Multiple Distant Amino Acid Residues Present in the Serpentine Region of the Follitropin Receptor Modulate the Rate of Agonist-induced Internalization*

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The amino acid sequences of the human (h) and rat (r) follitropin receptors (FSHR) are ~89% identical, but the half-time of internalization of agonist mediated by the rFSHR is ~3 times faster than that of the hFSHR. Chimeras of the hFSHR and the rFSHR showed that this difference in rate is dictated mostly by the serpentine domain. Further analysis identified six residues, two non-contiguous residues in the transmembrane helix 4 (Leu/Thr in the rFSHR and Met/Ile in the hFSHR), three non-contiguous residues in the third intracellular loop (Thr/Thr/Lys in the rFSHR and Ile/Asn/Arg in the hFSHR), and one in transmembrane helix 7 (Tyr in the rFSHR and His in the hFSHR) that are fully responsible for the difference in the rates of internalization of the hFSHR and the rFSHR.

Like many other GPCRs, the agonist-induced activation of the glycoprotein hormone receptors results in the internalization of the agonist-receptor complex via clathrin-coated pits (10, 11) by a pathway that requires dynamin (12–17). The follitropin, lutropin, and thyrotropin receptors (FSHR, LHR, and TSHR, respectively) are members of the rhodopsin/β₃-adrenergic-like subfamily of GPCRs (1, 2). They form a small sub-family 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 (3–5). 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 (6–9).

Like many other GPCRs, the agonist-induced activation of the glycoprotein hormone receptors results in the internalization of the agonist-receptor complex via clathrin-coated pits (10, 11) by a pathway that requires dynamin (12–17). The agonist-induced activation and phosphorylation of these receptors as well as their interaction with a non-visual arrestin can stand the structural features of these receptors that participate in internalization and trafficking. For example, studies utilizing LHR/TSHR chimeras have shown that the serpentine and intracellular domains of these receptors contain structural features that determine their routing to the recycling versus the degradation pathways (11). Studies utilizing LHR/TSHR and LHR/FSHR chimeras have also shown that the extracellular domains play a role in the rate and/or the extent of internalization (11, 15). Last, our analysis of chimeras of the hLHR and rLHR resulted in the identification of seven non-contiguous, non-phosphorylated intracellular residues of the LHR that dictate the rate of internalization of agonist mediated by the LHR from these two species (17). In the studies summarized herein we used chimeras of the rFSHR and hFSHR to identify amino acid residues that dictate the rate of internalization of this receptor. Surprisingly there is no overlap in the identity of the residues that dictate the internalization of the LHR and the FSHR, and there is little overlap in the topological location of these residues.

MATERIALS AND METHODS

Plasmids and Cells—Full-length cDNAs encoding for the hFSHR (19) and rFSHR (20) were subcloned into pCDNA 3.1 or pCDNAI/Neo, respectively. Six chimeras of these two receptors were constructed by taking advantage of convenient restriction sites or by using conventional PCR strategies. Individual amino acid mutations were done using PCR strategies. The identities of all chimeras and mutants were verified by automated DNA sequencing (performed by the DNA core of The Diabetes and Endocrinology Research Center of the University of Iowa). The six initial chimeras were constructed by exchanging the three principal receptor domains (the N-terminal extracellular domain, the middle serpentine domain, and the C-terminal intracellular domain), and the presence of an h or an r in a given position of each chimera indicates the origin of that region as shown in Fig. 1. For example, (rhh)F is a chimera in which the N-terminal extracellular domain was derived from the rFSHR, and the serpentine and C-terminal domains were derived from the hFSHR. The exact boundaries of the three major receptor domains used to prepare the chimeras are shown in Figs. 1 and 2.

Expression vectors for arrestin-2 and arrestin-3 (21) were generously provided by Dr. Jeff Benovic (Thomas Jefferson University). The expression of the encoded proteins has been previously documented (14). 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. Transient transfections were done using the calcium phosphate method of Chen and Okayama (22).
Cells were plated in 35-mm wells that had been coated with gelatin and transfected when 70–80% confluent. After overnight incubation, the cells were washed and incubated for an additional 24 h before use.

