Ligand Selectivity of Gonadotropin Receptors

ROLE OF THE β-STRANDS OF EXTRACELLULAR LEUINE-RICH REPEATS 3 AND 6 OF THE HUMAN LUTEINIZING HORMONE RECEPTOR

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The difference in hormone selectivity between the human follicle-stimulating hormone receptor (hFSH-R) and human luteinizing hormone/chorionic gonadotropin receptor (hLH-R) is determined by their ~350 amino acid-long N-terminal receptor exodomains that allow the mutually exclusive binding of human follicle-stimulating hormone (hFSH) and human luteinizing hormone (hLH) when these hormones are present in physiological concentrations. The exodomains of each of these receptors consist of a nine-leucine-rich repeat-containing subdomain (LRR subdomain) flanked by N- and C-terminal cysteine-rich subdomains. Chimeric receptors, in which the structural subdomains of the hFSH-R exodomain were substituted with those of the hLH-R, showed a similar high responsiveness to human chorionic gonadotropin (hCG) and hLH as long as they harbored the LRR subdomain of the hLH-R. In addition, these chimeric receptors showed no responsiveness to hFSH. The LRR subdomains of the gonadotropin receptor exodomains are predicted to adopt a horseshoe-like conformation, of which the hormone-binding concave surface is composed of nine parallel β-strands. Receptors in which individual β-strands of the hFSH-R were replaced with the corresponding hLH-R sequences revealed that hCG and hLH selectivity is predominantly determined by hLH-R β-strands 3 and 6. A mutant receptor in which the hFSH-R β-strands 3 and 6 were substituted simultaneously with their hLH-R counterparts displayed a responsiveness to hCG and hLH similar to that of the wild type hLH-R. Responsiveness to hFSH was not affected by most β-strand substitutions, suggesting the involvement of multiple low-impact determinants for this hormone.

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) stimulate the FSH receptor (FSH-R) and LH receptor (LH-R), respectively, which are expressed in different target cells (1). In some species, chorionic gonadotropin (CG) is also able to stimulate the LH-R. The coordinated interplay between the complementary and specific actions of FSH and LH/CG is required to guarantee successful reproduction. The interaction between these gonadotropins and their respective receptors is highly specific, and there is virtually no cross-reactivity between hormones and heterologous receptors (i.e. high receptor selectivity) except for LH and CG, which both act on the LH-R (2, 3).

The FSH-R and LH-R, together with the thyroid-stimulating hormone receptor, represent the structurally unique glycoprotein hormone receptor (GpHR) subfamily of the G protein-coupled receptor superfamily. Characteristically, GpHRs are composed of two approximately equally sized but functionally distinct domains: an extracellular N-terminal half (exodomain), which is responsible for the selective recognition and high-affinity binding of its corresponding hormone, and a typical G protein-coupled receptor domain, consisting of seven transmembrane o-helices, which transduces the specific signal of hormone binding to the exodomain across the membrane to activate intracellular signaling pathways (4). The GpHR exodomain is subdivided into three structural subdomains: an N-terminal cysteine-rich subdomain (NCR subdomain) followed by a nine contiguous imperfect leucine-rich repeat-containing subdomain (LRR subdomain) and a C-terminal cysteine-rich subdomain (CCR subdomain; see Figs. 1 and 2A).

LRR motifs have been recognized in a large variety of proteins, with repeats composed of 20–29 amino acids each (5). Crystal structure analyses of some of these LRR-containing proteins, such as the porcine ribonuclease inhibitor (6) and Listeria internalins (7), revealed that each LRR forms a right-handed structural unit that is composed of a short β-strand and a helical segment, which are positioned nearly antiparallel to each other and are linked by short loops. Tandem arrays of LRR units form a one- to three-quarter donut-like structure with the consecutive β-strands forming a parallel β-sheet at the concave surface, whereas the alternating helices are aligned next to each other to form the convex side of the structure. The concave surface of the ribonuclease inhibitor interacts with ribonucleases using multiple contact points (6).

