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A Follicle-Stimulating Hormone Receptor Ecto-Domain Epitope That Is a Target for Receptor Immunoneutralization Yet Does Not Affect Ligand Contact and Activation

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The follicle-stimulating hormone receptor (FSHR) large extracellular domain suggests that interaction of ligand with receptor is likely to be complex. Residues 265–296 of the FSHR are part of a sequence primarily nonhomologous with other glycoprotein hormone receptors. A reasonable hypothesis is that this sequence of the FSHR plays a role in binding FSH. Flow cytometry studies of this region revealed that antibody X179 against peptide R265–S296 binds to human FSHR expressed by CHO cells and can be competed against by preincubating the cells with hFSH. These results suggested that the region corresponding to residues 265–296 in the extracellular domain of the FSHR is involved in binding to hormone. To test this hypothesis 10 scanning alanine mutants of rFSHR at the 265–296 epitope were generated, and the binding characteristics of these mutants were studied. Their affinity constants for ^{125}I-hFSH did not deviate greatly from that of wild-type FSHR, in which some mutants exhibited an approximately two- to threefold reduction in K_a compared to wild-type receptor, and no mutation abolished signal transduction. These results lead to rejection of the hypothesis that this region contains residues critical for conveying hormone specificity and receptor-dependent hormone action.

Key Words: Receptor; ligand; interaction; mutagenesis; antipeptide antibody.

Introduction

Follicle-stimulating hormone receptor (FSHR) is a member of the G-protein-coupled receptor family and on hormone occupancy activates adenyl cyclase *(1)*. Defining the molecular basis for the glycoprotein hormone (GPH)-receptor (GPHR) interaction could have broad implications for determining whether or not there is a common pathway for activation of adenylate cyclase by G-protein receptors. Stabilization of active receptor conformers by ligand binding or by spontaneous activating mutations is believed to affect signal transduction *(2)*. Whereas other G-protein-coupled receptors have small extracellular domains, the large extracellular domains of the GPHR remain an enigma but likely perform functions in docking, as well as acting as conformational stabilizers.

The extent to which the large GPHR ectodomains are required for ligand binding has been studied. Rat luteinizing hormone receptor (LHR) truncated to as little as 206 residues (exons 1–8) binds LH with a twofold change in affinity and a thousandfold reduction in receptor concentration *(3)*. However, chimeric FSH/LH receptors, in which FSHR residues 1–140 are switched with LHR residues, bind neither LH nor FSH; when FSHR residues 1–257 are used, signal transduction is stimulated by FSH *(3)*. Full-length nonfusion ectodomains of rFSH residues 1–346 are trapped intracellularly but still bind FSH with a small reduction in affinity *(4)*. Full-length ectodomains of hFSHR (residues 1–358), expressed on the cell surface as fusion proteins, can act as functional antagonists in vivo *(5)*. Membrane-bound ovine FSH receptor variants with as little as 241 residues in the ectodomain bind FSH *(6)*. In summary, these data suggest that the N-terminal 250 residues are required for GPH binding to GPHR.

Peptide challenge and immunochemical studies have implicated the C-terminal of the extracellular domain of GPHR as being involved in hormone-receptor interaction. For example, four synthetic peptides corresponding to LH/human chorionic gonodotropin receptor (hCGR) primary sequences R21–P38, R102–T115, Y253–F272, and K573–K583 inhibited binding of LH to receptor *(7)*. Similarly, thyroid-stimulating hormone receptor (TSHR) peptides 12–30 and 324–344 were found to inhibit bovine TSH binding *(8)*. Inhibition of binding is presumed to occur via peptide-hormone interaction. In the case of

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FSHR, a C-terminal site has been delineated between residues 201 and 319 using bacterially expressed hFSHR peptides *(9)*. Also rFSHR peptide S9–N30 bound to ovine, bovine, and human FSH *(10)* and antisera raised against this peptide and peptide K19–R29 blocked FSH binding and/or activation of Sertoli cells in vitro *(11,12)*. The hCG receptor is constitutively activated when single-chain hCG is fused to the N-terminus of the hCG receptor, suggesting that the N-terminal of the hCG receptor is also important for signal transduction *(13)*. In summary, the notion that the amine terminal of the extracellular domain is important for hormone binding is supported by peptide challenge tests and immunochemical and protein engineering approaches. In this article, we have studied a highly accessible C-terminal epitope of the FSHR, and found that modification of this epitope does not greatly affect ligand binding or activation.

