Mutations of the Second Extracellular Loop of the Human Lutropin Receptor Emphasize the Importance of Receptor Activation and De-emphasize the Importance of Receptor Phosphorylation in Agonist-induced Internalization*

Received for publication, November 20, 2000
Published, JBC Papers in Press, December 15, 2000, DOI 10.1074/jbc.M010482200

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Alanine scanning mutagenesis of the second extracellular loop of the human lutropin receptor (hLHR) showed that mutation of most of the residues present in this region either enhance or impair the internalization of agonist. A more complete analysis of four mutants, two that enhanced internalization (F515A and T521A) and two that impaired internalization (S512A and V519A), showed that the two mutants that impaired internalization also show a decrease in the sensitivity for agonist-induced cAMP accumulation, whereas the two mutants that enhanced internalization show an increase in the sensitivity for agonist-induced cAMP accumulation. None of these mutants had an effect on the agonist-induced phosphorylation of the hLHR, however. We conclude that, in contrast to the prevailing view of the relative importance of receptor phosphorylation in the internalization of G protein-coupled receptors, the phosphorylation of the hLHR is less important than the agonist-induced activation of the hLHR in the process of internalization.

Internalization of G protein-coupled receptors (GPCRs) is a ubiquitous response that follows agonist activation (reviewed in Refs. 1–3). Although several GPCR internalization pathways can now be recognized (3), the most common and best understood pathway is dependent on the G protein-coupled receptor kinase (GRK)-catalyzed phosphorylation of GPCRs and the subsequent formation of a complex between the agonist-activated and phosphorylated GPCRs and a family of proteins known as the nonvisual arrestins or β-arrestins. The nonvisual arrestins (arrestin-2 and -3) target the activated and phosphorylated GPCRs to clathrin-coated pits by virtue of their ability to bind with high affinity to clathrin and to adaptor protein-2 (4, 5). Once localized to clathrin-coated pits, the GPCRs are internalized by a process that requires the participation of dynamin, a GTPase involved in the fission of clathrin-coated pits (6).

The follitropin, lutropin and thyrotropin receptors (FSHR, LHR, and TSHR, respectively) are members of the rhodopsin/β2-adrenergic-like subfamily of GPCRs (7, 8). They form a small subfamily of GPCRs, collectively known as the glycoprotein hormone receptors, that is characterized by the presence of relatively large extracellular domains composed of leucine-rich repeats (9–11). Additional leucine-rich repeat-containing G protein-coupled receptors that are homologous to the glycoprotein hormone receptors have been recently identified in mammals and other organisms, but their ligands and functions are not yet known (reviewed in Ref. 12).

Like many other GPCRs, the binding of agonist to the LHR triggers the internalization of the agonist-receptor complex via clathrin-coated pits by a pathway that is dependent on receptor activation and phosphorylation and requires the participation of the nonvisual arrestins and dynamin (13–17). Whereas the model derived from the large number of studies on the β2-adrenergic receptor emphasizes the importance of GPCR phosphorylation on the process of internalization (1–3), recent mutagenesis studies suggest that the agonist-induced activation and phosphorylation of the human (h) LHR may play redundant roles in the agonist-induced internalization of this receptor (17). Additional studies on the LHR, FSHR, and TSHR have also uncovered an unusual, but as yet unexplained, role for their extracellular domains on the rate of internalization and/or the fate of the internalized receptors (18, 19).

The studies presented herein describe a novel set of mutations of the LHR (all located in the second extracellular loop) that enhance or impair receptor activation but do not affect agonist-induced phosphorylation. These mutations affect the rate of internalization of agonist in a manner that parallels their effects on receptor activation and thus highlight the importance of receptor activation, as opposed to phosphorylation, in the agonist-induced internalization of the LHR.

MATERIALS AND METHODS

Plasmids and Cells—Full-length cDNAs encoding for the hLHR and rLHR (20, 21) were modified with the Myc epitope at the N terminus (17, 22) and subcloned into pcDNANeo (rLHR) or pcDNA 3.1(hLHR) for expression. Site-directed mutagenesis was performed using conventional PCR strategies. The identity of each mutant was verified by automated DNA sequencing of the mutated region (performed by the DNA core of the Diabetes and Endocrinology Research Center of the University of Iowa).