Binding, Internalization, and cAMP Assays—Cells were transfected using different amounts of expression vectors (0.06–1 μg) chosen to give equivalent receptor expression as described in the tables and figures. In preliminary experiments we found that there was not necessary to include an empty vector in the transfection mixtures to balance the total amount of DNA transfected. Thus, this was not done in most of the experiments presented. The expression of the different chimeras and mutants was ascertained by measuring the binding of a saturating concentration of 125I-hFSH (∼30 nM) 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 an excess of equine FSH as described before (13, 15). Because intact cells internalize and degrade hFSH, the binding reactions needed to quantitate receptor numbers should formally be done at 4 °C (to prevent internalization and degradation). The reliability of the binding data at this reduced temperature is questionable, however, because the affinity of hFSH for the rFSHR and hFSH is reduced substantially at 4 °C, and the nonspecific binding often accounts for more than 50% of the total binding obtained. Thus, the conditions used here for these assays were chosen as a compromise to preserve the high binding affinity and to slow down the internalization and degradation of the internalized hFSH.

Determinations of the rates of internalization of 125I-hFSH were done using at least five different data points collected at 4-min intervals after the addition of 125I-hFSH as described elsewhere (15, 23). The endocytotic rate constant (k_e) was calculated from the slope of the line obtained by plotting the internalized radioactivity against the integral of the surface-bound radioactivity (15, 23, 24). The half-time of internalization (t_{1/2}) is defined as 0.693/k_e.

Hormonal responsiveness was assessed by measuring cAMP accumulation in intact cells. Total cAMP was measured at the end of a 2-h incubation (37 °C) with a maximally effective concentration of hFSH (3 nM) or a maximally effective concentration of cholera toxin (0.6 nM) as described elsewhere (13, 17, 23).

Hormones and Supplies—Purified hFSH (AFP-5720D, prepared from human pituitaries) was kindly provided by Dr. A. Parlow and the National Hormone and Pituitary Agency of the NIDDK, National Institutes of Health, and purified recombinant hFSH (in this study we used both preparations, and the results were indistinguishable) was provided by Ares Serono (Randolph, MA). Partially purified equine FSH was kindly donated by Dr. George Bousfield (Wichita State University). 125I-hFSH was prepared as described previously (25). The 125I-cAMP used for the radiomimunoassays was prepared by the Iodination Core of the Diabetes and Endocrinology Research Center. 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

Six chimeras of the rFSHR and the hFSHR were prepared by exchanging the different domains shown in Fig. 1. Exchanging only the C-terminal cytoplasmic domains produced (rhr)F and (hrr)F, exchanging only the serpentine domains produced (rrh)F and (hrh)F, and exchanging the serpentine and C-terminal cytoplasmic domains produced (rhr)F and (hrh)F.

Preliminary experiments designed to measure the binding affinity and capacity of 293 cells transiently transfected with the rFSHR-wt or the hFSHR-wt showed that cells transfected with either receptor-bound hFSH with the same apparent affinity (K_d ∼ 1–3 nM, data not shown). When the amount of plasmid transfected was kept constant, however, the maximal binding capacity of cells transfected with the rFSHR-wt was 5–10-fold higher than that of cells transfected with the hFSHR-wt (data not shown). Thus, in all the experiments described below we attempted to equalize the expression of the different chimeras and mutants by transfecting the cells with different amounts of plasmids (as described under “Materials and Methods”) chosen to result in equivalent receptor expression (as measured by the binding of 125I-hFSH to intact cells, see “Materials and Methods”). In doing these experiments we considered a 2-fold difference in receptor expression to be acceptable, because in cells transiently transfected with the rFSHR-wt or hFSHR-wt such variations in receptor density have little or no effect on hFSH responsiveness as measured by cAMP accumulation (data not shown). Moreover, the rate of internalization of hFSH mediated by the rFSHR-wt is the same in transiently or stably transfected 293 cells or in stably transfected 293 cells expressing vastly different densities of receptors (13, 15, 23, 26).

