The Rate of Internalization of the Gonadotropin Receptors Is Greatly Affected by the Origin of the Extracellular Domain

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Previous results from this laboratory have shown that human kidney (293) cells transfected with the rat folliculin receptor (rFSHR) internalize agonist (i.e. human folliculin, hFSH) at a rate similar to that of other agonist-G protein-coupled receptor complexes while 293 cells transfected with the rat lutropin/choriogonadotropin receptor (rLHR) internalize agonist (human choriogonadotropin, hCG) at a rate that is about 1 order of magnitude slower. Taking advantage of this difference and the high degree of homology between the rLHR and rFSHR, we have now used chimeras of these two receptors to begin to delineate structural features that influence their internalization.

Analysis of six chimeras that exchanged only the transmembrane domains (designated FLF and LFL), only the COOH-terminal domains (FFL or LLF) or both domains (PLL or LFF) show that the origin of the extracellular domain is at least as important, if not more, than the origin of the transmembrane and COOH-terminal domains in determining the rate of internalization of the gonadotropin receptors. Thus, the rates of internalization of agonist internalization mediated by FFL, FLF, and LFL more closely resemble rFSHR than rLHR, while the rates of agonist internalization mediated by LLF, LFL, and LFF more closely resemble rLHR than rFSHR.

The importance of the extracellular domain was also evident even upon overexpression of arrestin-3, a protein that enhances the rate of internalization of the wild-type receptors and chimeras by binding to their intracellular regions.

Despite the abundance of knowledge about structural motifs that mediate endocytosis of receptors that span the membrane only once (11), little information is available about the structural motifs that mediate the endocytosis of the seven-transmembrane spanning GPCRs. Some studies utilizing C-terminal truncations as well as receptor chimeras have identified the C-terminal tail as being particularly important for endocytosis (12–17), but a discrete definition of these motifs is not yet available. Other studies indicate that a dileucine motif conserved in the C-terminal tail may be involved in the endocytosis of some (18) but not other GPCRs (17). A NPXY motif is also conserved in transmembrane helix seven of the GPCRs. Although mutations of this motif impair agonist-induced internalization they do so indirectly, by preventing the agonist-induced activation rather than by a direct participation in endocytosis (1).

The FSHR, LHR, and thyrotropin receptors, collectively known as the glycoprotein hormone receptors, make up a small subfamily of the large family of rhodopsin-like G GPCRs (19–21). They are among the largest members of this family (~700 residues long), and about half of these residues are located extracellularly. The extracellular amino-terminal domains of the glycoprotein hormone receptors are encoded by multiple exons and are responsible for the recognition and high affinity binding of their large (28–38 kDa) glycoprotein hormone ligands. Their transmembrane and cytoplasmic tails are encoded by a single exon and are involved in signal transduction. Amino acid sequence alignments among the glycoprotein hormone receptors from the same species (19, 20) reveal a substantial degree of overall identity (40–50%). As expected from their roles, there is a smaller degree of identity in the extracellular domains (~40%) than in the transmembrane domains (60–75%). Surprisingly, however, the COOH-terminal domains are the most divergent, displaying an amino acid sequence identity of only 20–30%.

Like other GPCRs the binding of agonists to the gonadotropin receptors (LHR or FSHR) triggers the endocytosis of the agonist-receptor complex by a pathway that is dependent on a nonvisual arrestin, clathrin, and dynamin (9, 22–30). Once internalized the agonist-LHR complex accumulates in the lysosomes, where both the agonist and the receptor are degraded (24–26). The fate of the internalized FSHR has not been investigated, but it is clear that the internalized FSHR also accumulates in the lysosomes where it is eventually degraded to single amino acids (22, 23).

In a recent series of experiments using a standardized assay to measure the internalization of the rLHR and rFSFR expressed in transfected 293 cells, we have reported that the agonist-rFSFR complex is internalized with a t1/2 of ~10 min (27, 28), while the agonist-rLHR complex is internalized with a t1/2 of ~100 min (5, 9, 29, 31, 32). In the present study we have analyzed six chimeras of the rFSFR and rLHR to begin to...
identify regions of these receptors that influence their rates of internalization.