Expression vectors for arrestin-3 and arrestin-3-(284–489) (23) were generously provided by Dr. Jeff Benovic (Thomas Jefferson University, Philadelphia, PA). An expression vector for dynamin-K44A (24) was donated by Dr. Sandra Schmid (Scripps Research Institute, La Jolla, CA). The expression of these constructs has been documented previously (25, 26).

Human embryonic kidney (293) cells were obtained from the American Type Culture Collection (CRL 1573) and maintained in Dulbecco’s...
modified Eagle’s medium containing 10 mM Hepes, 10% newborn calf serum, and 50 µg/ml gentamicin, pH 7.4. Cells were plated in 100-mm dishes or 35-mm wells that had been coated with gelatin and transfected with 0.5–1 µg of plasmid/35-mm well or 10 µg of plasmid/100-mm dish when 70–80% confluent using the calcium phosphate method of Chen and Okayama (27). After an overnight incubation, the cells were washed and incubated for an additional 24 h prior to use.

Binding, Internalization, and cAMP Assays—The methods used to measure the internalization of 125I-hCG have been described (18, 28). Single point assays were done using a 30-min incubation for cells expressing the rLHR or a 10-min incubation for cells expressing the hLHR. The results of these experiments are expressed as an internalization index (i.e. the ratio of internalized/surface-bound hormone. The length of the incubations was different because the half-time of internalization of 125I-hCG mediated by the rLHR (~120 min) is much slower than that mediated by the hLHR (~20 min) and the internalization index is linear as a function of time for ~60 min for the rLHR and for ~30 min for the hLHR (18, 28).

Determinations of the rates of internalization were done using at least five different data points collected at 3–10-min intervals after the addition of 125I-hCG (depending on the construct transfected). The endocytotic rate constant (k̄) was calculated from the slope of the line obtained by plotting the internalized radioactivity against the integral of the surface-bound radioactivity (18, 29–32). The half-time of internalization (t̄/2) is defined as 0.693/ k̄.

The hCG binding properties of the different receptor constructs were ascertained using intact cells cotransfected with dynamin-K44A to prevent internalization (see “Results”). Binding was measured in the cotransfected cells that had been incubated with seven different concentrations of 125I-hCG for 1 h at room temperature. All binding assays were corrected for nonspecific binding, which was measured in the presence of 50 IU/ml partially purified hCG (3,000 IU/mg). The binding data were fitted to a sigmoidal equation (33) using DeltaGraph® software (Delta Point, Monterey, CA) to calculate the maximal amount of cell-associated hormone and the concentration of hCG required to attain half of this value (apparent Kd).

Hormonal responsiveness was assessed by measuring cAMP accumulation in intact transfected cells plated in gelatin-coated 35-mm wells (see above). Total cAMP was measured at the end of a 2-h incubation (37 °C) with increasing concentrations of hCG as shown in Fig. 3 or with a single concentration of cholera toxin (0.6 nM) known to be maximally effective (17). The parameters that describe these dose responses (i.e. EC50 and maximal response) were calculated by fitting the data obtained to a sigmoidal equation (33) as described above.

Phosphorylation Assays—Cells were plated in 100-mm dishes that had been coated with gelatin and were transfected as described above. After an overnight incubation, the cells were washed, trypsinized, and plated in gelatin-coated 100-mm dishes and in gelatin-coated 35-mm wells that had been coated with gelatin and were transfected as described above. Total cAMP was measured as described under “Experimental Procedures.” Each bar represents the mean ± S.E. of at least three independent transfections. The absence of an error bar indicates that the S.E. is too small to be shown.

The internalization of hCG mediated by the mutants marked with an asterisk could not be analyzed because of their poor expression. The vertical line emphasizes the internalization index of the LHR-wt, and the arrows mark the four mutants that were chosen for further analysis.