Based on sequence homology, the exodomains of GpHRs have been structurally modeled using the crystal structure of porcine ribonuclease inhibitor (8–10). Consequently, the β-sheet at the inner circumference of the curved exodomain of GpHRs has been proposed to form the main hormone-binding site, with some additional hormone contact sites situated outside the LRR subdomain (11, 12). Each of the LRR β-strands is composed of a highly conserved X3YX2LX3LX4X5 motif (Fig. 1), in
which $X$ indicates any amino acid and $L$ refers to leucine, isoleucine, or other hydrophobic residues (5). The side chains of the conserved $L$ residues are directed toward the helical segment and form the hydrophobic core of the LRR structure, whereas the side chains of the $X$ residues are exposed to the surface of the presumed ligand-binding site (13–15).

Selective gonadotropin binding is determined exclusively by the exodomain of the LH-R and FSH-R. Studies using chimeric receptors revealed that the sequence NCR-LRR6 of the LH-R is important to confer LH/CG selectivity to the receptor, whereas the sequence NCR-LRR3 of the FSH-R, in conjunction with the FSH-R sequence LRR7-CCR, is important for FSH binding specificity (2, 3). However, these results may be somewhat biased because the junctions of these chimeric proteins were introduced arbitrarily, depending mainly on the presence of common endonuclease restriction sites rather than considering the exact structural borders within the exodomain. Taking advantage of the current knowledge of the structural conformation of the exodomain of GpHRs, we examined in more detail the contribution of the NCR, LRR, and CCR subdomains of the human LH-R (hLH-R) exodomain in conferring human chorionic gonadotropin (hCG) and/or human LH (hLH) selectivity when placed in the context of a human FSH-R (hFSH-R) background. Furthermore, the contribution of individual (and combinations of) hLH-R $\beta$-strands in directing hCG and hLH (hCG/hLH) selectivity to the receptor was studied systematically with mutant receptors generated by introducing hLH-R $\beta$-strands into the hFSH-R.

**EXPERIMENTAL PROCEDURES**

**Glycoprotein Hormones and Antibodies**—Human recombinant FSH (hFSH, Org32489E), human recombinant LH (hLH, 99M7019), and human recombinant chorionic gonadotropin (hCG, 01MZ010) were kindly provided by Dr. W. G. E. J. Schoonen (Organon Inc., Oss, The Netherlands). The high-affinity monoclonal anti-HA antibody and goat anti-rat IgG peroxidase conjugate were purchased from Roche Applied Science and Sigma, respectively.

**Construction of Mutant Receptor cDNAs**—The cDNAs encoding the human FSH-R and LH-R, kindly provided by Dr. T. Minegishi (Gunma University School of Medicine, Maebashi, Japan) and Dr. E. Milgrom (Institut National de la Santé et de la Recherche Médicale, Paris, France), respectively, were subcloned into the pcDNA3.1/V5-His-TOPO expression vector (Invitrogen). To quantify receptor cell surface expression by enzyme-linked immunosorbent assay (ELISA), an HA epitope (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala; derived from the influenza virus hemagglutinin) was introduced between the C termini of the signal peptide sequences and the N termini of the mature FSH-R ([i.e. between Gly17 and Cys18]) and the mature LH-R ([i.e. between Leu29 and Arg30]). HA epitope tagging did not significantly influence the ligand binding and signaling properties of the receptors compared with their wild type counterparts (data not shown).

HA-tagged receptor cDNAs were used as templates to generate hFSH-R and hLH-R exodomain chimeras using a fusion PCR-based...
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Fig. 2. Cell surface expression and ligand-induced cAMP production of wild type hFSH-R, wild type hLH-R, and chimeric hFSH-Rs transiently expressed in HEK-T 293 cells. A, schematic representation of the chimeric receptors (see “Experimental Procedures”). Human FSH-R amino acids are indicated by open circles and hLH-R amino acids by filled circles. Arrows indicate the nine β-strands in each receptor. B, cell surface expression of wild type or chimeric receptors as determined by HA tag ELISA. C and D, human CG- and hFSH-stimulated, cAMP-mediated reporter gene activity in HEK-T 293 cells transiently transfected with wild type or chimeric receptor constructs. The variable levels of basal cAMP signaling are related to different levels of receptor expression. Human LH and hCG stimulated the various (chimeric) receptors with a similar efficacy; for clarity, only the hCG-stimulated, cAMP-mediated reporter gene activity is shown. Results are shown as the mean ± S.E. of triplicate observations from a single representative experiment. Mean EC50 values are presented in Table I.