Results

Accessibility and Proximity of Accessible C-Terminal Epitope to the FSH Binding Site

In previous studies, we showed that antibodies raised against hFSH receptor peptide 265–296 bound to hFSHR and inhibited FSH binding and steroidogenesis *(14)*. In the present study, we used flow cytometry to show, in native receptor *in situ*, that the 265–296 epitope was accessible. Figure 1 illustrates the results of flow cytometric analysis, with the *x*-axis representing the green fluorescence intensity and the *y*-axis the relative number of cells. The FACS Scan flow cytometer was set to measure 5000 events. Nonimmune rabbit sera at a 1:400 dilution showed some nonspecific immunofluorescence (*see* Fig. 2). Antiserum X179 from rabbits immunized with the R265-S296 peptide bound to a high degree at a 1:400 dilution.

To approximate the spatial relationship of the hFSH binding site to the 265–296 epitope, control Chinese hamster ovary (CHO) cells and CHO cells expressing hFSH-R (CHOR) were incubated overnight with 1 μg of hFSH prior to the addition of a 1:40 dilution of immune rabbit serum (Fig. 2). As expected, nonspecific binding of nonimmune serum as well as X179 to CHO cells was not reduced by the addition of hFSH. Antipeptide serum X179 bound to CHOR cells displayed intense fluorescence. The binding of X179 to CHOR cells was reduced by 82% with the addition of hFSH.

Immune serum X179 was preincubated with CHOR cells to confirm whether the antibodies could inhibit binding of radioligand to receptor. As previously demonstrated *(14)*, antiserum X179 inhibited binding of ^{125}I-hFSH to hFSHR (Fig. 3).

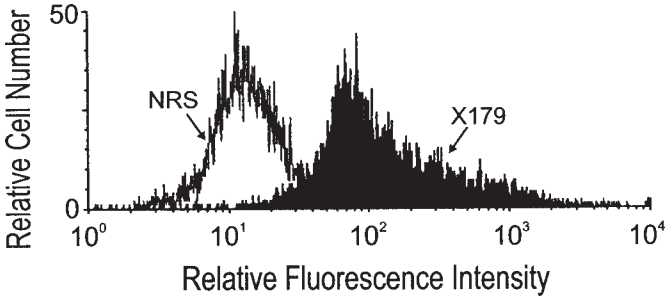

Fig. 1. Binding of FSHR peptide R265–S279 antiserum X179 to CHOR cells as determined by flow cytometry. CHOR cells (1×10^6/tube) were incubated for 1 h with a 1:400 dilution of antiserum X179 and preimmune rabbit serum (normal rabbit serum: NRS) followed by fluorescein-isothiocyanate–conjugated (FITC) goat antirabbit IgG. Data are representative of three independent experiments.

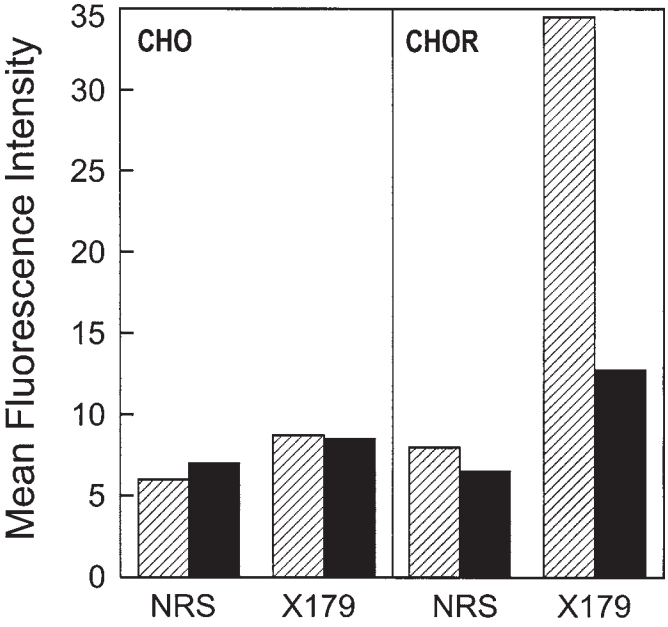

Fig. 2. Binding to CHOR and CHO (control) cells by preimmune serum (normal rabbit serum) and antiserum X179 in the presence or absence of hFSH was measured using flow cytometry. Cells (1×10^6/tube) were incubated overnight with (j) or without (▨) 1 μg of hFSH prior to the addition of 1:400 preimmune serum or antiserum X179, followed by the addition of FITC goat antirabbit IgG. Data are representative of three independent experiments.