![Fig. 1. Effect of mutations of the second intracellular loop of the rLHR and the hLHR on the internalization of 125I-hCG. Each of the 20 residues of the second extracellular loop of the rLHR (right panel) or the hLHR (left panel) was substituted with alanine as indicated. The resulting plasmids were transiently transfected into 293 cells (0.5 µg/35-mm well) and the internalization of 125I-hCG was measured as described under “Experimental Procedures.” Each bar represents the mean ± S.E. of at least three independent transfections. The absence of an error bar indicates that the S.E. is too small to be shown.](image)

The internalization of hCG mediated by the mutants marked with an asterisk could not be analyzed because of their poor expression. The vertical line emphasizes the internalization index of the LHR-wt, and the arrows mark the four mutants that were chosen for further analysis.

**Results**

Our interest on a potential role of the second extracellular loop of the LHR in the rate of internalization of hCG arose from two sets of studies. First, a recent analysis (35) of a truncation of the rLHR (at residue 653 in the C-terminal tail) that was initially reported to impair the internalization of hCG revealed that the decrease in internalization was due to an additional mutation (V497A in the second extracellular loop) that was inadvertently introduced (and went unnoticed) during the construction of the original C-termiinally truncated mutant. Second, other investigators have documented the potential involvement of this loop in modulation the hCG binding affinity and signaling properties of the rLHR (36). Alanine scanning mutagenesis of each of the 20 residues present in the second extracellular loop of the rLHR confirmed that the V497A mutation decreases the internalization of hCG in the context of the full-length receptor and showed that the mutation of many other residues present in this loop also enhance or decrease internalization (Fig. 1, right panel). Four mutants of this receptor region (Y468A, I491A, C492A, and P494A) could not be analyzed for internalization because the binding of 125I-hCG to cells transfected with these plasmids was very low or undetectable (these are marked with asterisks in Fig. 1). The reduced or undetectable expression of the I491A, C492A, and P494A mutants is in agreement with the previous study cited above (36) involving alanine scanning mutagenesis of this region. We disagree on the expression of the S484A mutant, however. Ryu and co-workers (36) reported that cells expressing the S484A mutant had no detectable 125I-hCG binding. In our experiments, however, the expression of this mutant was normal. Since the transient expression of the human (h) LHR is much more robust than that of the rLHR (16, 17) and the rate of internalization of hCG mediated by the hLHR is faster than that mediated by the rLHR (16), we reasoned that using the hLHR to examine the potential effect of mutations of the second extracellular loop may facilitate detection of changes in the rate of internalization and it would be a more amenable model for an analysis of the role of the second extracellular loop in the rate of internalization.
TABLE I
Half-times of internalization of hCG mediated by the hLHR-wt, and mutants thereof

| Receptor                  | t₁/₂ of internalization (min) |
|---------------------------|------------------------------|
|                           | pcDNA 3.1 | Arrestin-3-284-409 | Dynamin-K44A | Arrestin-3 |
| wt                        | 24 ± 1    | 67 ± 2*            | 201 ± 17b  | 3 ± 1b     |
| S512A                     | 162 ± 17a | 169 ± 15           | 272 ± 34c  | 29 ± 6b    |
| F515A                     | 10 ± 3a   | 55 ± 2b            | 146 ± 10e  | 3 ± 1c     |
| V519A                     | 118 ± 8a  | 117 ± 17           | 235 ± 17b  | 24 ± 3c    |
| T521A                     | 12 ± 1a   | 57 ± 8b            | 247 ± 27f  | 4 ± 1d     |

*p < 0.05 when compared to cells co-transfected with hLHR-wt and pcDNA 3.1.

system to examine these effects in more detail. The results of alanine-scanning mutagenesis of the second extracellular loop residues of the hLHR are shown in Fig. 1, left panel. Only one mutant of the hLHR (C514A, marked with an asterisk in Fig. 1) was not properly expressed and could not be analyzed for internalization. Alanine scanning of the remaining 19 residues, showed that only one residue (Glu520) had no effect on internalization. Of the remaining 18 residues, the mutation of 12 residues resulted in an increase in internalization and the mutation of 6 residues resulted in a decrease in internalization. Based on these results we chose two mutants that enhanced internalization and two that decreased internalization of the hLHR (see arrows in the left panel of Fig. 1) for more detailed studies.