method (16). Briefly, 5’- and 3’-cDNA fragments were generated using overlapping primers (Invitrogen and Isogen) in combination with specific primers demarcating the cDNA insert. These PCR fragments were then fused in a self-primer PCR taking advantage of the introduced overlapping sequence. The fusion products were then PCR-amplified using the specific primers demarcating the cDNA insert. All PCRs were performed using the Advantage-HF PCR kit (Clontech). Four different exodomain chimeras (Fig. 2A) were generated in which particular hFSH-R domains were substituted with either the entire exodomain of the hLH-R (generating the chimera LLL-hFSH-R), or with the LRR subdomain in combination with either the NCR subdomain or the CCR subdomain of hLH-R (LLF-hFSH-R and FLL-hLH-R), or with only the LRR subdomain in combination with either the NCR subdomain or the CCR subdomain of the hLH-R (generating the chimera LLL-hFSH-R), or with the LRR subdomain of hFSH-R (FLF-hFSH-R). In a similar way, a set of mutant receptors was generated in which the residues indicated with D, human CG- and hFSH-stimulated, cAMP-mediated reporter gene activity in HEK-T 293 cells transiently transfected with wild type or chimeric receptor constructs. The variable levels of basal cAMP signaling are related to different levels of receptor expression. Human LH and hCG stimulated the various (chimeric) receptors with a similar efficacy; for clarity, only the hCG-stimulated, cAMP-mediated reporter gene activity is shown. Results are shown as the mean ± S.E. of triplicate observations from a single representative experiment. Mean EC50 values are presented in Table I.

Detection of Ligand-induced cAMP Production—The receptor-mediated stimulation of cAMP-induced reporter gene activity was assayed according to Chen et al. (18) with minor modifications as described previously (17). Briefly, 2 days after transfection the cells were stimulated for 6 h with various concentrations of hFSH, hCG, and hLH in 25 μl of Hepes-modified Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin and 0.1 mM 3-isobutyl-1-methylxanthine (all from Sigma). Ligand-induced changes in β-galactosidase activity (conversion of o-nitrophenyl-β-D-galactopyranoside into o-nitrophenol) were measured at 405 nm and related to the forskolin-induced changes (10 μM) in each 96-well plate. Therefore, the results are expressed as arbitrary units related to the forskolin-induced cAMP-mediated reporter gene activation. All experiments were repeated at least three times using cells from independent transfections, each performed in triplicate.

Receptor Binding Assay—Competition ligand binding assays were carried out on purified cell membranes from HEK-T 293 cells expressing (mutant or chimeric) receptors. Two days after transfection, HEK-T 293 cells were rinsed with Dulbecco’s phosphate-buffered saline (Sigma), subsequently harvested in ice-cold Tris buffer (10 mM Tris-HCl, 5 mM MgCl₂, 6 mM H₃O, pH 7.4), and centrifuged at 200 × g at 4 °C for 10 min. The pellet was resuspended in ice-cold Tris buffer containing 250 mM sucrose and homogenized by 40 strokes in a Dounce homogenizer at 4 °C, and the cell suspension was centrifuged at 15,000 × g at 4 °C for 30 min. The pellet was resuspended in Tris buffer. Cell membranes (∼50 μg of protein) were incubated for 18–20 h in 300 μl of Tris buffer containing 0.1% sodium dodecyl sulfate (SDS), 100 μM [3H]CG, and 100 nM of the unlabeled ligand.
Cyclic AMP production upon stimulation with hCG, hLH, and hFSH was measured in HEK-T 293 cells, transiently cotransfected with various receptor constructs and a plasmid (pCRE/β-gal) containing a β-galactosidase gene under control of a promoter containing five cAMP-response elements. The identity of the exodomain subdomains present in the chimeric hFSH-Rs is indicated by capital F or L for each of the three subdomains if they were derived from the hFSH-R or the hLH-R, respectively (see text). The EC50 values presented are the calculated mean ± S.E. of EC50 values derived from at least three independent experiments. Human LH and hCG yielded a similar efficacy as defined for every construct in at least two independent assays; for clarity, only the hCG data are shown. EC50 values were calculated by dividing the EC50 for the wild type hFSH-R with the wild type hLH-R or chimeric receptor EC50 values, ND, not detectable.