Follitropin-Binding Activity of Wild-Type and Mutant FSHRs

Ten alanine triple mutants spanning the 265–296 region of rFSHR were generated (Table 1) and expressed in Sf9 cells. These mutants were tested in a radioreceptor assay (RRA) to determine binding activity compared with the

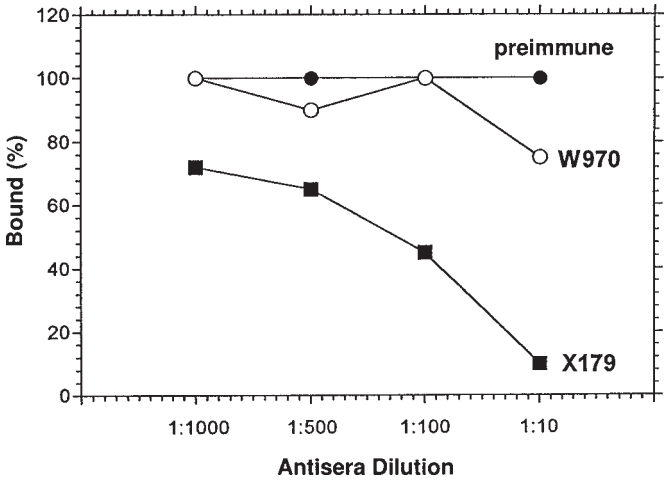

Fig. 3. Inhibition of ^{125}I-hFSH binding to CHOR cells by antibody X179. Increasing concentrations of preimmune serum or X179 and W970 sera were incubated with CHOR cells (5×10^5) overnight at 4°C. As a negative control, we used antibody W970 (against FSHR peptide G150-L183), which we previously have shown not to interfere with hormone-receptor interaction. Radioligand (150,000 cpm at specific activity of 70.0 μCi/μg) was then added and incubation was continued at room temperature overnight. Nonspecific binding was assessed by the addition of 1 μg of unlabeled FSH to the reaction. Radioactive counting was performed the following day.

wild-type rFSHR. We analyzed the data utilizing the LIGAND program *(15)*. The affinity constants of receptors encoding mutations M3 and M8 were 1.8- and 1.7-fold, M6 was 2.4-fold, and M7 3.4-fold lower than that of the wild-type receptor (Table 2). As determined by B_{max}, the concentration of functional receptor on Sf9 cells was 4-fold lower for M2, 2.8-fold lower for M4, 2-fold lower for M10, and 22-fold lower for M1 when compared to wild-type receptor. The markedly lower receptor expression of mutation M1 raised concern that the accuracy of determination of K_a would be compromised. We showed by Western blotting that this mutation caused a significant reduction in protein expression (Fig. 4). Therefore, to have a comparable number of receptors for M1 in the RRA, we used 10 times more cells for M1 than for the other receptors. Under this condition, no significant difference in affinity constant was detected for M1 (Table 2). As expected, M4, the mutation that disrupted the glycosylation sequence at N276, showed reduced cell surface expression but enhanced hormone binding affinity.

Effect of rFSHR Mutations on Signal Transduction

Cyclic adenosine monophosphate (cAMP) produced in response to FSH by Sf9 cells expressing wild-type or mutant rFSHRs was measured to determine whether any of the mutations abolished signal transduction. Sf9 cells infected by the wild-type rFSHR virus and each of the 10 mutant viruses were stimulated with 10 ng of hFSH (1.5 nM, approximate ED_{50} of the wild type) for 1.5 h at room temperature *(14)*. Simultaneously, RRAs were performed with cells from the same culture batch. The data in Table 3 show that all mutant rFSHR molecules were coupled to adenylate cyclase following hFSH treatment. None of these rFSHR mutants demonstrated loss of signal transduction. These data should be considered qualitative because the data with a single dose of FSH did not provide sufficient rationale to conduct a full dose response curve.

Discussion

Considerable progress has been made in understanding the structure of the GPHRs *(16)*. The extracellular domain exhibits <40% homology over the three pituitary GPHRs *(17)* and has been shown to be the region for hormone binding for all the GPHs, including TSH *(18)*.