MATERIALS AND METHODS

Plasmids and Cells—Full-length cDNAs encoding for the rFSHR and rLHR (33, 34) were subcloned into the pcDNAI/Neo (Invitrogen) for expression. Six chimeras of these two receptors were constructed using PCR 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 each receptor was subdivided into three domains, NH2-terminal extracellular, transmembrane, and COOH-terminal intracellular, and the presence of an L or and F in a given position of each chimera indicates the origin of that region. For example, LLF is a chimera in which the NH2-terminal extracellular and the transmembrane domains are derived from the rLHR and the COOH-terminal intracellular domain is derived from the rFSHR.

An expression vector for arrestin-3 (35) was generously provided by Dr. Jeff Benovic (Thomas Jefferson University).

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% new born calf serum and 50 μg/ml gentamicin, pH 7.4. Transient transfections were done using the calcium phosphate method of Chen and Okayama (36). Cells were plated in 100-mm dishes and transfected with 10 μg of plasmid when 70–80% confluent. After an overnight incubation the cells were washed, trypsinized, and replated in 35-mm wells (5–10 cells/well), and used 24 h later.

Internalization Assays—The endocytosis of 125I-hCG and 125I-hFSH were measured as follows (37–39). Cells (plated in 35-mm wells) were preincubated in 1 ml of Waymouth’s MB752/1 containing 1 mg/ml bovine serum albumin and 20 mM Hepes, pH 7.4, for 30–60 min at 37 °C. Each well then received 10 ng/ml 125I-hCG or 40 ng/ml 125I-hFSH (these concentrations are equivalent to the K0 for the hCG–rLHR and the hFSH–rFSHR interaction, respectively), and the incubation was continued at 37 °C. At the times indicated cells were placed on ice and washed two to three times with 2-ml aliquots of cold Hanks’ balanced salt solution containing 1 mg/ml bovine serum albumin. The surface-bound hormone was then released by incubating the cells in 1 ml of cold 50 mM glycine, 150 mM NaCl, pH 3, for 2–4 min (24). This buffer was removed and the cells were washed once more. The acid buffer washes were combined and counted, and the cells were solubilized with 100 μl of 0.5 N NaOH, collected with a cotton swab, and counted to determine the amount of internalized hormone.

Determinations of the rates of internalization were done using at least five different data points collected at 3–10-min intervals (depending on the chimera used). The endocytotic rate constant (k1) was calculated from the slope of the line obtained by plotting the internalized radioactivity against the integral of the surface-bound radioactivity (37–40). The half-life of internalization (t1/2) is defined as 0.693/k1.

Hormones and Supplies—Purified hFSH (AFP-5720D), hCG (CR-127), and pregnant mare serum gonadotropin were kindly provided by the National Hormone and Pituitary Agency of the National Institute of Diabetes and Digestive and Kidney Diseases. 125I-hFSH and 125I-hCG were prepared as described elsewhere (41). 127I-cAMP 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 and Life Technologies, Inc., respectively. All other chemicals were obtained from commonly used suppliers.

RESULTS

Construction and Signaling Properties of Gonadotropin Receptor Chimeras—Six chimeras of the rLHR and the rFSHR were constructed as shown in Fig. 1. Exchanging only the COOH-terminal cytoplasmic domains produced LLF and FFL; exchanging only the transmembrane domains produced LFF and FLL; and exchanging the transmembrane and COOH-terminal cytoplasmic domains produced LFL.