The signaling properties and ligand-induced internalization of the FSHR chimeras expressed in transiently transfected 293 cells is shown in Table I. The basal and hFSH-induced cAMP response was measured in cells incubated with buffer only or with a maximally effective concentration of hFSH (3 nM), and the results obtained were corrected by normalization to an internal control obtained by measuring the cAMP response of the transfected cells to a maximally effective concentration (0.6 nM) of cholera toxin (see column labeled “Response ratio” in Table I). These data show that all chimeras display a low basal

![Endocytic Motifs in the Glycoprotein Hormone Receptors](Image)

FIG. 1. Structure of the rFSHR/hFSHR receptor chimeras. The overall structure of the rFSHR and the hFSHR are shown in dark and light gray, respectively. Chimeras were constructed by exchanging one or two of the three major receptor domains (N-terminal extracellular, middle serpentine, and C-terminal intracellular) as indicated. Note that the mature rFSHR and hFSHR are 675 and 677 residues long, respectively. The apparent gap shown in each junction is artificially caused by differences in the numbering of amino acids as explained in the legend to Fig. 2.
293 cells were plated in 35-mm wells and transiently transfected with the indicated plasmids (amounts used in μg are indicated in parentheses). 125I-hFSH binding and the t½ of internalization were measured as described under “Materials and Methods.” Total cAMP accumulation was measured during a 2-h incubation with buffer only or the indicated concentrations of hFSH or cholera toxin as described under “Materials and Methods.” A response ratio was calculated for each experiment by dividing the hFSH-induced response by the cholera toxin-induced response. Each value represents the mean ± S.E. of 3–4 independent transfections.

### TABLE I

**Properties of FSH receptor chimeras**

| Plasmid transfected | 125I-hFSH bound | t½ of internalization | cAMP | Cholera toxin (0.6 nM) | Response ratio |
|---------------------|-----------------|-----------------------|------|----------------------|---------------|
| Plasmid transfected | fmol/10^6 cells | min                   | pmol/10^6 cells | Response ratio |
| rFSHR-wt (0.20)    | 158 ± 22        | 14 ± 1                | 9 ± 2 | 197 ± 26           | 2.5 ± 0.3     |
| (rhF)F (0.50)      | 171 ± 9         | 12 ± 2                | 10 ± 2 | 149 ± 30           | 1.7 ± 0.1     |
| (rrh)F (0.06)      | 161 ± 47        | 73 ± 1                | 9 ± 1  | 187 ± 14           | 2.0 ± 0.4     |
| (rhr)F (0.08)      | 146 ± 31        | 51 ± 2                | 7 ± 1  | 152 ± 6            | 1.9 ± 0.4     |
| hFSHR-wt (1.00)    | 115 ± 3         | 43 ± 3                | 10 ± 1 | 164 ± 21           | 2.6 ± 0.1     |
| (hhr)F (0.30)      | 133 ± 37        | 74 ± 3                | 10 ± 1 | 192 ± 13           | 2.6 ± 0.5     |
| (hhr)F (1.00)      | 90 ± 9          | 13 ± 1                | 12 ± 2 | 99 ± 21            | 1.3 ± 0.1     |
| (hhr)F (0.50)      | 112 ± 6         | 22 ± 1                | 10 ± 1 | 164 ± 21           | 2.1 ± 0.4     |

The potential lack of contribution of the divergent residues in the C-terminal tail to the differences in the rate of agonist internalization mediated by the rFSHR-wt and hFSHR-wt suggested by the chimeras (cf. Table I) was independently confirmed by comparing the behavior of C-terminal deletions of the two receptor species (Fig. 3). A truncation of the C-terminal tail of the FSHR at residue 659 or the hFSHR at residue 677 (see the dashed vertical lines in Fig. 2 for the exact location) had little or no effect on the rate of agonist internalization, whereas truncations at residue 644 of the rFSHR or residue 662 of the hFSHR (see the dashed vertical lines in Fig. 2 for the exact location) enhanced the rate of internalization of hFSH mediated by either receptor. Thus, whereas the C-terminal tail of the FSHR does contribute to the rate internalization of agonist, its role is the same in both receptor species, and we can safely conclude that the divergent residues removed by these truncations of the C-terminal tail of the FSHR do not contribute to the differences in the rate of agonist internalization mediated by the rFSHR-wt and hFSHR-wt. The reasons behind the enhanced internalization of truncated forms of the hFSHR and rFSHR were not examined further in these studies.