Our laboratory has been interested in the hFSHR region 250–300, because this region has two potential glycosylation sites, which suggests that the region is on the surface. Sequence 265–296 also represents a region of helix-turn-helix motif and exhibits low homology with LHR. In addition, this sequence is encoded by exon 10 of the FSHR gene, which also encodes the transmembrane domains, intervening extracellular and intracellular domains, and the terminal cytoplasmic domain.

Several potential hormone-binding regions in the LH, FSH, and TSH receptors have been identified *(7,8,10,11)*, but the 265–296 region of the FSHR has not been studied in detail before. Roche et al. *(7)* showed that LHR peptide spanning regions 239–272 inhibited binding of LH to its receptor. LHR and FSHR sequences diverge at the junction between exon 9 and 10 of FSHR; thus we reasoned that these sequences might be involved in ligand binding.

Antibodies against rFSHR peptide R265–S296 inhibited ^{125}I-hFSH binding of rFSHR expressed in insect cells and inhibited hFSH-stimulated adenylate cyclase activity of the rFSHR. Fixed CHOR cells could also be stained using this antipeptide antibody, and FSH binding to Y1 cells expressing hFSHR could be blocked by this antibody *(14)*.

The present study is a closer investigation of the 265–296 region of FSHR in relationship to hormone binding. Antibody X179 from a rabbit immunized with R265–S296 peptide had low background binding to CHO cells not expressing the receptor but had high median fluorescence intensity staining of CHOR cells. We have established that this binding could be prevented by prior incubation with hFSH. In addition, we have shown that preincubation of

Table 1
Primary Sequences of rFSHR Residues 265–296 and 10 Mutant Forms of rFSHR[a]

Mutation		Sequence
rFSHR (wild type)	265-296	KRQISELHPICNKSILRQDIDDMTQIGDQRVS
rFSHR M1		AAAISELHPICNKSILRQDIDDMTQIGDQRVS
rFSHR M2		KRQAAALHPICNKSILRQDIDDMTQIGDQRVS
rFSHR M3		KRQISEAAPACNKSILRQDIDDMTQIGDQRVS
rFSHR M4		KRQISELHPICAAAILRQDIDDMTQIGDQRVS
rFSHR M5		KRQISELHPICNKSAAAQDIDDMTQIGDQRVS
rFSHR M6		KRQISELHPICNKSILRAAADDMTQIGDQRVS
rFSHR M7		KRQISELHPICNKSILRQDIAAATQIGDQRVS
rFSHR M8		KRQISELHPICNKSILRQDIDDMAAAGDQRVS
rFSHR M9		KRQISELHPICNKSILRQDIDDMTQIAAARVS
rFSHR M10		KRQISELHPICNKSILRQDIDDMTQIGDQAAA

[a]Mutations were made as triple mutations of residues to alanine. For example, mutant M1 was made by changing KRQ/AAA.

Table 2
Characterization of ^{125}I hFSH-Binding Properties of 10 rFSHR Mutants[a]

Mutations		K_a ($10^9/M$)	B_{max} (functional receptors/cell)
Wild type	None	5.82 ± 0.61	163,756 ± 0.20
M1[b]	^{265}KRQ^{267}/AAA	4.22 ± 0.33	7,163 ± 0.10
M2	^{268}ISE^{270}/AAA	4.33 ± 0.53	40,091 ± 0.13
M3	^{271}LHPI^{274}/AAPA	3.25 ± 0.52	99,880 ± 0.50
M4	^{276}NKS^{278}/AAA	7.27 ± 0.56	57,242 ± 0.13
M5	^{279}ILR^{281}/AAA	3.97 ± 0.60	95,163 ± 0.10
M6	^{282}QDI^{284}/AAA	2.44 ± 0.22	195,145 ± 0.10
M7	^{285}DDM^{287}/AAA	1.69 ± 0.22	114,316 ± 0.09
M8	^{288}TQI^{290}/AAA	3.43 ± 0.45	107,381 ± 0.12
M9	^{291}GDQ^{293}/AAA	4.71 ± 0.50	173,787 ± 0.09
M10	^{294}RVS^{296}/AAA	4.82 ± 0.28	82,348 ± 0.10

[a]Mutations were made in sequence (R265–S296) exon 10 of rFSHR.Data are the average of five independent experiments for each receptor (duplicate determinations). The data resulting from binding assays were analyzed using the computer program LIGAND. K_a represents the affinity constant calculated assuming a one-site model.
[b]This experiment was performed with 1×10^5 cells/tube with the exception of M1, in which 1×10^6 cells/tube was used.

receptor with antibody X179 prevented hFSH from binding to receptor. These observations lead to the preliminary hypothesis that antibodies to peptide R265–S296 are directed toward an epitope that is present in the receptor binding site.