| Receptor constructs | hCG EC50 | EC50/wt/mut | hFSH EC50 | EC50/wt/mut |
|---------------------|----------|-------------|-----------|-------------|
| Wild type hFSH-R    | 397 ± 22.6 | 1.00 | 0.106 ± 0.019 | 1.00 |
| Wild type hLH-R     | 0.536 ± 0.093 | 740 | ND | ND |
| LLL-hFSH-R          | 0.603 ± 0.086 | 658 | ND | ND |
| LLF-hFSH-R          | 0.534 ± 0.031 | 1121 | ND | ND |
| FLL-hFSH-R          | 27.1 ± 17.3 | 14.6 | ND | ND |
| FLF-hFSH-R          | 1.71 ± 0.275 | 231 | ND | ND |

**Legend:**

| a | Groups sharing the same letter were not significantly different.

**Results**

**Hormone Specificity of Mutant β-Strand hFSH Receptors**—To identify which of the nine β-strands of the hLH-R confers hCG/hLH selectivity to its LRR subdomain, all X residues of individual hFSH-R β-strand motifs (with the consensus sequence X3.X2LX’LXX’X’Y) were mutated to their corresponding hLH-R residues (see Table II). Next, each of the mutant β-strand hFSH-Rs was transiently expressed in HEK-T 293 cells and analyzed for cell surface expression as well as hCG-, hLH-, and hFSH-dependent cAMP production. Alternatively, this strategy may also lead to the identification of β-strands of the hFSH-R that usually are involved in repelling hCG and/or hLH from its LRR subdomain. Most of the mutant β-strand hFSH-Rs were expressed at the cell surface, with levels ranging from 21 to 147% of wild type hFSH-R expression (Fig. 3, A and D). However, hFSH-R/Lβ4 and hFSH-R/Lβ6 were expressed at lower levels (4 and 15%, respectively), whereas hFSH-R/Lβ1 and hFSH-R/Lβ7 were undetectable in the anti-HA tag ELISA (Fig. 3, A and D).

The hFSH-R/Lβ2, hFSH-R/Lβ3, hFSH-R/Lβ5, hFSH-R/Lβ6, and hFSH-R/Lβ9 constructs responded to hFSH with EC50 values (ranging from 0.064 to 0.223 ng/ml; p < 0.05) similar to that of wild type hFSH-R (Fig. 3, C and F, and Table II). This finding indicates that these hFSH-R α-strands, when replaced by hLH-R α-strands, are not crucial in conferring hFSH selectivity to the hFSH-R exodomain. In contrast, the responsiveness to hFSH was decreased significantly in hFSH-R/Lβ8 (5-fold; p < 0.05), hFSH-R/Lβ4 (14-fold; p < 0.001), hFSH-R/Lβ7 (130-fold; p < 0.001), and hFSH-R/Lβ1 (260-fold; p < 0.001),
Cyclic AMP production upon stimulation with hCG, hLH, and hFSH was measured in HEK-T 293 cells, transiently cotransfected with various receptor constructs and a plasmid containing a β-galactosidase gene under control of a promoter containing five CAMP-response elements (pCRβ3-gal). Residues that were introduced into the hFSH-R β-strands are underlined. The EC50 values presented are the calculated mean ± S.E. of EC50 values derived from at least three independent experiments. Human LH and hCG yielded a similar efficacy as determined for every construct in at least two independent assays; for clarity, only the hCG data are shown. EC50 values were calculated by dividing the EC50 for the wild type hFSH-R with the mutant receptor EC50 values. ND, not detectable.

