the loops between the first and second, third and fourth, and fifth and sixth transmembrane regions, as well as the carboxy-terminal tail, are exposed intracellularly. In the LH/CG receptor, as well as other G protein-coupled receptors, these intracellular regions contain serines, threonines, and tyrosines which may represent potential phosphorylation sites. In addition, the cytoplasmic tail of the rat LH/CG receptor also contains two clusters of basic amino acids which represent potential sites for tryptic cleavage (6, 9). In the present study we have examined the contributions

of the residues in the cytoplasmic tail of the rat LH/CG receptor in mediating the binding of hCG, the activation of adenylyl cyclase activity in response to hCG, and the internalization of receptor-bound hormone by creating mutants of the receptor with progressively larger deletions of the C-terminal cytoplasmic tail. Thus, rLHR-t653, rLHR-t631, and rLHR-t616 lacked 21, 43, and 58 amino acids or 30%, 62%, and 84% the C-terminal cytoplasmic tail, respectively.

Although rLHR-t653 and rLHR-t631 were found to be expressed on the cell surface of transfected cells at levels comparable to cells transfected with cDNA encoding for the full-length receptor, most of the binding activity in cells expressing rLHR-t616 was found to be located intracellularly. Thus, although cells transiently expressing rLHR-t616 exhibited barely detectable cell surface binding of [^{125}I]hCG, high levels of high affinity binding activity were observed in detergent extracts of these cells. This mutant terminates before a pair of cysteines (residues 621 and 622), of which at least one is conserved throughout all G protein-coupled receptors characterized thus far. It has been shown that the comparable cysteine residue in the β_2-adrenergic receptor is palmitoylated (22). If the LH/CG receptor were similarly acylated, and if this acylation were necessary for the receptor to be incorporated into the plasma membrane correctly, then this might account for the low expression of rLHR-t616 on the cell surface.

LH/CG receptor mutants t631 and t653 were expressed on the cell surface and bound [^{125}I]hCG with the same high affinity as the full-length LH/CG receptor. Cells expressing either of these mutants, however, were about 2-fold more responsive to hCG in terms of the maximal stimulation of cAMP production than cells expressing the full-length receptor. Experiments with other G protein-coupled receptors suggest that regions within the intracellular loops and proximal portion of the cytoplasmic tail are involved in the coupling of these receptors to G proteins (11–14). Possible interpretations of our data include the following: 1) the C-terminal 43 amino acids of the cytoplasmic tail may sterically inhibit the interaction of other intracellular regions of the receptor with Gs; 2) this region may confer some ability of the receptor to interact with Gi; 3) the distal portion of the cytoplasmic tail may be involved in the functional uncoupling or desensitization of the receptor-Gs interaction; if so, removal of this region would diminish the desensitization and thereby augment the stimulation of Gs; and 4) this region, or a portion of it, may interact with cytoskeletal components. As such removal of this region may allow the receptor a greater mobility in the plasma membrane, which might allow it to interact with more Gs molecules. Further experiments will be required to determine the molecular basis of this phenomenon.

The 293 cells expressing the full-length LH/CG receptor were found to internalize receptor-bound hCG, although with a relatively slow rate (an estimated half-life of internalization of 11 h). One of the best characterized systems in which hCG internalization has been examined is MA-10 cells, a clonal line of murine Leydig tumor cells that express functional LH/CG receptors (20). In MA-10 cells the internalization of hCG occurs much more rapidly, with an estimated half-life of 20–50 min (18, 21, 23). In these cells it has been shown that the internalized hormone-receptor complex does not dissociate within the acidic endosome compartment, and that both the hormone and the receptor are delivered to the lysosome compartment, where they both are presumably degraded (17). As such, even in the continuous presence of hCG, there is a significant loss of cell surface receptors (or down-regulation) within 6 h (17, 24). In contrast, the experiments presented with transfected 293 cells indicate no down-regulation of cell surface LH/CG receptors during a 6-h incubation with hCG (*cf.* Fig. 2) and only a 40% loss of cell surface receptors after a 12-h incubation (*cf.* Fig. 5). This slow rate of loss of cell surface receptors in the continuous presence of hormone suggests that receptor recycling may be occurring in these cells; however, more direct experimentation will be needed to confirm this. Similar results were also observed in stably transfected 293 cells, in COS-7 cells transiently expressing the LH/CG receptor, and in primary cultures of rat luteal cells, which express the endogenous LH/CG receptor gene (data not shown). However, CHO cells stably transfected with the LH/CG receptor internalize hCG with a faster rate, similar to that observed in MA-10 cells (Wang, Z., and M. Ascoli, personal communication). Clearly, it will be important to identify which parameters determine the rate of internalization of the hormone-occupied full-length LH/CG receptor in different cells.