A potential involvement of the 12 divergent residues present in the serpentine domain was examined by first comparing 14 additional mutants in which the divergent residues present in each of the seven divergent receptor regions (i.e. TM1, TM2, TM4, TM5, TM6, and TM7, see bold residues marked with asterisks in Fig. 2) were exchanged as discrete groups between the two receptor species. For example, in the rFSHR-hTM1/ hFSHR-rTM1 pair we exchanged the three divergent residues in TM1 rFSHR (i.e. T-TV in the rFSHR and I-I/I) in the hFSHR, see Fig. 2); in the rFSHR-hiL2/hFSHR-ril2 pair we exchanged the single divergent residue in iL2 (i.e. Glu in the rFSHR and Asp in the hFSHR, see Fig. 2) and so on.

The internalization of hFSH mediated by each of these 14 exchange mutants was analyzed, and a given receptor region was considered to contribute to the difference in the t½ of internalization only if its exchange had an opposite effect on the rate of internalization of the rFSHR and hFSHR. Based on this analysis (Fig. 4), we concluded that TM4, iL3, and TM7 contained important residues. Thus, when these regions of the hFSHR were grafted onto the rFSHR, they shortened the t½ of internalization, and when the same regions of the rFSHR were grafted onto the hFSHR, they shortened the t½ of internalization (see gray bars in Fig. 4). We then prepared an additional pair of mutants, designated rFSHR(hTM4+hiL3+hTM7) and hFSHR(rTM4+ril3+rTM7) in which the three regions identified above were simultaneously exchanged between the hFSHR and the rFSHR. As shown by the black bars in Fig. 4, the exchange of these regions fully transformed the short t½ of
internalization of agonist mediated by the rFSHR into the long 
1/2 of agonist internalization mediated by the hFSHR and

vice versa.

Since the 1/2 of internalization of agonist mediated by the
rFSHR is lengthened by mutations of the rFSHR that impair
activation (13) and since the serpentine region of GPCRs is
important for receptor activation (1, 2, 13), we also tested some
of the exchange mutants described above for their ability to
stimulate cAMP accumulation. The results of these experi-
ments are summarized in Table II and show that most of the
mutations in question had little or no effect on the ability of the
hFSHR or rFSHR to stimulate cAMP accumulation. Cells ex-
pressing the hFSHR-rTM7 mutant did display an impairment
in hFSH-induced cAMP accumulation. This impairment in sig-
naling is unlikely to be responsible for the effect of the hFSHR-
rTM7 mutation on internalization because this mutant inter-
nalizes hFSH faster than hFSHR-wt, and impairments
in signaling lengthen rather than shorten the half-times of
internalization (13).

The rFSHR is an unusual member of the GPCR family in
that agonist-induced stimulation results in the phosphoryla-
tion of S/T residues present in the first and third intracellular
loops (23, 27). Since in the rFSHR-hiL3 a T/T/K group was
substituted for an I/N/R group (cf. Fig. 2), such a substitution
could impair receptor phosphorylation and internalization.
Previous studies from this laboratory have shown, however,
that despite the impairment in agonist-induced phosphoryla-
tion resulting from the mutation of all Ser and Thr residues
present in iL3 of the rFSHR, such a mutant internalizes hFSH
with a 1/2 that is shorter than that of rFSHR-wt (23). Thus, the
phosphorylation state of the rFSHR-hiL3 mutant was not ex-
amined because the lengthening of the 1/2 of internalization of

![Fig. 2. Amino acid sequence alignment of the rat and human FSHR and overall structure of the rFSHR/hFSHR chimeras.](image)