| Receptor constructs | Wild type | Mutant | hCG EC50 | EC50 | hFSH EC50 | EC50 |
|---------------------|-----------|--------|----------|-------|-----------|-------|
|                     | XXXXXX    | XXXXXX | ng/ml    |       | ng/ml     |       |
| Wild type hFSH-R    |           |        | 397 ± 226  | 1.00  | 0.106 ± 0.019  | 1.00  |
| LLL-hFSH-R          |           |        | 0.603 ± 0.008  | 658  | ND        | ND    |
| hFSH-R/Lβ1          | IELRFLV   | TRFLFAY | ND       | ND    | 280 ± 62.2  | 0.0003 |
| hFSH-R/Lβ2          | KRIEQG    | KRIEQG  | 568 ± 64.3  | 0.70  | 0.088 ± 0.030  | 1.20  |
| hFSH-R/Lβ3          | HEIRIEK   | SEITLON | 6.55 ± 1.46  | 60    | 0.064 ± 0.011  | 1.66  |
| hFSH-R/Lβ4          | QQILSN    | QYILSCN | >30000     | <0.15 | 1.53 ± 0.102  | 0.07  |
| hFSH-R/Lβ5          | VLILIDQ   | FIELIDQ | >20000     | <0.20 | 0.232 ± 0.025  | 0.46  |
| hFSH-R/Lβ6          | VILMLNK   | VILMLNG | 3.86 ± 0.383  | 102  | 0.145 ± 0.008  | 0.73  |
| hFSH-R/Lβ7          | DELNLSD   | TGLBLKE | ND        | ND    | 13.7 ± 2.37b  | 0.0077 |
| hFSH-R/Lβ8          | VILDISR   | RTLDISN | 773 ± 179a  | 0.50  | 0.533 ± 0.232  | 0.20  |
| hFSH-R/Lβ9          | KIIRARS   | QRIIATG | 151 ± 22.5a  | 2.60  | 0.073 ± 0.018  | 1.45  |
| hFSH-R/Lβ3,Lβ6      | HEIRIEK   | SEITLON | 0.589 ± 0.106b  | 673  | 0.186 ± 0.031  | 0.57  |
|                     | VILMLNK   | VILMLNG |          |       |            |       |
| hFSH-R/Lβ4,Lβ5      | QQILSN    | QYILSCN | 354 ± 75.3  | 1.12  | 0.322 ± 0.097  | 0.33  |
|                     | VLILIDQ   | FIELIDQ | 824 ± 332  | 0.48  | 0.133 ± 0.096  | 0.80  |
| hFSH-R/Lβ4-C133S    | QQILSN    | QYILSCN | 1491 ± 949  | 0.26  | 0.116 ± 0.010  | 0.91  |
|                     | VLILIDQ   | FIELIDQ |          |       |            |       |

a,b Groups sharing the same letter were not significantly different.

1. Ligand efficacy was too low to calculate EC50 values; therefore, the estimated mean EC50 values are presented.
Cell Surface Expression of the hFSH-R Is Sensitive to Mutations in β-Strands 1 and 7—The mutant hFSH-R/Lβ1 and hFSH-R/Lβ7 receptors displayed completely impaired cell surface expression (Fig. 3, A and D), together with a 2600- and 130-fold decreased hFSH responsiveness, respectively (Fig. 3, C and F, and Table II), and no response to hCG or hLH (Fig. 3, B and E, and Table II). To test whether the observed reduction in responsiveness is related directly to reduced cell surface receptor expression, HEK-T 293 cells were transfected with wild type, chimeric, or mutant β-strand hFSH-R constructs. Human LH and hCG stimulated all constructs with a similar efficacy; for clarity, only the hCG-induced, cAMP-mediated reporter gene activity is shown. Results are shown as the mean ± S.E. of triplicate observations from a single representative experiment. Mean EC₅₀ values are presented in Table II. ND, not detectable.

![Fig. 3](image-url)  
**FIG. 3.** Cell surface expression and ligand-induced cAMP production of wild type, chimeric, or mutant hFSH-Rs transiently expressed in HEK-T 293 cells. A, D, and G, cell surface expression of wild type, chimeric, or mutant β-strand hFSH-Rs as determined by HA tag ELISA. B, E, and H, human CG-stimulated, cAMP-mediated reporter gene activity in HEK-T 293 cells transiently transfected with wild type, chimeric, or mutant β-strand hFSH-R constructs. C, F, and I, human FSH-stimulated, cAMP-mediated reporter gene activity in HEK-T 293 cells transiently transfected with wild type, chimeric, or mutant β-strand hFSH-R constructs. Results are shown as the mean ± S.E. of triplicate observations from a single representative experiment. Mean EC₅₀ values are presented in Table II. ND, not detectable.

