Seven Non-contiguous Intracellular Residues of the Lutropin/Choriogonadotropin Receptor Dictate the Rate of Agonist-induced Internalization and Its Sensitivity to Non-visual Arrestins*

(Received for publication, September 20, 1999, and in revised form, October 15, 1999)

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The amino acid sequences of the human (h) and rat (r) lutropin/choriogonadotropin receptors (LHR) are 87% identical, but the rate of agonist-induced internalization of the hLHR is ~7 times faster than that of the rLHR. Chimeras of the hLHR and the rLHR showed that this rate is dictated by the serpentine domain and the cytoplasmic tail. Further mutational analysis identified seven residues, two adjacent residues in the second intracellular loop (Val/Gln in the rLHR and Ile/His in the hLHR), four non-contiguous residues in the third intracellular loop (Arg/Gln/Thr/Pro in the rLHR and Lys/Arg/Met/Thr in the hLHR), and one in the C-terminal tail (Leu in the rLHR and Phe in the hLHR), that are necessary and sufficient to impart the slow rate of internalization of the rLHR and the fast rate of internalization of the hLHR. The internalization of the rLHR and the hLHR display different sensitivities to the non-visual arrestins. Therefore, we also tested if the simultaneous exchange of these seven residues resulted in the exchange of this property. Since this was found to be the case, we propose that these seven residues identified here form a non-visual arrestin-binding site.

Agonist binding to G protein-coupled receptors (GPCR) is quickly followed by the internalization of the receptor or the agonist-receptor complex. This process is facilitated by the GPCR phosphorylation that follows agonist-induced activation, and it usually requires the formation of a binary complex between the agonist-activated GPCR and the non-visual arrestins. The non-visual arrestins bind clathrin with high affinity and serve as adaptor molecules that target agonist-activated phosphorylated GPCRs to clathrin-coated pits (1–4).

The LHR is a member of the rhodopsin-like subfamily of GPCRs (5), and the agonist-LHR complex is internalized (6–8) via clathrin-coated pits (9) following agonist-induced activation. Like other GPCRs, the agonist-induced LHR activation and phosphorylation, as well as the interaction of the LHR with a non-visual arrestin, can now be recognized as important steps in agonist-induced internalization of the LHR. Thus, the rate of internalization of the free LHR is slower than the rate of internalization of the agonist-receptor complex (8, 10) and the rate of internalization of a complex formed by the LHR and a weak partial agonist is slower than that of the complex formed by the LHR and a full agonist (11). Likewise, mutations of the LHR that impair signal transduction or induce constitutive activation impair or enhance agonist-induced internalization, respectively (10, 12). On the other hand, mutations of the LHR that do not affect agonist-induced activation, but impair agonist-induced LHR phosphorylation, also impair agonist-induced internalization (13, 14). Finally, the internalization of the agonist-LHR complex can be inhibited by co-transfection with dominant-negative mutants of the non-visual arrestins or by co-transfection with a dominant-negative mutant of dynamin (14, 15). Whereas most internalized GPCRs recycle quickly to the plasma membrane (3), the internalized agonist-LHR complex does not (8, 16). This internalized complex traverses the endosomal compartment without dissociation and is routed to the lysosomes in an intact form, where both the ligand and the receptor are ultimately degraded (7, 9). This process of internalization and lysosomal accumulation of the agonist-LHR complex is an essential component of the termination of agonist-stimulated cAMP accumulation (10, 17) and the agonist-induced down-regulation of the LHR (16, 18).

By taking advantage of the finding that the hLHR and rLHR are highly homologous but internalize agonist at different rates, we have constructed and analyzed rLHR/hLHR chimeras, and we have used them as a starting point for the identification of intracellular residues of the LHR that influence internalization. Based on the results obtained with these chimeras we prepared and analyzed additional point mutations and multiple substitutions that ultimately resulted in the identification of seven intracellular residues of the LHR that dictate the rate of agonist-induced internalization and the sensitivity of this rate to the non-visual arrestins.