We first considered the possibility that the mutants in question were internalized by pathways that do not require the participation of the visual arrestins and/or dynamin. This was tested by measuring the rates of internalization of hCG in cells cotransfected with arrestin-3(384–409), a dominant-negative mutant of the nonvisual arrestins (23), or with dynamin-K44A, a dominant-negative mutant of dynamin (24). These constructs have been previously shown to slow the t₁/₂ of internalization of hCG mediated by the hLHR-wt (16, 17). As shown in Table I, dynamin-K44A was an effective inhibitor of the internalization of hCG mediated by the hLHR-wt and the four mutants tested. In contrast, arrestin-3(284–409) inhibited the internalization of the hLHR-wt and the two mutants with a fast t₁/₂ of internalization (F515A and T521A) but had little or no effect on the two mutants with a slow t₁/₂ of internalization (S512A and V519A). Thus, it appears that the slow mutants internalize hCG by a pathway that is still dependent on dynamin but independent of the nonvisual arrestins. The data presented in Table I also show, however, that the slow rate of internalization of hCG mediated by these mutants can be rescued by overexpression of arrestin-3.2

Note that the amino acid sequences of the second extracellular loops of the rLHR and the hLHR are identical except for two residues (Leu693 and Ser699 in the rLHR are replaced by Phe543 and Thr541 in the hLHR). Additionally, note that the different numbers assigned to equivalent residues is artificially caused by differences in the numbering of amino acids. Since the N terminus of the mature hLHR is not known, residue number 1 is taken to the be the methionine present at the N terminus of the signal peptide. In contrast, since the N terminus of the mature rLHR is known, this residue (which corresponds to residue 23 of the hLHR) is taken to be residue number 1.
T521A were reported to reduce the $K_d$ for hCG binding whereas mutations equivalent to F515A and V519A were reported to have no effect on hCG binding affinity.

A representative experiment documenting the ability of cells expressing each of these four mutants to respond to hCG with increases in cAMP accumulation is shown in Fig. 3 and a summary of several experiments is presented in Table III. As pointed out before (17), there is some inherent variability associated with measuring the cAMP responses of transiently transfected cells and we attempted to correct for it by normalizing the basal and hCG-induced cAMP responses mediated by the different mutants to the cholera toxin-induced cAMP response measured in the same cells (see columns labeled basal/cholera toxin and hCG/cholera toxin in Table III). Thus, instead of using the absolute levels of cAMP, the responsiveness of cells expressing the different mutants shown in Table III is best compared by using these ratios.

The results presented in Table III show that the basal levels of cAMP were normal in cells transfected with any of the four mutants selected (see basal/cholera toxin response ratio in Table III). These results also show that the two mutants that lengthen the $t_{1/2}$ of internalization (SS12A and V519A, cf. Table I) display a rightward shift in the $EC_{50}$ whereas the two mutants that shorten the $t_{1/2}$ of internalization (F515A and T521A) display a leftward shift in the $EC_{50}$. The maximal cAMP response to hCG was also reduced in cells expressing the F515A or T521A mutants (see hCG/cholera toxin response ratio in Table III). These results are also somewhat different than those reported for mutations of the rLHR (36). The four equivalent mutations of the rLHR mutations were reported to increase the $EC_{50}$ and to have little or no effect on the maximal cAMP response.

### Table II

| Receptor | Apparent $K_d$ (nM) | Maximal binding (molecules/cell) |
|----------|---------------------|---------------------------------|
| wt       | 2.2 ± 0.6           | 188,000 ± 16,000                |
| S512A    | 8.5 ± 2.3*          | 172,000 ± 31,000                |
| F515A    | 4.3 ± 1.5           | 188,000 ± 32,000                |
| V519A    | 1.1 ± 0.2           | 141,000 ± 16,000                |
| T521A    | 1.2 ± 0.2           | 188,000 ± 47,000                |

*Statistically different ($p < 0.05$) from hLHR-wt.