The amino acid sequences of the rFSHR and hFSHR were taken from Sprengel et al. (20) and Minegishi et al. (19), respectively. The complete sequence of the rFSHR is shown using the single letter amino acid code. A dash under a given FSHR residue indicates that the corresponding hFSHR residue is identical. Residues that are not identical are identified with the appropriate single letter code. Dots indicate gaps introduced for optimal alignment. The transmembrane regions are labeled TM1-TM7, and the three intracellular and extracellular loops are labeled iL1-iL3 and eL1-eL3, respectively. The boundaries of these regions were taken from the most recent models for the rhodopsin/\(b_2\)-adrenergic receptor subfamily of GPCRs (53) and the hLHR (54, 55). The boundaries of the extracellular and serpentine domains used in the construction of the six chimeras shown in Fig. 1 are labeled by the brackets, and the location of the C-terminal truncations used in Fig. 3 are labeled by the vertical dashed lines. The divergent amino acids present in the serpentine or C-terminal domains are shown in bold and marked with an asterisk. The residues of this group that are also shaded are the six residues identified here as the determinants of the different rates of internalization of hFSH mediated by the rFSHR and the hFSHR.

![Fig. 3. Half-times of internalization of 125I-hFSH by C-terminal truncations of the rFSHR and hFSHR.](image)

293 cells were plated in 35-mm wells and were transiently transfected with the indicated plasmids (see Fig. 2 for the exact nature of the residues removed by the indicated truncations). The rFSHR plasmids were transfected at 0.2 \(\mu\)g/well, and the hFSHR plasmids were transfected at 1 \(\mu\)g/well to give equivalent receptor expression (cf. Table I), and the 1/2 of internalization of 125I-hFSH was measured as described under “Materials and Methods.” Each value bar represents the mean \(\pm\) S.E. of 3–4 independent transfections.

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Fig. 4. Half-times of internalization of $^{125}$I-hFSH by selected exchange mutants of the rFSHR and the hFSHR. 293 cells were plated in 35-mm wells and were transiently transfected with the indicated plasmids. The amounts of plasmid transfected were chosen to give equivalent levels of $^{125}$I-hFSH binding as shown in Table II, and the $t_{1/2}$ of internalization of $^{125}$I-hFSH was measured as described under "Materials and Methods." The mutant designated rFSHR-hTM1 refers to a construct in which Thr$^{360}$, Thr$^{365}$, and Val$^{369}$ in TM1 of the rFSHR were simultaneously mutated to the corresponding human residues (Ile, Ile, and Ile, respectively). The mutant designated hFSHR-rTM1 refers to a construct in which Ile$^{381}$, Ile$^{382}$, and Ile$^{386}$ of the hFSHR were simultaneously mutated to the corresponding human residues (Ile, Ile, and Ile, respectively). The mutant designated hFSHR-rTM1 refers to a construct in which His$^{614}$ of the hFSHR was mutated to the corresponding human residue (His). The mutant designated hFSHR-rTM7 refers to a construct in which Tyr$^{597}$ of the rFSHR was mutated to the corresponding human residue (Thr). The mutant designated rFSHR-hTM7 refers to a construct in which Met$^{575}$ of the hFSHR was mutated to the corresponding human residue (Met). The mutant designated hFSHR-hTM7 refers to a construct in which Thr$^{549}$, Thr$^{554}$, and Lys$^{563}$ of the hFSHR were simultaneously mutated to the corresponding rat residues (Thr, Thr, and Lys, respectively). The mutant designated rFSHR-hTM6 refers to a construct in which Met$^{545}$ and Ile$^{546}$ of the rFSHR was mutated to the corresponding human residue (Met and Ile, respectively). The mutant designated rFSHR-hiL3 refers to a construct in which Ile$^{381}$, Ile$^{382}$, and Ile$^{386}$ of the hFSHR were simultaneously mutated to the corresponding rat residues (Thr, Thr, and Val, respectively). The mutant designated rFSHR-hiL3 refers to a construct in which Thr$^{532}$, Thr$^{542}$, and Lys$^{551}$ of the rFSHR were simultaneously mutated to the corresponding rat residues (Thr, Thr, and Lys, respectively). The mutant designated hFSHR-riL3 refers to a construct in which Ser$^{532}$ of the rFSHR was mutated to the corresponding human residue (Ser). The mutant designated hFSHR-riL3 refers to a construct in which Ser$^{532}$ of the rFSHR was mutated to the corresponding human residue (Ser). The mutant designated rFSHR-riL3 refers to a construct in which Thr$^{532}$, Thr$^{542}$, and Lys$^{551}$ of the rFSHR were simultaneously mutated to the corresponding human residues (Thr, Thr, and Lys, respectively). The mutant designated rFSHR-rTM5 refers to a construct in which Asp$^{477}$ of the hFSHR was mutated to the corresponding human residue (Asp). The mutant designated hFSHR-rTM5 refers to a construct in which Glu$^{460}$ of the hFSHR was mutated to the corresponding human residue (Asp). The mutant designated hFSHR-riL2 refers to a construct in which Asp$^{477}$ of the hFSHR was mutated to the corresponding human residue (Asp). The mutant designated hFSHR-riL2 refers to a construct in which Asp$^{477}$ of the hFSHR was mutated to the corresponding human residue (Asp). The mutant designated rFSHR-hTM5 refers to a construct in which Asp$^{477}$ of the hFSHR was mutated to the corresponding human residue (Asp). The mutant designated hFSHR-rTM5 refers to a construct in which Asp$^{477}$ of the hFSHR was mutated to the corresponding human residue (Asp). The mutant designated hFSHR-riL2 refers to a construct in which Asp$^{477}$ of the hFSHR was mutated to the corresponding human residue (Asp). The mutant designated hFSHR-riL2 refers to a construct in which Asp$^{477}$ of the hFSHR was mutated to the corresponding human residue (Asp).