MATERIALS AND METHODS

Plasmids and Cells—Full-length cDNAs encoding for the hLHR and rLHR (19, 20) were subcloned into pcDNA1/Neo (rLHR) or pcDNA3.1(hLHR) for expression. Six chimeras of these two receptors were constructed by simple subcloning or by using polymerase chain reaction strategies. Their identity was verified by automated DNA sequencing (performed by the DNA core of The Diabetes and Endocrinology Research Center of the University of Iowa). The overall structure of these chimeras and the exact location of the junctions are shown in Fig. 1. In order to name them we adopted a nomenclature in which the LHR was divided into three domains, an N-terminal extracellular domain, a middle serpentine domain, and a C-terminal intracellular domain. The presence of an h or an r in a given position of each chimera indicates the

* This work was supported in part by National Institutes of Health Grant CA-40629 (to M. A.). The services and facilities provided by the Diabetes and Endocrinology Research Center of the University of Iowa were supported by National Institutes of Health Grant DK-25295. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Supported in part by a fellowship from the Lalor Foundation.
‡ Supported in part by a fellowship from the Lalor Foundation.
§ Supported in part by a fellowship from the Lalor Foundation.

The abbreviations used are: GPCR, G protein-coupled receptors; h, human; r, rat; LHR, lutropin/choriogonadotropin receptors; hCG, human choriionic gonadotropin; TM, transmembrane.
Endocytic Motifs in G Protein-coupled Receptors

origin of that region. For example, rrh is a chimera in which the N-terminal extracellular and the serpine domains are derived from the rLHR and the C-terminal cytoplasmic tail is derived from the hLHR. Individual amino acid mutations were accomplished using polymerase chain reaction strategies. Expression vectors for arrestin-2, arrestin-3, and arrestin-2 (219–418) (21) were generously provided by Dr. Jeff Benovic (Thomas Jefferson University). An expression vector for dynamin-K44A (22) was generously provided by Dr. Sandra Schmid (Scripps Research Institute).

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% newborn calf serum, and 50 μg/ml gentamicin, pH 7.4. Transient transfections were done using the calcium phosphate method of Chen and Okayama (23). Cells were plated in 35-mm wells that had been coated with gelatin. The cells were transfected with 1–2 μg of plasmid when 70–80% confluent. After an overnight incubation, the cells were washed and incubated for an additional 24 h prior to use.

Binding, Internalization, and cAMP Assays—The expression of the different receptor constructs was ascertained by measuring the binding of a single, saturating concentration of 125I-hCG to intact cells during a 1-h incubation at room temperature. All binding assays were corrected for nonspecific binding which was measured in the presence of 50 IU/ml of partially purified hCG (3,000 IU/mg).

To measure the internalization of 125I-hCG have been described (15, 24). 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 endocytic rate constant (k_i) was calculated from the slope of the line obtained by plotting the internalized radioactivity against the integral of the surface-bound radioactivity (8, 24–27). The half-time of internalization (t_1/2) is defined as 0.693/k_i.

Hormonal responsiveness was assessed by measuring cAMP accumulation in intact cells. Total cAMP was measured at the end of a 15-min incubation (37 °C) with maximally effective concentrations of hCG (100 ng/ml) or at the end of a 2-h incubation with a maximally effective concentration of cholera toxin (500 ng/ml) as described elsewhere (10, 12–14, 28, 29).

Hormones and Supplies—Purified hCG (CR-127, ~13,000 IU/mg) was kindly provided by the National Hormone and Pituitary Agency of the NIDDK, National Institutes of Health. 125I-hCG was prepared as described elsewhere (30). Partially purified hCG (~3,000 IU/mg) was purchased from Sigma, and it was used only for the determination of nonspecific binding (see above). 125I-hAMP and cell culture medium were obtained from the Iodination Core and the Media and Cell Production Core, respectively, of the Diabetes and Endocrinology Research Center of the University of Iowa. Other cell culture supplies and reagents were obtained from Corning Glass and Life Technologies, Inc., respectively. All other chemicals were obtained from commonly used suppliers.