### Table III

| Receptor | Basal cAMP (pmol/10^6 cells) | hCG-induced response | Maximal chola toxin-induced response | Response ratio |
|----------|-------------------------------|-----------------------|-------------------------------------|----------------|
|          | $EC_{50}$ (pmol cAMP/10^6 cells) | Maximal | $EC_{50}$ (pmol cAMP/10^6 cells) | Basal/cholera toxin | hCG/cholera toxin |
| wt       | 33 ± 5 | 104 ± 2 | 695 ± 53 | 189 ± 32 | 0.18 ± 0.04 | 3.57 ± 0.29 |
| S512A    | 30 ± 5 | 312 ± 23* | 655 ± 96 | 174 ± 8 | 0.17 ± 0.03 | 3.38 ± 0.67 |
| F515A    | 37 ± 4 | 70 ± 5* | 483 ± 56 | 212 ± 24 | 0.17 ± 0.02 | 2.21 ± 0.02* |
| V519A    | 30 ± 5 | 278 ± 18* | 745 ± 107 | 191 ± 9 | 0.16 ± 0.03 | 3.37 ± 0.28 |
| T521A    | 38 ± 7 | 70 ± 2* | 544 ± 36 | 201 ± 20 | 0.19 ± 0.02 | 2.64 ± 0.11* |

*Statistically different ($p < 0.05$) from hLHR-wt.

### DISCUSSION

Studies performed with rhodopsin as well as the adrenergic and muscarinic receptors have shown that the GRK-catalyzed phosphorylation of these GPCRs is dependent on receptor activation (1–3) and that arrestin binding is in turn exquisitely dependent on the GRK-catalyzed phosphorylation of these GPCRs (37–39). One would thus predict that the agonist-induced internalization of GPCRs that are internalized by a nonvisual arrestin-dependent pathway would also be dependent on receptor activation and phosphorylation. Such dependence can be readily demonstrated with the $\beta_2$-adrenergic ($\beta_2$AR) and the $\mu$-opioid receptors. Thus, mutation of the phosphorylation sites of the $\beta_2$AR inhibits agonist-induced internalization 2–3-fold and an activation impaired mutant (Y326A in the transmembrane helix-7) inhibits agonist-induced internalization 3–6-fold (40, 41). In transfected cells, the agonist-induced phosphorylation and internalization of $\beta_2$AR-Y326A are decreased by ~90% but they can both be rescued by cotransfection with GRK2, provided that the phosphorylation sites are intact (41). These results suggest that the agonist-provoked phosphorylation of the $\beta_2$AR is more important than receptor activation in the process of internalization. More recent studies (42, 43) have also nicely documented the importance of phosphorylation on the agonist-induced internalization of the $\mu$-opioid receptor. When expressed in transfected cells, the $\mu$-opioid receptor becomes phosphorylated and internalized upon exposure to etorphine, but not upon exposure to morphine even though both compounds are full agonists that can activate the...
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μ-opioid receptor to the same extent. In this case, again, the phosphorylation and internalization of the morphine-activated μ-opioid receptor can be induced by cotransfection with GRK2.

Unlike the studies summarized above, however, our recent work with the hLHR suggests that the agonist-induced activation and the phosphorylation of this GPCR play a redundant role in internalization (17). This conclusion was based on the following findings. First, an activation-competent but phosphorylation-impaired mutant of the hLHR (constructed by mutation of the phosphorylation sites) lengthens the $t_{1/2}$ of internalization of the hLHR less than 2-fold. Second, three different activation-impaired mutants of the hLHR with intact phosphorylation sites are resistant to agonist-induced phosphorylation and lengthen the $t_{1/2}$ of internalization 5–7-fold. Third, the already long $t_{1/2}$ of internalization of these activation-impaired mutants cannot be further lengthened by mutation of their phosphorylation sites. Fourth, the phosphorylation of these activation-impaired mutants and the long $t_{1/2}$ of internalization can be rescued by overexpression of GRK2 but only if the phosphorylation sites are intact. It appears then that, although the agonist-induced internalization of the hLHR is readily impaired by mutations that impair both activation and phosphorylation, the agonist-induced internalization of the LHR is minimally impaired when activation is intact and phosphorylation is impaired or when activation is impaired and phosphorylation is intact.