The identity of the rLHR/rFSHR chimeras was confirmed by sequencing (see “Materials and Methods”) and by measuring their ability to bind hCG and hFSH and to respond to them with increased cAMP accumulation (Table I), because these properties should be entirely dependent on the origin of the extracellular domain (42, 43). In these experiments the levels...
of cell surface expression of the different chimeras containing the extracellular domains of the rLHR or the rFSHR were matched (as measured by binding of their respective agonists, see column labeled “Ligand-bound” in Table I), and the cAMP responses mediated by the chimeras were corrected by normalizing the data to an internal control obtained by measuring the cAMP response of the transfected cells to cholera toxin (see methods used here (5, 29, 31, 32). Degradation products of 125I-hCG begin to be released within 45 min (data not shown), and the longest time point used for the experiments utilizing 125I-hFSH was 18 min (Fig. 2D). As shown in Fig. 2, E and F, this time course is about twice as long as the 1/2 of internalization of the 125I-hFSH.

The internalization of 125I-hCG and 125I-hFSH mediated by the different chimeras was next analyzed using transiently transfected 293 cells. In contrast to the agonist-induced cAMP responses, which are dependent on receptor density, the rate of internalization of agonist was found to be independent of receptor density (data not shown, also see “Discussion”). In the experiments presented in Fig. 3, however, the levels of cell surface expression of the different chimeras containing the extracellular domains of the rLHR or the rFSHR were matched as described for the experiments presented in Table I. The internalization data summarized in Fig. 3 are grouped based on the origin of the extracellular domain, because all chimeras containing the extracellular domain of the rFSHR showed a substantial reduction in this response when compared with rLHR. The cAMP response to hFSH was minimal in all the chimeras containing the extracellular domain of the rLHR.

Internalization of hFSH and hCG—When 293 cells transiently transfected with the rLHR are exposed to 125I-hCG they internalize the bound hormone slowly (Fig. 2, top panels) while cells transiently transfected with the rFSHR internalize the bound 125I-hFSH quickly (Fig. 2, bottom panels). Using these experimental conditions one can calculate an endocytic rate constant (kε) from the slopes of two kinds of plots as shown in Fig. 2, B and E (39), or in Fig. 2, C and F (37, 38, 40). In either case the half-life of internalization (t1/2) is defined as 0.693/kε. While both plots give similar results (compare Fig. 2, B and C, and Fig. 2, E and F), we routinely used plots like those shown in Fig. 2, C and E, as opposed to those shown in Fig. 2, B and E, to calculate rates of internalization because they tended to be linear with more data points, and the results obtained were more reproducible.

In doing these experiments care must be taken to avoid the loss of the internalized ligand (which occurs as consequence of its degradation and subsequent release of degradation products into the medium) during the time course of measurement (39, 40). Since degradation products of 125I-hCG begin to be released within 90 min (24, 31), the longest time point used to estimate the t1/2 of internalization of 125I-hCG was 60 min (see Fig. 2A). As shown in Fig. 2, B and C, the t1/2 of internalization of 125I-hCG is ~100 min, and since the 60-min time course used is shorter than the calculated t1/2 of internalization, it can be argued that using this method to estimate the t1/2 of internalization may yield erroneous results. We don't believe that is the case, however, because measurements of the rate of internalization of 125I-hCG in 293 cells transfected with the rLHR using other methods that follow the fate of the bound 125I-hCG for up to 4 h have resulted in estimates of the t1/2 of internalization that are similar (i.e. 80–140 min) to those obtained with the method used here (5, 29, 31, 32). Degradation products of 125I-hFSH begin to be released within 45 min (data not shown), and the longest time point used for the experiments utilizing 125I-hFSH was 18 min (Fig. 2D). As shown in Fig. 2, E and F, this time course is about twice as long as the t1/2 of internalization of the 125I-hFSH.