RESULTS

Signaling Properties and Internalization of hCG Mediated by rLHR/hLHR Chimeras—In a recent study we attempted to define intracellular regions of the LHR that participate in endocytosis by analyzing chimeras of the rLHR and the highly related rat follitropin receptor (24). Since such chimeras revealed a dramatic influence of the extracellular domain on the rate of internalization (24), their usefulness in identifying intracellular residues that participate in endocytosis is limited. We thus hypothesized that the use of rLHR/hLHR chimeras would provide a better starting point for this analysis for several reasons. First, whereas the rFSHr/hLHR chimeras used previously bound different ligands (i.e., hCG or FSH) depending on the origin of their extracellular domains (24), all rLHR/hLHR chimeras would bind the same ligand (i.e., hCG). Second, the rates of internalization of hCG by the hLHR and rLHR differed by a factor of ~7 (see below). Finally, there is a high degree of amino acid sequence identity between these two receptors, ~90% in the N-terminal extracellular domain and the serpine domain and ~70% in the C-terminal cytoplasmic tail (19, 20).

We thus prepared six chimeras of the rLHR and the hLHR as shown in Fig. 1. Exchanging only the C-terminal cytoplasmic domains produced rrh and hrr; exchanging only the serpine domains produced rhr and hrh; and exchanging the serpine and C-terminal cytoplasmic domains produced rhh and hrr. The identity of the six rLHR/hLHR chimeras was confirmed by sequencing (see “Materials and Methods”), and their hormone binding and signaling properties were assessed in transiently transfected 293 cells. The apparent K_d for 125I-hCG binding to the hLHR and the rLHR are ~2 and ~3 μM, respectively, and all three chimeras containing the extracellular domain of the hLHR bound hCG with an apparent K_d comparable to that of the hLHR (~2 μM). Only two of the three chimeras containing the extracellular domain of the rLHR (rhr and rhh) bound hCG with an apparent K_d comparable to that of the rLHR (~3 μM), however. The rrh chimera bound hCG with an apparent K_d (~1 μM) that was higher than that of the rLHR but lower than that of the hLHR.

The signaling properties and ligand-induced internalization of the rLHR/hLHR chimeras were analyzed in transiently transfected 293 cells expressing an equivalent amount of each chimera (as measured by binding of hCG, see column labeled Ligand-bound in Table I). This was accomplished by varying the amount of plasmid transfected. The basal and hCG-induced cAMP response mediated by the different chimeras was measured in cells incubated with buffer only or with a maximally effective concentration of hCG, and the results obtained were corrected by normalization to an internal control obtained by measuring the cAMP response of the transfected cells to cholera toxin (see column labeled Response Ratio in Table I). These data show that all chimeras display a low basal level of cAMP and respond well to hCG.

The internalization of 125I-hCG mediated by the different chimeras was also analyzed using 293 cells transiently transfected with the levels of plasmid known to result in equivalent receptor expression. Like the transferrin and EGF-receptor complexes (25, 26, 31), the 125I-hCG-LHR complex remains associated during internalization (7, 9), and internalization of the complex can be conveniently assessed by following the distribution of the bound 125I-hCG between the cell surface and the interior of the cell (see “Materials and Methods”). The results presented in Table I show that grafting only the C-terminal domain of the rLHR onto the hLHR (i.e. the hrh chimera), only the serpine domain (i.e. the hrh chimera), or both domains (i.e. the hrh chimera) lengthens the t_1/2 of internalization mediated by the hLHR toward that of the rLHR and that the combined substitution of both domains is more effective than their individual substitution. Conversely, the hrr chimera internalized hCG with a t_1/2 that was ~2-fold longer than that of the rLHR. Conversely, grafting only the C-terminal domain of the rLHR onto the rLHR (i.e. the rrh chimera), only the serpine domain (i.e. the rrh chimera), or both domains (i.e. the rrh chimera) shortens the t_1/2 of internalization of the rLHR toward that of the hLHR. Again, the combined substitution of both domains is more effective than their individual substitution (Table I). In parallel with the data obtained with the hrr chimera (see above), the rrh chimera internalized hCG with a t_1/2 that was ~2-fold shorter than that of the hLHR.

Although the chimeras described above do not fully reproduce the rates of internalization of hCG mediated by the rLHR and the hLHR, they do show that both the serpine and the C-terminal intracellular domains of this receptor affect the rate of internalization.