Studies with other receptors have shown that regions within the cytoplasmic tails confer the ability of the receptor to be internalized when occupied by hormone (25–31). Our studies show that removal of up to 43 amino acids from the C-terminus of the cytoplasmic tail of the LH/CG receptor had no detrimental effects on receptor-mediated internalization of hCG. Surprisingly, in fact, progressive deletions of the cytoplasmic tail up to the removal of 43 amino acids led to a progressively faster rate of receptor-mediated internalization of hCG (*cf.* Fig. 3). In addition, the hCG that is internalized by cells expressing these truncated receptors is subsequently degraded at a progressively faster rate (*cf.* Fig. 4). This finding suggests that in addition to an increase in the rate of internalization, the removal of these 43 amino acids leads to an increase in the rate at which the hormone is delivered to the lysosomes. Although in MA-10 cells it is the hormone-receptor complex (as opposed to the dissociated hormone) that is delivered to the lysosomes, we do not yet know if this is also the case in the 293 cells transfected with the wild type or mutant receptors. Likewise, since we cannot yet directly quantitate the rate of degradation of the receptor *per se*, we cannot conclude whether the faster rate of degradation of internalized hormone is associated with a faster rate of degradation of the internalized receptor. It is important to note, however, that quantitatively the

overall fate of the hCG bound to the rLHR-t631 receptor closely resembles that characterized in MA-10 cells.

As mentioned earlier, the cytoplasmic tail of the rLH/CG receptor contains two clusters of basic amino acids which may represent tryptic cleavage sites. The mutant rLHR-T631 terminates at one of these sites, that which is located further from the seventh transmembrane helix. Using an antibody raised against a peptide corresponding to the 14 most C-terminal amino acids, the 93-kilodalton mature LH/CG receptor can be detected on Western blots and the receptor can be detected by indirect immunofluorescence in permeabilized rat luteal cells (9). These studies suggest that at least some of the mature receptor is not normally proteolytically cleaved in the cytoplasmic tail. However, one cannot rule out the possibilities that some of the mature LH/CG receptor may be cleaved or that proteolytic cleavage may occur in response to hormonal stimulation. The studies presented suggest that if such a cleavage were to occur physiologically, the truncated receptor would internalize hormone more rapidly.

It is important to point out the similarities of our findings with the rLH/CG receptor with those reported for the avian β-adrenergic receptor. As compared to the mammalian β-adrenergic receptor, the turkey β-adrenergic receptor has an extended C-terminal cytoplasmic tail. Whereas mammalian forms of this receptor are internalized and down-regulated, the avian form is not (32). It has been demonstrated, however, that the removal of 18 amino acids from the C-terminus of the avian receptor results in a receptor which now is internalized and down-regulated in response to agonist (32, 33). It was also observed that, similarly to the rLH/CG receptor, the truncated avian β-adrenergic receptor had an enhanced ability to activate adenylyl cyclase in response to agonist as compared to the full-length receptor (33). Unlike cells expressing the truncated rLH/CG receptors, however, cells expressing the truncated avain β-adrenergic receptor also exhibited higher levels of basal cAMP (33). Reconstitution studies of the full-length *vs*. truncated avian receptors with Gs, however, demonstrated that the activation of Gs in response to agonist in the two receptor forms was the same (33).