DISCUSSION

The internalization of GPCRs is a ubiquitous response to agonist-induced activation that occurs within the same time frame as the activation of G proteins and their cognate effectors (reviewed in Refs. 28–32). Although the agonist-induced internalization of GPCRs can occur by several distinct pathways (28, 33), the best understood internalization pathway is one that involves clathrin-coated pits. The GPCRs that are internalized by this pathway are targeted to clathrin-coated pits via adaptor proteins such as the non-visual arrestins (31, 32, 34), and the fission of the coated pits into coated vesicles requires the participation of dynamin (29). Since the agonist-induced internalization of the LHR and the FSHR is sensitive to inhibition by dominant-negative mutants of dynamin or by dominant-negative mutants of the non-visual arrestins (13, 15–17, 26), we can readily conclude that they are internalized by the same, non-visual arrestin and dynamin-sensitive pathway. The involvement of clathrin-coated pits in the agonist-induced internalization of the LHR has also been formally demonstrated (10).

Although much has been learned recently about the adaptor proteins that target GPCRs to clathrin-coated pits, less is known about the structural features of GPCRs that participate in endocytosis. By analogy with what is known about the endocytosis of other membrane proteins (34, 36–39), we have proposed (17) that the agonist-induced internalization of GPCRs via the non-visual arrestin/clathrin/dynamin-dependent pathway is ultimately mediated by discrete GPCR motifs that interact with clathrin-adaptor proteins such as the non-visual arrestins (40) or AP-2 (41). Serine and/or threonine residues present in the intracellular regions of GPCRs that become phosphorylated upon agonist stimulation would represent such a motif because the phosphorylation of these residues enhances the formation of the GPCR-non-visual arrestin complex (42, 43), and the mutation of these residues often impairs the agonist-induced internalization of GPCRs (29–32). Clearly, however, the formation of the GPCR/non-visual arrestin complex requires the participation of additional intracellular GPCR residues that are not phosphate acceptors (42–45), and the involvement of several intracellular non-phosphorylatable residues in the agonist-induced internalization of GPCRs can be readily documented by mutagenesis (see Refs. 16 and 46–48).

We have recently devised an approach that exploits the high degree of amino acid sequence identity of the LHR and FSHR and their divergent rates of internalization (15, 17) to identify...
in intracellular residues of these receptors that participate in agonist-induced internalization. Our initial studies utilized chimeras of the rLHR and rFSHR and highlighted a dominant role for the extracellular domain of these receptors on the rate of internalization (15). Although these results were novel and interesting, they proved to be of limited value for the identification of intracellular GPCR residues that affect internalization. The use of chimeras of the LHR derived from two different species (rat and human), however, proved to be a more useful approach, and it eventually resulted in the identification of seven non-contiguous intracellular residues of the LHR that dictate the rate of agonist-induced internalization and its sensitivity to non-visual arrestins (17). The experiments presented herein took advantage of the same approach to identify intracellular residues of the FSHR that dictate the rate of agonist-induced internalization of this receptor and its sensitivity to non-visual arrestins.