Further Identification of Intracellular Residues of the LHR That Affect Internalization—A comparison of the amino acid sequences of the serpine and C-terminal intracellular domains of the rLHR and hLHR is presented in Fig. 2. This alignment shows that 289 of the 333 residues located in these
regions are identical between the LHR from these two species. Of the 44 amino acid residue differences in these regions of the rLHR and hLHR, only 28 (shown in bold in Fig. 2) are predicted to be located intracellularly. The remaining 16 are located in the transmembrane (TM) helices (2 in TM-I, 2 in TM-III, 5 in TM-IV, 3 in TM-V, and 2 in TM-VII) or in extracellular loop two (2 residues), which is located between TM-IV and TM-V. Since the aim of this study was to identify intracellular regions of the LHR that participate in endocytosis, the mutants described below were designed to probe the involvement of the 28 non-conserved intracellular residues of the rLHR and hLHR.

Eighteen of the 28 non-conserved residues are located in the extreme C-terminal tail of the LHR (cf. Fig. 2), and their involvement in the internalization of hCG was tested by analyzing two C-terminal truncations of the hLHR and the rLHR that remove these residues. Two C-terminal truncations of the rLHR (at residues 643 or 664) that remove some or all of these 18 non-conserved residues (cf. Fig. 2) shortened the \( t_{1/2} \) of internalization of the rLHR minimally, from 122 ± 6 min to 113 ± 5 and 108 ± 4 min, respectively. Similar C-terminal truncations of the hLHR (at residues 684 and 663, cf. Fig. 2) also shortened the \( t_{1/2} \) of internalization of the hLHR minimally.
from 18 ± 1 min to 14 ± 1 and 10 ± 1, respectively. Thus it appears that the 18 non-conserved residues eliminated by these truncations do not contribute much to the difference in the rates of internalization of hCG mediated by the rLHR and hLHR.

The remaining 10 non-conserved residues present in the intracellular regions are located in the second intracellular loop located between TM-III and TM-IV (2 residues), the third intracellular loop located between TM-V and TM-VI (4 residues), and the proximal region of the C-terminal tail (4 residues). In the next series of experiments we chose to examine the involvement of the non-conserved residues present in the second and third intracellular loops by mutating the hLHR residues to those present in the rLHR and vice versa. The data presented in Fig. 3 show that exchanging the two non-conserved residues present in the second intracellular loop or the four non-conserved residues present in the third intracellular loop of the rLHR for corresponding hLHR residues shortens the half-time of internalization of the rLHR (i.e. the rLHR(h2) and rLHR(h3) mutants in Fig. 3A), whereas exchanging these two residues of the hLHR for the rLHR residues lengthens the half-time of internalization of the hLHR (i.e. the hLHR(r2) and hLHR(r3) mutants in Fig. 3A).

In the experiments presented in Fig. 3A we included one additional pair of mutants that exchanged an L/F pair that is present in the second and third intracellular loops by mutating the hLHR residue (F) also shortens the half-time of internalization of the rLHR (i.e. the rLHR(L/F) mutant in Fig. 3A), whereas exchanging the hLHR residue (L) for the rLHR residue (F) also lengthens the half-time of internalization of the rLHR (i.e. the rLHR(L/F) mutant in Fig. 3B). The data presented in Fig. 3 also show that a combination of these three exchanges fully imposes the slow rate of internalization of the rLHR into the hLHR (i.e. the hLHR(r2 + r3 + F/L) mutant in Fig. 3B).

Although combinations of only the h2/r2 + h3/r3 exchanges were not tested, combinations of only the r3 + F/L and h3 + L/F exchanges were found to be less effective than the combination of the three exchanges shown in Fig. 3 (data not shown). Finally, it should be noted that due to the results presented in Fig. 3 the involvement of three additional residues present in the C-terminal tail (Ala/Thr, Arg/Lys, and Glu/Asp; cf. Fig. 2) on the agonist-induced internalization of the LHR was not tested.

We conclude that only 7 (shown in bold and shaded in Fig. 2) of the 28 intracellular amino acid residues that are different between the rLHR and the hLHR control the rate of internalization of agonist mediated by these two receptors.