TABLE I

| Receptor | Ligand | Ligand-bound | cAMP | Response ratio |
|----------|--------|--------------|------|---------------|
|          | ng/10^6 cells | pmol/10^6 cells | Buffer | hFSH (100 ng/ml) | hCG (100 ng/ml) | Cholera toxin (500 ng/ml) | |
| rFSHR    | 125I-hFSH | 4.4 ± 0.5 | 4 ± 3 | 540 ± 62 | 8 ± 1 | 1340 ± 234 | 0.49 ± 0.04 |
| FFL      | 125I-hFSH | 3.3 ± 0.4 | 4 ± 1 | 365 ± 31 | 13 ± 1 | 1102 ± 207 | 0.32 ± 0.03 |
| PLL      | 125I-hFSH | 3.8 ± 0.5 | 4 ± 2 | 431 ± 35 | 17 ± 7 | 1150 ± 197 | 0.40 ± 0.05 |
| FLL      | 125I-hFSH | 4.8 ± 0.6 | 2 ± 1 | 517 ± 68 | 13 ± 5 | 1001 ± 69 | 0.50 ± 0.09 |
| rLHR     | 125I-hCG  | 1.2 ± 0.2 | 3 ± 1 | 7 ± 2 | 313 ± 66 | 1001 ± 96 | 0.26 ± 0.07 |
| LFF      | 125I-hCG  | 1.2 ± 0.2 | 2 ± 1 | 3 ± 3 | 52 ± 3 | 1175 ± 89 | 0.04 ± 0.003 |
| LFL      | 125I-hCG  | 0.9 ± 0.2 | 2 ± 1 | 2 ± 2 | 113 ± 26 | 690 ± 22 | 0.16 ± 0.04 |
| LFF      | 125I-hCG  | 0.9 ± 0.2 | 2 ± 0 | 3 ± 1 | 79 ± 23 | 840 ± 98 | 0.09 ± 0.02 |

a Indicates statistically significant difference (p < 0.05) from rFSHR.
b Indicates statistically significant differences (p < 0.05) from rLHR.
nalization. The combined substitution of both of these domains (i.e. LFF) was only slightly more effective in shortening the $t_{1/2}$ of internalization than the individual substitution of the transmembrane domain, however.

Previous results from this laboratory have shown that overexpression of arrestin-3 enhances the internalization of agonists mediated by the rLHR or the rFSHR and that the magnitude of this effect is more pronounced with $^{125}$I-hCG than with $^{125}$I-hFSH (28–30). This phenomenon is illustrated by the results presented in Table II. Overexpression of arrestin-3 shortens the $t_{1/2}$ of internalization of $^{125}$I-hFSH mediated by rFSHR less than 2-fold, but it shortens the $t_{1/2}$ of internalization of $^{125}$I-hCG mediated by rLHR ~4-fold. Since the arrestins are known to bind to the intracellular regions of GPCRs (reviewed in Ref. 2), we expected that the overexpression of arrestin-3 would have a greater effect on the internalization of agonist mediated by FLL than on the internalization of agonist mediated by LFF. As shown in Table II this was in fact found to be the case. Arrestin-3 shortened the $t_{1/2}$ of internalization of agonist mediated by FLL and by LFF ~7- and ~4-fold, respectively. Even in cells cotransfected with arrestin-3, the $t_{1/2}$ of internalization of agonist mediated by LFF was till ~4-fold longer than the $t_{1/2}$ of agonist internalization mediated by FLL, however.

Thus, all results presented show that the origin of the extracellular domain has a substantial effect on the rate of internalization of the gonadotropin receptors.

**DISCUSSION**

Six chimeras of the rLHR and rFSHR were used in experiments designed to determine the structural basis of the different rates of agonist internalization mediated by the gonadotropin receptors.