Numerous studies have been performed with other cell surface receptors toward the identification of the sequence(s) that mediate receptor internalization (see Refs. 25–31 for examples). An excellent compilation and analysis of the these sequences has recently been put forth by Canfield *et al*. (31). In all cases, the regions important for mediating internalization are located within the cytoplasmic tail, generally within approximately 30 amino acids from the plasma membrane. Although it is clear that no one common sequence *per se* is responsible for receptor internalization, a more general motif appears to be common. This appears to be an aromatic residue and a hydrophobic amino acid flanking two to four amino acids, where in most (but not all) cases one of these interior amino acids is a basic residue. In examining the rLH/CG receptor sequences 629–634, the sequence of which is YRRKEF. The mutant rLHR-t631 discussed herein was truncated between the lysine and arginine residues of this sequence. If this sequence corresponds to a motif important for internalization of the rLH/CG receptor, then further truncation of the cytoplasmic tail should yield a receptor impaired in its ability to internalize hormone. Experiments are underway to test this hypothesis.

MATERIALS AND METHODS

Construction of LH/CG Receptor cDNA Mutants

pCLHR, the expression vector containing the rat luteal LH/CG receptor cDNA, has been previously described (6). Mutant cDNAs were created using the polymerase chain reaction to splice out or alter regions as described by Pease and coworkers (34, 35). rLHR-t616, which is truncated after Leu^{616}, was created by deleting those nucleotides encoding for amino acids 617–674. rLHR-t631 and rLHR-t653, which encode for receptors terminating after R^{631} and Q^{653}, respectively, were prepared by creating stop codons immediately after the codons encoding those residues. The sequence of the region of each mutant cDNA generated by DNA synthesis was verified directly by dideoxy sequencing (36).

Transfections

Human embryonic kidney 293 cells were obtained from the American Tissue Culture Collection (Rockville, MD; CRL 1573) and were maintained in growth medium (Dulbecco's modified Eagle's medium containing 50 μg/ml gentamicin and 10% heat-inactivated horse serum) in a 37 C humidified atmosphere containing 5% CO_2.

Transient transfections were initiated when the cells (plated in 35-mm dishes) were 75% confluent following the procedure of Chen and Okayama (37). Eighteen hours later the cells were washed two times with Waymouth MB752/1 media containing 50 μg/ml gentamicin and 1 mg/ml BSA (assay media), the growth medium was replaced, and the cells were returned to a 5% CO_2 incubator. The cells were used for experiments 24 h later.

[^{125}I]hCG Binding to Intact Cells

Cells were washed twice with 2-ml portions of cold assay media without sodium bicarbonate and then placed in 1 ml of the same medium containing increasing concentrations of [^{125}I] hCG in the absence or presence of an excess of crude hCG (final concentration, 50 IU/ml). The binding assay was performed overnight at 4 C and was finished by scraping the cells into a small volume of cold Hank's balanced salt solution containing 1 mg/ml BSA (wash buffer), centrifuging (1500 × *g*, 20 min), and washing the pellet in 2 ml of the same buffer. All measurements were done in duplicate and were corrected for nonspecific binding. Data were analyzed by Scatchard analysis.

cAMP Measurements

Cells were washed twice with 2-ml portions of warm assay media and placed in 1 ml of the same medium containing 0.5 mM isobutylmethylxanthine. After 15 min at 37 C, hCG was added (final concentrations, 0.1–300 ng/ml), and the incubation was continued for 30 min at 37 C. The cells were then placed on ice, and the total cAMP accumulated (in the cells

acid containing 360 μg/ml theophylline and then measured by RIA.

Parameters describing hCG-stimulated adenylyl cyclase activation (*i.e.* EC_{50}, the concentration of hormone required to produce half-maximal stimulation, and R_{max}, the maximal amount of cAMP produced) were determined using the Allfit computer program (38).