**Effects of Non-visual Arrestins on the Internalization of the LHR** — As is the case with the agonist-induced internalization of many other GPCRs, the internalization of hCG mediated by the rLHR can be inhibited by co-transfection with dominant-negative mutants of the non-visual arrestins or by co-transfection with dominant-negative mutants of dynamin (14–16). The dynamin construct used here (designated dynamin-K44A) behaves as a dominant-negative mutant because it is deficient in GTPase activity (22). The dominant-negative mutant of the non-visual arrestins used here, designated arrestin-2-(319–418), is an arrestin-2 construct that lacks the receptor-binding domain but retains the clathrin-binding domain (21). In addition, the internalization of hCG mediated by the rLHR can be enhanced by co-transfection with arrestin-2 or arrestin-3, but at maximally effective plasmid concentrations, the magnitude of the effect of arrestin-3 is much more pronounced than that of arrestin-2 (16, 24).

The data presented in Table II show a comparison of the effects of co-transfection of the non-visual arrestins, a dominant-negative mutant of the non-visual arrestins, and a dominant-negative mutant of dynamin on the internalization of hCG mediated by the rLHR and the hLHR. In agreement with
rLHR(h2) refers to a construct in which Val450 and Gln451 in the second intracellular loop of the rLHR were mutated to the corresponding rat residues (Arg, Gln, Thr, and Pro, respectively). The mutant designated rLHR(L/F) refers to a construct mutated to the corresponding rat residues (Val and Gln, respectively). The mutant designated rLHR(h3) refers to a construct in which Arg526, Gln532, Thr537, and Pro539 in the third intracellular loop of the rLHR were mutated to the corresponding rat residues (Val and Gln, respectively). The mutant designated rLHR(h2) refers to a construct in which Ile472 and His473 in the second intracellular loop of the rLHR were mutated to the corresponding rat residues (Leu). The mutant designated hLHR(r2) refers to a construct in which Ile472 and His473 in the second intracellular loop of the hLHR were mutated to the corresponding human residues (Ile and His, respectively). The mutant designated hLHR(r3) refers to a construct in which Lys548, Arg554, Thr559, and Pro560 in the third intracellular loop of the hLHR were mutated to the corresponding rat residues (Arg, Gln, Thr, and Pro, respectively). The mutant designated hLHR(r2) refers to a construct in which Arg296, Gln302, Thr307, and Pro308 in the third intracellular loop of the hLHR were mutated to the corresponding human residues (Lys, Arg, Met, and Thr, respectively). The mutant designated hLHR(r3) refers to a construct in which Lys298, Arg304, Met305, and Thr309 in the third intracellular loop of the hLHR were mutated to the corresponding rat residues (Arg, Gln, Thr, and Pro, respectively). The mutant designated rLHR(h2) refers to a construct in which Val302, Gln308, Lys313, and Pro314 in the third intracellular loop of the hLHR was mutated to the corresponding human residue (Phe). The mutant designated hLHR(F/L) refers to a construct in which Phe630 in the C-terminal tail of the hLHR was mutated to the corresponding rat residue (Leu). The mutants designated rLHR(h2 + h3 + L/F) and hLHR(r2 + r3 + F/L) harbor a combination of all three mutations. See Fig. 2 for the amino acid sequence alignment of the hLHR and the rLHR. Each value represents the mean ± S.E. of 3–4 independent transfections.

The differential effect of the non-visual arrestins and arrestin-2(319–418) on the internalization of hCG mediated by the rLHR and hLHR allowed us to test further if the amino acid exchanges described above impart a rat “phenotype” to the hLHR and a human phenotype to the rLHR. In fact, the data presented in Table II show that this is indeed the case. Thus, in contrast to rLHR-wt, where arrestin-3 is more efficacious than arrestin-2 in enhancing internalization, the rLHR(h2 + h3 + L/F) mutant behaves like hLHR-wt in that arrestin-2 and arrestin-3 have a comparable effect on the internalization of hCG. Conversely, the hLHR(r2 + r3 + F/L) mutant behaves like rLHR-wt in that arrestin-3 is more efficacious than arrestin-2 in enhancing the internalization of hCG (Table III). Likewise, the effect of arrestin-2(319–418) on the hLHR(r2 + r3 + F/L) mutant more closely resembles the effect of this construct on rLHR than on hLHR. Conversely, the effect of arrestin-2(319–418) on rLHR(h2 + h3 + L/F) more closely resembles the effect of this construct on hLHR than on rLHR (Table II). Finally, co-transfection with dynamin-K44A slowed down the internalization of hCG by all these constructs to a limiting t½ of 200–300 min.