The studies presented herein characterize a novel class of mutations of second extracellular loop residues of the hLHR that continue to emphasize a remarkable correlation between the state of activation of the LHR and the rate of agonist internalization (17, 44–46) but de-emphasize the importance of receptor phosphorylation in this process. We show that two mutations of the second extracellular loop that impair receptor activation (S512A and V519A) also impair internalization (Table I and III). In contrast to all previously described signaling-impaired mutations that have been studied in detail (D405N, Y546F, and I625K, all of which are in the transmembrane helices; see Ref. 17), the S512A and V519A mutants reported here are the first signaling-imparing mutations of the hLHR that impair internalization without affecting receptor phosphorylation (Fig. 2). The differential effect of these two classes of mutations on receptor phosphorylation may be related to the extent of impairment in signaling. The second extracellular loop mutants result in only a 2–3-fold rightward shift in the $EC_{50}$ for cAMP accumulation (see Table III), whereas the previously examined transmembrane mutants result in a 10–50-fold rightward shift in this $EC_{50}$ (17, 47).

Despite the milder effects of the extracellular loop mutants on activation and the lack of effect on phosphorylation, the extent of impairment in internalization induced by them is similar to that detected with the transmembrane mutants that induce a more drastic change in activation and block phosphorylation (see Table I and Ref. 17). The slow rate of internalization of both sets of mutants also becomes insensitive to inhibition by a dominant-negative mutant of the nonvisual arrestins but can be rescued by overexpression of arrestin-3 (Table I and Ref. 17).

Previous studies have also shown that several distinct mutations of the rLHR or the hLHR (L435R, D578G, D578Y, and D578H, all of which are located in the transmembrane helices) that induce constitutive activity also enhance the internalization of hCG in transfected cells (17, 46, 48). The results presented here show, for the first time, that two mutations of the second extracellular loop that have no effect on basal cAMP levels but enhance receptor activation by hCG (F515A and T521A) also enhance internalization (Tables I and III). As with

The signaling-imparing mutations, the increased internalization of hCG mediated by the F515A and T521A mutants occurs independently of changes in receptor phosphorylation (Fig. 2). Based on these results, we must now conclude that, in contrast to the model derived from the study of the β2-AR and the μ-opioid receptors (see above), for the hLHR the agonist-induced activation is more important than phosphorylation in the process of internalization of this receptor. A similar conclusion may be drawn from recent studies on the FSHR and the parathyroid hormone receptor. In the case of the parathyroid hormone receptor, it has been shown that the agonist-induced phosphorylation of a signaling-impaired mutation can be rescued by cotransfection with GRK2 but such treatment does not rescue the rate of internalization of agonist (49). In the case of the parathyroid hormone receptor, it has been shown that the agonist-induced internalization of this receptor is not affected by mutation of its phosphorylation sites (50).

The emphasis that these results have placed on receptor activation (as opposed to phosphorylation) on the agonist-induced internalization of the hLHR agrees well with recent studies showing that the mutation (17) or removal of the phosphorylation sites of the hLHR have a relatively small effect on internalization (16). They also complement the finding that the rate of internalization of hCG mediated by the hLHR can be drastically reduced by mutation of a few intracellular residues that do not become phosphorylated upon agonist stimulation (16). Finally, whereas we have shown previously that extracellular residues can have a dramatic effect on the rate of internalization of the LHR without affecting receptor activation (18), the studies presented here highlight the importance of a discrete extracellular region of the LHR, the second extracellular loop, in modulating the agonist-induced internalization of the hLHR. As documented here the involvement of this extracellular region in the process of internalization seems to be indirectly mediated by changes in receptor activation. The importance of this region is underscored by the finding that the mutation of just about every residue in this loop either enhances or impairs internalization (Fig. 1B).

Acknowledgments—We thank Dr. Deborah L. Segaloff for critically reading this manuscript, Dr. Jeff Benovic for providing us with the expression vectors for arrestin-3 and arrestin-3 (284–409), and Dr. Sandra Schmid for providing us with a plasmid coding for the hLHR. We gratefully acknowledge the services and facilities provided by the Diabetes and Endocrine Research Center of the University of Iowa (supported by National Institutes Health Grant DK-25295).

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