![Fig. 4](image-url)  
**FIG. 4.** Ligand binding to membranes prepared from HEK-T 293 cells transiently transfected with wild type or mutant β-strand hFSH-R constructs. Displacement of [¹²⁵I]hFSH binding to membranes prepared from HEK-T 293 cells transiently transfected with wild type or mutant β-strand hFSH-Rs by various concentrations of unlabeled hCG (A) or unlabeled hFSH (B). Results are shown as the mean ± S.E. of triplicate observations from a single representative experiment. Mean Kᵢ values are presented in Table III.

Cell Surface Expression of the hFSH-R Is Sensitive to Mutations in β-Strands 1 and 7—The mutant hFSH-R/Lβ1 and hFSH-R/Lβ7 receptors displayed completely impaired cell surface expression (Fig. 3, A and D), together with a 2600- and 130-fold decreased hFSH responsiveness, respectively (Fig. 3, C and F, and Table II), and no response to hCG or hLH (Fig. 3, B and E, and Table II). To test whether the observed reduction in responsiveness is related directly to reduced cell surface receptor expression, HEK-T 293 cells were transfected with 10, 100, and 1000 ng of wild type HA-tagged hFSH-R construct. Although these amounts of transfected construct appeared to be expressed at different levels (3 and 23% and set at 100%, respectively) on the cell surface, the production of cAMP in response to hFSH, hCG, and hLH stimulation appeared to be only slightly, although significantly, affected by the different receptor numbers (Fig. 5 and Table IV); a 33-fold reduction in cell surface receptor expression was accompanied by a less than 5-fold reduction in ligand responsiveness. To determine whether replacing hFSH-R β-strands 1 or 7 with the corresponding hLH-R β-strands either affected hFSH binding directly or severely disrupted the exodomain conformation, the hLH-R-specific residues in hFSH-R/Lβ1 and hFSH-R/Lβ7 were
TABLE IV
Summary of the ligand-binding properties of membranes of HEK-T 293 cells transiently transfected with wild type and mutant hFSH-R constructs

| Receptor constructs | hCG $K_i$ | $K_i^{\text{expr}}$ | hFSH $K_i$ | $K_i^{\text{expr}}$ |
|---------------------|-----------|---------------------|-----------|---------------------|
| Wild type hFSH-R    | 1357 ± 28.3 ng/ml | 1.00 | 0.864 ± 0.119 ng/ml | 1.00 |
| hFSH-R/Lβ3          | 8.08 ± 0.841 ng/ml | 168 | 0.798 ± 0.093 ng/ml | 1.08 |
| hFSH-R/Lβ6          | 6.91 ± 0.549 ng/ml | 196 | 1.03 ± 0.119 ng/ml | 0.83 |
| hFSH-R/Lβ3/Lβ6      | 0.415 ± 0.006 ng/ml | 3273 | 0.718 ± 0.195 ng/ml | 1.20 |

$K_i^{\text{expr}}$ values were obtained from competitive binding experiments of $[^{125}\text{I}]$hFSH to membranes prepared from HEK-T 293 cells, transiently expressing wild type and mutant hFSH receptors, in the presence of various concentrations of unlabeled hCG or hFSH. The $K_i$ values presented are the calculated mean ± S.E. of $K_i$ values derived from two independent assays. $K_i^{\text{expr}}$ values were calculated by dividing the $K_i$ for the wild type receptor with the mutant receptor $K_i$ values.

\(1,010,016 \text{ ng/ml}\)

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**Fig. 5.** Effect of different amounts of hFSH-R construct, transiently transfected into HEK-T 293 cells on receptor cell surface expression and ligand responsiveness. HEK-T 293 cells were transiently transfected with 0.01, 0.1, and 1 μg of expression vector containing the wild type hFSH-R cDNA insert. A, cell surface expression of hFSH-R as determined by HA tag ELISA. B and C, human CG- and hFSH-stimulated, cAMP-mediated reporter gene activity in HEK-T 293 cells transiently transfected with different amounts of hFSH-R. Human LH and hCG stimulated all constructs with a similar efficacy; for clarity, only the hCG-induced, cAMP-mediated reporter gene activity is shown. Summary of the ligand-induced intracellular cAMP production in HEK-T 293 cells transiently transfected with different quantities of wild type hFSH-R construct

| Amount of hFSH-R transfected | hCG EC$50$ | EC$50^{\text{wt/red}}$ | hFSH EC$50$ | EC$50^{\text{wt/red}}$ |
|------------------------------|-----------|---------------------|-----------|---------------------|
| μg                           | ng/ml     | ng/ml               | ng/ml     | ng/ml               |
| 1                            | 397 ± 22.6 $b$ | 1.00 | 0.106 ± 0.019 $b$ | 1.00 |
| 0.1                          | 849 ± 905 $c$ | 0.47 | 0.148 ± 0.008 $c$ | 0.72 |
| 0.01                         | 1615 ± 96.4 $d$ | 0.25 | 0.470 ± 0.094 $d$ | 0.23 |