We conclude that the seven residues identified above as being responsible for controlling the rate of internalization of hCG mediated by the rLHR or the hLHR also control the ability of the non-visual arrestins to modulate this process.

**DISCUSSION**

The agonist-induced internalization of most GPCRs is mediated by the non-visual arrestins. These proteins bind clathrin with high affinity (32) and target the agonist-activated and phosphorylated GPCRs to clathrin-coated pits for subsequent internalization (1–4).

Although much has been recently learned about the structural features of the arrestins that participate in GPCR binding and the structural features of the non-visual arrestins that bind clathrin (21, 33, 37), little is known about the structural features of GPCRs that participate in endocytosis and/or bind arrestins. By analogy with what is known about the endocytosis of other membrane proteins (38–42), it is reasonable to propose that the agonist-induced internalization of GPCRs is ultimately mediated by endocytic sorting signals present in their intracellular regions that interact directly with clathrin or indirectly via clathrin-adaptor proteins such as the non-visual arrestins (32) or AP-2 (43). The only GPCR sorting signal that has been identified so far, however, consists of the serine and/or threonine residues that are phosphorylated in response to agonist stimulation. The mutation of such residues often impairs the agonist-induced internalization of GPCRs because their phosphorylation promotes the physical interaction of the GPCRs with clathrin-coated pits for subsequent endocytosis (1–4). Since the binding of the non-visual arrestins to GPCRs requires the participation of other GPCR domains that are not phosphorylated (1, 33, 44), one would predict the presence of non-phosphorylated GPCR residues that also participate in the binding of non-visual arrestins and GPCR internalization. In fact, some mutagenesis studies have suggested the existence of other GPCR endocytic signals that act independently of phosphorylation and/or agonist-induced activation. For example, experiments with progressor C-terminal truncations or receptor chimeras have identified the C-terminal tail of some GPCRs as being particularly important for agonist-induced endocytosis (45–50), but a discrete definition of the endocytic signals present in the C-terminal tail of GPCRs is not available. In addition, mutation of known endocytic signals (41) present in GPCRs have not always been informative. Thus, mutation of a conserved tyrosine-based motif in transmembrane helix seven (TM-VII) of the GPCRs generally impairs agonist-induced in-
293 cells were plated in 35-mm wells and were transiently transfected with plasmid DNA mixtures containing 1 µg of the indicated arrestin or dynamin constructs, different amounts of a given receptor plasmid chosen to give equivalent levels of 125I-hCG binding (i.e., ~1.5 pmol/10^6 cells as shown in Table I), and different amounts of pCDNA3.1 to give a total of 2 µg of plasmid DNA per well. The rate of internalization of hCG was measured 2 days later as described under “Materials and Methods.” Each value represents the mean ± S.E. of 3–15 independent transfections.

<table>
<thead>
<tr>
<th>Plasmid co-transfected</th>
<th>rLHR</th>
<th>hLHR</th>
<th>rLHR</th>
<th>hLHR</th>
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<tbody>
<tr>
<td>pCDNA 3.1</td>
<td>122 ± 19</td>
<td>18 ± 1</td>
<td>158 ± 7</td>
<td>11 ± 1</td>
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<td>Arrestin-2</td>
<td>68 ± 10</td>
<td>8 ± 1</td>
<td>79 ± 7</td>
<td>6 ± 1</td>
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<tr>
<td>Arrestin-3</td>
<td>19 ± 6</td>
<td>5 ± 1</td>
<td>17 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Arrestin-2-(319–418)²</td>
<td>168 ± 10</td>
<td>87 ± 11</td>
<td>239 ± 23</td>
<td>64 ± 5</td>
</tr>
<tr>
<td>Dynamin-K44A³</td>
<td>255 ± 69</td>
<td>202 ± 17</td>
<td>310 ± 27</td>
<td>255 ± 4</td>
</tr>
</tbody>
</table>