Results on the specificity of gonadotropin responsiveness show that the abilities of the different chimeras to recognize and respond to a given ligand (i.e. hCG or hFSH) are entirely dictated by the origin of the extracellular domain. Thus, all rLHR/rFSHR chimeras that have the extracellular domain of the rLHR display a robust cAMP response to hCG and a minimal response to hFSH, while those that have the extracellular domain of the rFSHR display a robust cAMP response to hFSH and a minimal response to hCG (Table I).
sented here were conducted using transiently transfected cells with matched receptor density. It should be emphasized, however, that the rates of internalization of agonist mediated by the rLHR or the rFSHR are independent of receptor density and of the mode of transfection (i.e. transient versus stable transfections). Previous experiments from this laboratory have shown that the $t_{1/2}$ of internalization of 125I-hFSH is 10 min in clonal lines of 293 cells stably expressing the rFSHR at a density of 40,000 to 400,000 molecules/cell (27, 28). The experiments reported here for the rFSHR were done using transiently transfected 293 cells expressing an average density of 40,000 molecules/cell and the $t_{1/2}$ of internalization of 125I-hFSH was also 10 min (Figs. 2 and 3 and Table I). Increasing the expression of the rFSHR to 400,000 molecules/cell did not affect the rate of internalization, however. Likewise, the $t_{1/2}$ of internalization of 125I-hFSH mediated by the rLHR is 100 min in clonal lines of 293 cells stably expressing the rLHR at a density of 3,000–200,000 molecules/cell (5, 29, 31, 32). This value compares well with the $t_{1/2}$ of internalization of 125I-hCG mediated by the rLHR reported here (Figs. 2 and 3 and Table II) for transiently transfected 293 cells expressing rLHR at an average density of 40,000 molecules/cell.

Like many other GPCRs (2, 3), the agonist-induced activa-

tion of the LHR and FSHR are necessary for the efficient endocytosis of the agonist-receptor complex. Thus, the rate of internalization of the hCG-LHR complex is faster than that of the free LHR (5, 37); the rate of internalization of an antagonist-LHR complex is slower than that of the agonist-LHR complex (44); signaling impairing mutations of the LHR (5, 31) or the FSHR (28) decrease internalization of the agonist-receptor complex; and activating mutations of the LHR enhance the internalization of the free and/or agonist-occupied receptor (5).

Cyclic AMP accumulation per se is not needed for internalization, however, because the slow rate of internalization of the antagonist-LHR complex is not enhanced by the addition of cAMP analogs, and the internalization of the hCG-LHR complex is not impaired in cells that express a cAMP-resistant phenotype (44). Likewise, the internalization of the hFSH-rFSHR complex is also not impaired in cells that express a cAMP-resistant phenotype.2

All the chimeras used here are capable of being activated by their appropriate agonists, as judged by cAMP accumulation (Table I). While their ability to become activated in response to agonist binding may vary, there is no correlation between this parameter and the rate of internalization of agonist. If the extent of agonist-induced activation was an important determinant of the rate of internalization of a given chimera one would expect that the chimeras that do not respond well with an increase in cAMP accumulation would display a long $t_{1/2}$ of internalization. This is certainly not the case, as shown by the finding that the $t_{1/2}$ of internalization of agonist mediated by FFL and FLL are both longer than the $t_{1/2}$ of internalization of agonist mediated by rFSHR (Fig. 3), yet only the agonist-induced activation of FFL is reduced compared with that of rFSHR (Table I). Likewise, the $t_{1/2}$ of internalization of agonist mediated by LLF is similar to that of rLHR (Fig. 3), but the agonist-induced activation of LFF is substantially lower than that of rLHR (Table I). Last, the $t_{1/2}$ values of internalization of agonist mediated by LFL and LLF are both shorter than that of rLHR (Fig. 3), but the agonist-induced activation of LFL is similar to that of rLHR, while the agonist-induced activation of LFF is less than that of rLHR (Table I).

One reason why there is a correlation between the agonist-

induced activation and internalization of some GPCRs is that the agonist-activated GPCRs are better substrates for the G protein-coupled receptor kinases (GRKs) than the free recep-

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2 M. Ascoli, unpublished observations.