The results obtained with the initial six chimeras and with the truncations of the C-terminal tail (Table I and Fig. 3) showed that the difference in the rates of internalization of the rFSHR and the hFSHR is dictated mostly by the serpentine region. Further mutational analysis involving exchanges of clusters of divergent residues resulted in the identification of two non-contiguous residues present in TM4, three non-contiguous residues present in iL3, and one residue present in TM7 that fully account for the slow rate of internalization of hFSH mediated by the hFSHR and the fast rate mediated by the rFSHR (Figs. 2 and 4). The internalization of hFSH mediated by the rFSHR and hFSHR also differ in their sensitivities to the non-visual arrestins. Overexpression of arrestin-3 is more effective than overexpression of arrestin-2 in enhancing the agonist-induced internalization of the hFSHR, but arrestin-2 or -3 are equally effective in enhancing the agonist-induced internalization of the rFSHR (Table III). The six residues that control the rate of internalization of hFSH-rFSHR or hFSH-hFSHR complexes also confer this differential sensitivity of the complexes to the non-visual arrestins (Table III).

Since all amino acid residues that affect internalization of membrane proteins have been found to be located in the intracellular regions of these proteins (34, 36–39), we expected that only intracellular residues of the FSHR would be involved in dictating the rate of internalization. Only three of the six residues identified here are present in the intracellular regions of the hFSHR (i.e. the T/T/K or I/N/R groups in iL3 of the rFSHR and hFSHR, respectively, see Fig. 2), however. These residues could affect internalization by directly promoting or preventing the interaction of this FSHR with intracellular components of the endocytic machinery such as the non-visual arrestins or AP-2 (34, 36–39). The finding that iL3 residues affect the internalization of the FSHR is in agreement with other reports documenting the involvement of iL3 residues in the internalization of other GPCRs (17, 49–51). Our proposal for the involvement of these residues on arrestin binding is also in agreement with the finding that non-visual arrestins can bind to synthetic peptides corresponding to iL3 of several other GPCRs (44, 45).

The other three residues that affect internalization of the FSHR are located in TM4 or TM7 (see Fig. 2). If these residues are exposed to the membrane environment, they may affect internalization by directly promoting or preventing the interaction of the FSHR with other transmembrane proteins. Conversely, if they are oriented toward other helices or toward the intrahelical space, their effect on internalization may be indirect (i.e. a reflection of a more global conformational change).

Since we have recently identified seven non-contiguous residues that are determinants of the rate of internalization of the highly related LHR (17), a comparison of the results obtained here with those of the LHR are in order. Thus, an alignment of the amino acid sequences of the serpentine and C-terminal tails of the rLHR, hLHR, rFSHR, and hFSHR is shown in Fig. 5. The residues that dictate the different rates of internalization of the rLHR/hLHR and rFSHR/hFSHR pairs are identified

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**Table II**

| Plasmid transfected | 125I-hFSH bound | cAMP |
|----------------------|-----------------|------|
|                      | fmol/10^6 cells | pmol/10^6 cells | Buffer | hFSH (3 nM) | Cholera toxin (0.6 nM) | Response ratio |
| rFSHR-wt (0.15)      | 175 ± 66        | 38 ± 5          | 492 ± 136 | 596 ± 131 | 0.81 ± 0.05 |
| rFSHR-hTM4 (0.10)    | 127 ± 26        | 38 ± 6          | 460 ± 132 | 708 ± 230 | 0.66 ± 0.08 |
| rFSHR-hiL3 (0.12)    | 130 ± 14        | 43 ± 6          | 394 ± 68  | 668 ± 190 | 0.65 ± 0.18 |
| rFSHR-hTM7 (0.20)    | 147 ± 54        | 38 ± 5          | 581 ± 173 | 640 ± 120 | 0.87 ± 0.09 |
| hFSHR-wt (0.50)      | 125 ± 14        | 44 ± 5          | 472 ± 82  | 509 ± 109 | 0.96 ± 0.12 |
| hFSHR-rTM4 (0.50)    | 123 ± 15        | 47 ± 5          | 483 ± 115 | 561 ± 145 | 0.88 ± 0.13 |
| hFSHR-rIL3 (1.00)    | 79 ± 10         | 56 ± 5          | 461 ± 83  | 412 ± 60  | 1.15 ± 0.22 |
| hFSHR-rTM7 (0.20)    | 131 ± 27        | 41 ± 2          | 333 ± 83  | 634 ± 101 | 0.51 ± 0.05 |