² The amounts of plasmid transfected (1 µg/35-mm well) were chosen based on their ability to induce maximal stimulation (arrestin-2 and arrestin-3) or maximal inhibition (arrestin-2-(319–418) and dynamin-K44A) of internalization. The expression of all these products was verified by Western blotting (data not shown) as described elsewhere (16, 58).

ternalization but does so indirectly, by preventing agonist-induced activation rather than by a direct participation in endocytosis (51), whereas mutations of conserved leucine-based motifs in the C-terminal tail of GPCRs have not revealed a consistent involvement of this motif in internalization. For example, the mutation of a leucine-based motif present in the C-terminal tail of the β2-adrenergic receptor impairs agonist-induced internalization (52), and the mutation of the equivalent leucine-based motif of the thromboxane A2 (50) or the rat follicitropin receptor² has no effect on agonist-induced internalization, whereas the mutation of the equivalent leucine-based motif of the rLHR enhances agonist-induced internalization (15).

The experiments described herein were designed to identify intracellular residues of the LHR, a member of the rhodopsin-like subfamily of GPCRs (5), that participate in agonist-induced internalization. In order to accomplish this goal, we utilized chimeras of the LHR derived from two different species (rat and human). The rLHR and the hLHR display an 87% identity in the amino acid sequence of their serpentine and C-terminal regions and bind the same ligand, but they internalize it with very different half-times, ~20 min for the hLHR and ~130 min for the rLHR.

By taking advantage of these properties, we constructed six rLHR/hLHR chimeras that exchanged the major receptor domains (i.e., N-terminal extracellular, middle serpentine, and C-terminal intracellular) while retaining agonist-induced responsiveness (Table I). The results obtained with these chimeras showed that grafting a combination of the serpentine and intracellular domains of the rLHR onto the hLHR imparts a slow rate of internalization on the hLHR, while grafting a combination of the serpentine and intracellular domains of the hLHR onto the rLHR imparts a fast rate of internalization on the rLHR (Table I). Further mutational analysis involving C-terminal truncations (see “Results”) or exchanges of a few amino acids between the hLHR and the rLHR (Fig. 3) resulted in the identification of seven residues that fully account for the slow rate of internalization of the agonist-rLHR complex and the fast rate of internalization of the agonist-hLHR complex. These residues do not represent a linear sequence, however. Two of them are located in the second intracellular loop, four in the third intracellular loop, and one in the C-terminal cytoplasmic tail (Fig. 2). The agonist-induced internalization of the rLHR and the hLHR also differs in their sensitivities to the non-visual arrestins. Thus, overexpression of arrestin-3 is more effective than overexpression of arrestin-2 in enhancing the agonist-induced internalization of the rLHR, whereas a C-terminal construct of arrestin-2 is minimally effective in inhibiting this process (Table II and Refs. 14–16 and 24). In contrast, arrestin-2 or -3 are equally effective in enhancing the agonist-induced internalization of the hLHR and a C-terminal construct of arrestin-2 is very effective in inhibiting this process (Table II). The seven residues that control the rate of internalization of the agonist-rLHR and -hLHR complex also confer this differential sensitivity of the complexes to the non-visual arrestins (Table II).

Taken together these results suggest that the seven non-contiguous residues of the LHR identified here form a site that is involved in the binding of the non-visual arrestins to the LHR. Although a direct interaction of the LHR with the non-visual arrestins has not yet been assessed, such an interaction can be readily inferred by the functional assay (i.e. agonist-induced internalization) used here.