$a-d$ Groups sharing the same letter were not significantly different.

Ligand Selectivity of Gonadotropin Receptors

Summary of the ligand-selectivity of gonadotropin receptors expressed in HEK-T 293 cells transiently transfected with wild type and mutant hFSH-R constructs. $K_i$ values were obtained from competitive binding experiments of $[^{125}\text{I}]$hFSH to membranes prepared from HEK-T 293 cells, transiently expressing wild type and mutant hFSH receptors, in the presence of various concentrations of unlabeled hCG or hFSH. The $K_i$ values presented are the calculated mean ± S.E. of $K_i$ values derived from two independent assays. $K_i^{\text{expr}}$ values were calculated by dividing the $K_i$ for the wild type receptor with the mutant receptor $K_i$ values.

Mammalian glycoprotein hormones are bound with high affinity and high selectivity by the exodomains, which are highly related and are thought to share a similar structural conformation of their corresponding receptors. Nevertheless, sequences in the exodomains of these receptors have diverged sufficiently to generate the above-mentioned selectivity toward their respective glycoprotein hormones. Taking advantage of their structural similarity, the hormone selectivity of the hLH-R was studied in the present study by substituting hFSH-R-specific sequences with their corresponding hLH-R sequences. In contrast to previous studies (2, 3, 21), chimeric junctions were designed to coincide with the presumed structural borders of the extracellular subdomains (i.e., the NCR, LRR, and CCR subdomains, as well as the β-strands within the LRR subdomain).

Receptor chimeras in which the extracellular NCR, LRR, and/or CCR subdomains of the hFSH-R were substituted by their homologous hLH-R counterparts indicated that the determinants involved in ligand selectivity are confined mainly to the LRR subdomain (cf. LLL-hFSH-R, LLF-hFSH-R, and hLH-R). Similar to other extracellular LRR-containing proteins, it is thought that the hydrophobic core of the LRR subdomain of gonadotropin receptors is protected from the solvent at both ends by its flanking NCR and CCR subdomains (5). Despite the conservation of four almost equally spaced Cys residues, which are presumably disulfide-bonded and essential for correct folding (10, 20), the NCR subdomain of the hFSH-R was not compatible with the LRR subdomain of hLH-R as revealed by the severely impaired cell surface expression of the PLL-hFSH-R and FLF-hFSH-R chimeras.
Ligand Selectivity of Gonadotropin Receptors

Cyclic AMP production upon stimulation with hCG, hLH, and hFSH was measured in HEK-T 293 cells, transiently cotransfected with various receptor constructs and a plasmid containing a β-galactosidase gene under control of a promoter containing five CAMP-response elements (pCRE/β-gal). Residues that were introduced into the hFSH-R β-strands are underlined. The EC₅₀ values presented are the calculated mean ± S.E. of EC₅₀ values derived from at least three independent experiments. Human LH and hCG yielded a similar efficacy for all constructs as determined for every construct in at least two independent assays; for clarity, only the hCG data are shown. EC₅₀ values were calculated by dividing the EC₅₀ for the wild type hFSH-R with the mutant receptor EC₅₀ values. ND, not detectable.

| Receptor constructs | Wild type | Mutant | hCG EC₅₀ | hFSH EC₅₀ | EC₅₀ ratio |
|---------------------|-----------|--------|-----------|-----------|------------|
| Wild type hFSH-R    | 397 ± 22.6 | ND     | 1.00      | 0.106 ± 0.019 | 1.00 |

Fig. 6. Cell surface expression and ligand-induced cAMP production of wild type or β-strand 1 or 7 mutant hFSH-Rs transiently expressed in HEK-T 293 cells. A and D, cell surface expression of wild type or β-strand 1 or 7 mutant hFSH-Rs as determined by HA tag ELISA.