**Table III**

| Plasmid co-transfected | rFSHR | hFSHR | rFSHR (hTM4 + hiL3 + hTM7) | hFSHR (rTM4 + rIL3 + rTM7) |
|-------------------------|-------|-------|---------------------------|---------------------------|
|                         |       |       |                           |                           |
| pcDNA 3.1               | 17 ± 1| 48 ± 2| 57 ± 6                    | 13 ± 1                    |
| Arrestin-2              | 8 ± 1 | 17 ± 1| 25 ± 2                    | 6 ± 1                     |
| Arrestin-3              | 6 ± 1 | 8 ± 1 | 10 ± 1                    | 4 ± 0                     |

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Endocytic Motifs in the Glycoprotein Hormone Receptors

293 cells were plated in 35-mm wells and transiently transfected with the indicated plasmids (amounts used, in μg, are indicated in parentheses). ¹²⁵I-hFSH binding and the t½ of internalization were measured as described under "Materials and Methods." Total cAMP accumulation was measured during a 2-h incubation with buffer only or the indicated concentrations of hFSH or cholera toxin as described under "Materials and Methods." A response ratio was calculated for each experiment by dividing the hFSH-induced response by the cholera toxin-induced response. Each value represents the mean ± S.E. of 3–4 independent transfections.
by an asterisk and are shown in bold and shaded in Fig. 5. The residues highlighted in the top two lines are responsible for the different half-times of internalization of hCG mediated by the rLHR and hLHR, and those highlighted in the bottom two lines are responsible for the different half-times of internalization of hFSH mediated by the rFSHR and the hFSHR. This figure shows that the residues that determine the rates of internalization of these highly related GPCRs are not contained in linear sequences and are distributed among five receptor regions, iL2, TM4, iL3, TM7, and the juxtamembrane region of the C-terminal tail. Moreover, these results show that only one of the regions that contributes to internalization, iL3, is common to both receptor pairs. The iL3 residues that contribute to the rate of internalization are not equivalent among the two receptor pairs, however. It is also interesting to note that for the most part, when a given residue affects the internalization of a receptor pair, the same position of the other receptor pair is invariant (Fig. 5). The rat (V-Q) and human (I-H) sequences in iL2 that contribute to the internalization of the LHR are represented by invariant residues (G/L) in the rLHR and hLHR. In iL3, the rat (R/Q/T/P) and human (K/R/M/T) residues that contribute to the internalization of the FSFR are represented by invariant residues (H/R/V/S) in the rFSHR and hFSHR, whereas the rat (T/T/K) and human (I/N/R) residues that contribute to the internalization of the FSFR are represented by invariant residues (I/E/K) in the rLHR and hLHR. Last, the rat (Tyr) and human (His) residues in TM7 that contribute to the internalization of the FSFR is represented by an invariant Tyr in the rLHR and the hLHR; and the rat (Leu) and human (Phe) residues in the C-terminal tail that contribute to the internalization of the LHR are represented by an invariant Phe in the rFSHR and hFSHR.

In summary, the results presented here for the FSHR as well as those previously reported for the LHR (17) and the m2 muscarinic receptor (52) show that the internalization of GPCRs is affected by a number of distant amino acid residues present in distinct topological domains including iL2, iL3, TM4, TM6, and TM7 and/or the C-terminal cytoplasmic tail, with iL3 being the only region common to all three receptors examined in detail. These results appear to stand in contrast to those obtained with receptors that have a single transmembrane-spanning domain whose internalization is dictated by discrete and short linear amino acid sequences (34, 36–39).

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