There are at least two primary sites in GPCRs that participate in arrestin binding, a phosphorylation recognition site and an activation recognition site (33–35). The phosphorylation recognition site is composed of the GPCR residues that become phosphorylated by G protein-coupled receptor kinases in response to agonist stimulation, but the activation recognition sites have not been identified. The existence of the activation recognition site is supported by the finding that the non-visual arrestins can bind in vitro to GPCR fragments, such as the third intracellular loop of the m3 muscarinic receptor and the LHR (53, 54) that do not become phosphorylated upon agonist activation. Likewise, synthetic peptides derived from the second and third intracellular loops of rhodopsin can compete for the binding of visual arrestin to light-activated rhodopsin in vitro (44). For the rLHR, the phosphorylation recognition domain is comprised of 4 serine residues located in the C-terminal tail (Ser635, Ser636, Ser649, and Ser652, labeled with an asterisk in Fig. 2) because the simultaneous mutation of these 4 residues drastically impairs the agonist-induced phosphorylation and internalization of the rLHR (13, 14, 55). Further studies utilizing individual mutations of these residues have in fact shown that while all four residues become phosphorylated, only the phosphorylation of Ser635, Ser636, and Ser649 is needed for agonist-induced internalization (14). Although the phosphorylation sites of the hLHR have not been identified, three of the four serine residues that are phosphorylated in the rLHR (Ser635, Ser639 and Ser652) are conserved in the hLHR (cf. Fig. 2). The fourth residue, Ser649 of the rLHR, is substituted by an asparagine in the hLHR, but the adjacent rLHR residue

² H. Kishi and M. Ascoli, unpublished observations.
(Ala) is substituted by a serine in the hLHR (cf. Fig. 2). Thus it is likely that, similar to the phosphorylation recognition site of the rLHR, the phosphorylation recognition site of the hLHR is located in this portion of the C-terminal tail. Since none of the seven non-contiguous residues identified here are phosphate acceptors, and they are located in regions of the LHR that do not become phosphorylated (i.e. the second and third intracellular loops and the proximal portion of the C-terminal tail), they may represent the activation-recognition site of the LHR that participates in arrestin binding.

Our proposal for the involvement of the second and third intracellular loops of the LHR in arrestin binding is in agreement with the finding that peptides corresponding to the equivalent intracellular loops of rhodopsin can compete for the binding of visual arrestin to light-activated rhodopsin in vitro (44). This proposal is also in agreement with the finding that peptides corresponding to the third intracellular loop of several GPCRs, including the porcine LHR, bind arrestin-2 and/or -3 in vitro (53, 54). Finally, although the binding of arrestin-2 to a synthetic peptide derived from the second intracellular loop of the porcine LHR could not be demonstrated (54), such a finding is not necessarily in conflict with our proposal because the low binding affinity of arrestins to receptor fragments (53) makes these interactions difficult to detect.

The influence of the L/F pair present on the C-terminal tail of the LHR (cf. Fig. 2) on the rate of agonist-induced internalization (Fig. 3) has been noted before (15). This finding is interesting because these residues are in a hydrophobic stretch of the LHR containing a leucine-based endocytic signal that is known to participate in the clathrin-mediated endocytosis of several membrane proteins presumably by a direct interaction of this motif with AP-2, another clathrin adaptor (38–42). Moreover a recent report by Laporte et al. demonstrated that the non-visual arrestins can interact directly with the β2-subunit of AP-2 and that this interaction leads to the formation of an agonist-induced multimeric complex containing the β2-adrenergic receptor, a non-visual arrestin, and AP-2. Thus, the involvement of the L/F pair in the agonist-induced internalization of the LHR could reflect the involvement of these residues in the association of the LHR with a non-visual arrestin or the interaction of these residues with AP-2.

In summary the results presented here have identified an endocytic motif composed of a non-linear sequence of 7 intracellular residues of the LHR and suggest that this motif participates in the formation of a complex between the LHR and the non-visual arrestins.

Acknowledgments—We thank Dr. Deborah L. Segall for critically reading this manuscript; Dr. Jeff Benovic for providing us with the expression vectors for arrestin-2, arrestin-3, and arrestin-2 (319–418); and Dr. Sandra Schmid for providing us with the expression vector for the porcine LHR. We also thank Ares Serono for providing us with a plasmid coding for the hLHR.
Seven Non-contiguous Intracellular Residues of the Lutropin/Choriogonadotropin Receptor Dictate the Rate of Agonist-induced Internalization and Its Sensitivity to Non-visual Arrestins
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doi: 10.1074/jbc.275.1.241

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