The current knowledge of the putative spatial arrangement of the LRR subdomain of GpHRs (13, 14) was used to systematically exchange only those residues that were expected to have their side chains directed toward the presumed hormone-binding site (i.e. the X amino acid residues of the nine β-strand motifs arranged in parallel, each with the consensus sequence X₁X₂LX₃LX₄X₅X₆X₇X₈X₉). Human FSH, hCG, and hLH binding selectivity is very likely associated with the ~73% divergence of these X residues between hFSH-R and hLH-R. Identical X residues (e.g. the highly conserved Asp at position X² in β-strand 5) may be involved in common hormone-receptor contacts (10). Substitution of individual β-strands of the hFSH-R with the corresponding hLH-R β-strands allowed the identification of β-strands 3 and 6 as modules containing hCG- and hLH-selective determinants, as these mutant receptors mediate a 60 (β-strand 3)- to 100-fold (β-strand 6), respectively, enhanced responsiveness to hCG as well as to hLH. Moreover, both β-strands appeared to act synergistically, as a mutant receptor (i.e. hFSH-R/β₃LθLβ₆) displayed a responsiveness to hCG and hLH similar to that of wild type hLH-R. Hence, hLH-R selectivity toward hCG and hLH can be confined mainly to just these two β-strands. In fact, none of the other β-strands ap-
peared to confer additional hCG/hLH selectivity to a major extent. These results are consistent with previous studies (2, 3) in which hCG-selective determinants were predicted to be localized within the NCR–LRR6 sequence of the LH-R.

Most of the mutant hFSH-R constructs that harbored hLH-R β-strands did not exhibit severely impaired hFSH responsiveness, indicating that no essential hFSH-selective determinants are present in the corresponding hFSH-R β-strands. Moreover, this means that mutations in these β-strands did not alter the ligand-stimulated capacity of the receptors to induce cAMP production. Hence, the observed efficacy of hFSH, hCG, and hLH to stimulate receptor-mediated signaling correlates with the respective hormone binding affinities of these receptors as confirmed by ligand binding studies. Although some mutant receptors that were expressed at low levels (e.g. hFSH-R/Lβ3,Lβ6 and hFSH-R/Lβ5) exhibited efficient responsiveness to ligand stimulation, reduced responsiveness to hFSH stimulation was often related to severely impaired surface expression of mutant β-strand hFSH-Rs (e.g. hFSH-R/Lβ1, hFSH-R/Lβ4, and hFSH-R/Lβ7). This was likely because of disrupted protein folding/conformation, because reduced numbers (i.e. transfecting different concentrations of wild type hFSH-R constructs) of correctly folded receptors on the cell surface had only minor effects (<5-fold) on the level of ligand responsiveness. Also hFSH-R/Alaβ1 and hFSH-R/Alaβ7 exhibited disrupted cell surface expression and, as a consequence, reduced ligand responsiveness. However, Ala substitution of the individual residues in β-strands 1 and 7 revealed that these β-strands are not involved the binding of hFSH, hCG, or hLH.

In conclusion, the primary objective of this study was to identify hCG/hLH selective determinants in the extracellular N terminus of the LH-R by generating potential gain-of-function mutant receptors in the context of an hFSH-R background. Using this strategy, hCG/hLH selectivity was tracked down to hLH-R β-strands 3 and 6. Alternatively, the results observed may also be explained by the loss of hCG/hLH repulsion in these mutant hFSH-Rs. Surprisingly, hFSH signaling (and binding) was hardly affected by the introduction of most hLH-R β-strands, suggesting that selective hFSH responsiveness, in contrast to the responsiveness to hCG and hLH, appears to be determined by numerous hFSH-R β-strands, each, however, with a relatively low contribution. The identification of the amino acid residues in β-strands 3 and 6 that are critically involved in the molecular mechanism determining hCG/hLH selectivity is currently under investigation.

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Ligand Selectivity of Gonadotropin Receptors: ROLE OF THE $\beta$-STRANDS OF EXTRACELLULAR LEUCINE-RICH REPEATS 3 AND 6 OF THE HUMAN LUTEINIZING HORMONE RECEPTOR

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