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**TABLE II**

**Effect of arrestin-3 on the internalization of agonist mediated by selected rFSHR/rLHR chimeras**

| Chimera | Half-life of internalization $t_{1/2}$ (min) |
|---------|---------------------------------------------|
| rFSHR  | $12 \pm 1$ | $7 \pm 2^a$ |
| FLL    | $28 \pm 1$ | $4 \pm 1^a$ |
| rLHR   | $107 \pm 12$ | $24 \pm 4^a$ |
| LFF    | $67 \pm 6$ | $16 \pm 4^a$ |

*a Indicate statistically significant differences ($p < 0.05$) from the same construct without arrestin-3.
tors. The GRK-phosphorylated GPCRs in turn have a higher affinity for a family of proteins called arrestins and some of these (i.e. the nonvisual arrestins) are known to act as adapter molecules bridging the phosphorylated GPCR to clathrin for subsequent endocytosis via coated pits (2, 3). This general pathway applies to the gonadotropin receptors as illustrated by the following findings. First, dominant-negative mutants of GRK2, nonvisual arrestins or dynamin inhibit internalization (28–30). Second, cotransfections with arrestin-3 or several members of the GRK family (i.e. GRK2, GRK4α, or GRK6) enhance the internalization of agonists mediated by the rLHR and/or rFSHR (28–30). Last, the slow internalization of hFSHR mediated by signaling-impaired mutants of the FSHR can be rescued by cotransfection with arrestin-3 (28).

Since agonist-induced activation promotes the phosphorylation of serine residues present at the COOH-terminal cytoplasmic tail of the rLHR (29, 32) or serine and threonine residues present in the first and third intracellular loops of the rFSHR (27, 28), and the arrestin binding sites of GPCRs have been mapped to intracellular regions (2), we predicted that the structural determinants for endoeyosis of the gonadotropin receptors must reside in their intracellular loops and/or COOH-terminal cytoplasmic tails. The analysis of the six rLHR/rFSHR chimera show indeed that the transmembrane and/or cytoplasmic domains of these receptors affect the rate of internalization (Fig. 3). It appears that the cytoplasmic tail of the rLHR is more important for internalization than the transmembrane domain because the FLF chimera internalized agonist at about the same rate as the rFSHR, while the FFL chimera internalized agonist slower than the rFSHR. Some kind of interaction between these two domains of the rLHR may occur during internalization, however, because grafting the cytoplasmic and transmembrane domains of the rLHR onto the extracellular domain of the rFSHR resulted in a chimera, FLL that internalized agonist at a slower rate than FFL or FLF. In contrast, the transmembrane domain of the rFSHR seems to be more important for internalization than the cytoplasmic tail because the LLF chimera internalized agonist at about the same rate as rLHR, while the LFL chimera internalized agonist at a slightly faster rate than rLHR wild type. Moreover, the combined substitution of both of these domains resulted in a chimera, FLF that internalized agonist at about the same rate as the chimera containing only the transmembrane domain.

While these findings provide a necessary first step in defining structural motifs that modulate the rate of internalization of agonist mediated by the gonadotropin receptors, the most interesting and surprising finding reported here is that the extracellular domain is at least as important, if not more, in determining the rate of agonist internalization. Thus, while comparing the rLHR to that of rFSHR (−7 min) than to that of LFF (−16 min). Conversely, the t1/2 of agonist internalization mediated by LFF (−16 min) is closer to that of rFSHR (−7 min) than to that of LLF (−24 min) than to that mediated by rFSHR (−7 min). These findings contrast those of previous studies that have shown that the structural features of other GPCRs that determine the rate of endocytosis are located in their COOH-terminal and transmembrane domains (13, 15, 16).

In summary, the results presented here underscore the uniqueness of the process of agonist-induced activation and agonist-induced internalization of the two gonadotropin receptors (and perhaps the three glycoprotein hormone receptors in general) when compared with other GPCRs and argue for the existence of a major conformational change that starts with the binding of agonist to the extracellular domain and is relayed to the transmembrane and cytoplasmic domains. Interactions among these domains have been previously proposed to explain signal transduction by the glycoprotein hormone receptors (21, 45). The data presented here clearly show that such interactions are equally important in determining the fate of the agonist-activated receptor. Our data also provide the basis for additional mutagenesis experiments designed to define structural motifs present in the intracellular regions of these receptors that affect internalization.

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