Multiple Rounds of Endocytosis

Cells were washed twice with 2-ml portions of warm assay media and incubated with 1 ml of the same medium containing a saturating concentration of [^{125}I]hCG (final concentration, 100 ng/ml) in the absence or presence of an excess of crude hCG (final concentration, 50 IU/ml). Cells were either processed immediately (t = 0), or incubated for up to 6 h at 37 C. At the times indicated, the cells were placed on ice and quickly washed five times with 2-ml portions of cold wash buffer. Receptor-bound hormone was quantitatively released by incubating the cells with 1 ml cold 50 mM glycine, 100 mM NaCl, pH 3, for 5 min on ice and washing them again with 1 ml of the same solution (18). The acid washes were collected and combined. The cells were then solubilized with 50 ml 0.5 N NaOH and collected with a cotton swab.

The radioactivity that was removed by the acid treatment was taken as a measure of the surface-bound hormone; the radioactivity that remained associated with the cells after acid treatment was considered to be internalized hormone (18). The efficiency of the acid treatment in releasing surface-bound hormone was verified by performing the binding assay overnight at 4 C (conditions in which hormone internalization does not occur), washing the cells, and then treating them with acid. In all cases, over 95% of the counts per min bound could be released.

One Round of Endocytosis

Cells were placed on ice and washed twice with 2-ml portions of cold assay media and incubated with 1 ml assay media containing a saturating concentration of [^{125}I]hCG (final concentration, 100 ng/ml) in the absence or presence of an excess of crude hCG (final concentration, 50 IU/ml) for 5 min at 37 C. At the end of this incubation, the cells were placed on ice and quickly washed five times with 2 ml ice-cold wash buffer to remove unbound hormone. Cells were either processed immediately (t = 0) or allowed to incubate up to 6 h at 37 C in medium without added hormone.

At the times indicated, the cells were placed on ice, the medium was removed and saved, and the cells were washed once with 2 ml cold wash buffer. The medium was precipitated with 10% trichloroacetic acid, and the soluble and insoluble radioactivity were considered to be degraded and undegraded hormone, respectively (39).

Rate of Degradation of Internalized Hormone

Cells were placed on ice and washed twice with 2-ml portions of cold assay media and incubated with 1 ml assay media containing a saturating concentration of [^{125}I]hCG (final concentration, 100 ng/ml) in the absence or presence of an excess of crude hCG (final concentration, 50 IU/ml) for 1 h at 37 C. After this incubation, the cells were placed on ice, washed five times with 2-ml portions of cold wash buffer, incubated for 5 min on ice with cold 50 mM glycine, 100 mM NaCl, pH 3 (to remove the surface-bound hormone), washed once with the same solution, and once with assay media. The cells then were placed in 2 ml warm assay media and were either processed immediately (t = 0), or incubated at 37 C for up to 4 h in the absence of added hormone. At the times indicated, the cells were placed on ice, the medium was collected, and the cells were solubilized with 0.5 N NaOH as described above to determine the amount of radioactivity that remained internalized. The medium was precipitated with 10% trichloroacetic acid to determine the amounts of degraded and undegraded hormone released.

Receptor Down-Regulation

Cells were washed twice with 2 ml cold assay media and incubated in 1 ml of the same medium with or without a saturating concentration of hCG (final concentration, 100 ng/ml) for 12 h at 37 C. After this incubation, the cells were placed on ice, washed five times with 2-ml portions of cold wash buffer (to remove unbound hormone), and treated with acid (see above) to remove receptor-bound hormone. The cells were then washed two times with 2-ml portions of cold Wa/BSA without bicarbonate and placed in 1 ml of the same medium containing a saturating concentration of [^{125}I]hCG (final concentration, 100 ng/ml) in the absence or presence of an excess of crude hCG (final concentration, 50 IU/ml). The binding assay was done overnight at 4 C and finished as described above (see binding to intact cells).

Hormones and Supplies

Highly purified hCG (CR-123 and 125) was kindly provided by the National Hormone and Pituitary Agency of the National Institute of Diabetes and Digestive and Kidney Diseases (Baltimore, MD) and was iodinated as described previously (40). Crude hCG was obtained from Sigma Chemical Co. (St. Louis, MO). Tissue culture plastic ware and reagents were from Corning (Corning, NY) and GIBCO (Grand Island, NY), respectively.

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