To investigate further the involvement of the 265–279 FSHR region in hormone-receptor interaction, we generated 10 triple alanine scanning mutations. Alanine mutagenesis addresses issues of side-chain function while preserving backbone structure *(19)*. Data from binding studies indicate that residues within the 265–279 region of FSHR have no significant impact on binding affinity, although some mutations greatly affected protein processing.

Regarding signal transduction, these data are consistent with results obtained using chimeric FSH-LH/CG receptors containing residues (1–258) of the FSHR and residues (259–674) of LH/CG receptor *(3)*. Amino acids changed within this region, 265–296 of FSHR, do not appear to be required for receptor activation. Again, these data should be considered only qualitative.

Not all contact residues at the hormone-receptor contact site are critical for binding. It has been reported *(20)* that alanine-scanning mutagenesis of residues at a site of interface between human growth hormone (hGH) and the extracellular domain of its receptor (hGHbp) did not affect binding kinetics, affinity, or overall structure, implying that only a small set of contacts are crucial for hGH-receptor

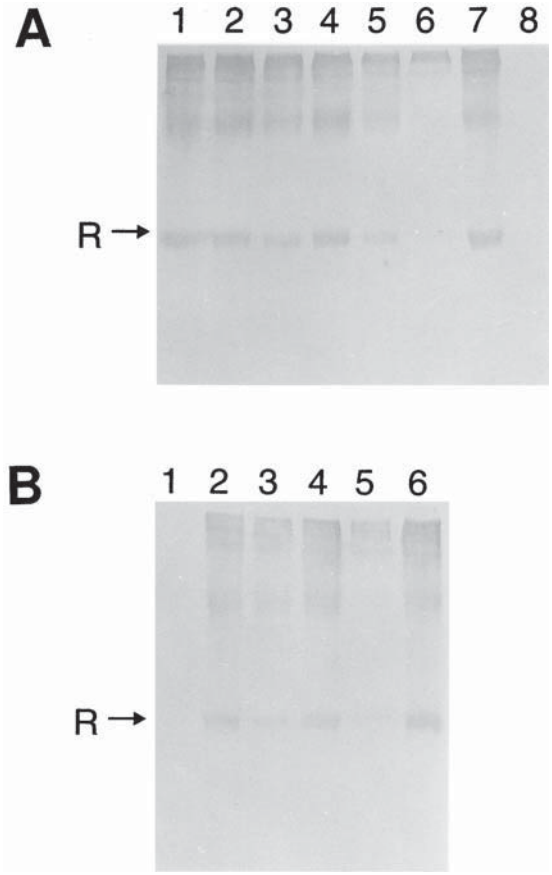

Fig. 4. Assessment of total cellular content of wild-type and mutant rFSHR by Western blot. The blots were probed with W971 antisera (1:1000) against rFSHR peptide 15–44 as previously described. Lysates of 5×10^4 Sf9 cells infected by each recombinant virus were loaded on each lane. **(A)** Lane 1, M10; lane 2, M5; lane 3, M4; lane 4, M3; lane 5, M2; lane 6, M1; lane 7, wild type; lane 8, control virus. **(B)** Lane 1, control virus; lane 2, wild type; lane 3, M6; lane 4, M7; lane 5, M8; lane 6, M9. R indicates receptor band.

interaction. Here, a similar situation could be envoked: if in fact FSHR residues 265–279 are part of a hormone contact site, then reduction in hormone-receptor contacts in this region is tolerated with little effect on affinity. Other experiments *(21–23)* suggest that hot spots of binding energy exist at protein-protein interfaces. Thus, mutations at inert contact residues reveal interfaces that are less important for affinity and therefore would make poor targets for rational drug design.

Care must be taken in the interpretation of the antipeptide antibody inhibition experiments. FSH may prevent the binding of antibody to receptor through steric hindrance and vice versa. Whether the 265–279 region of FSHR is in close proximity to a hormone binding site, as steric hindrance would indicate, or these residues are part of a hormone-receptor interface, is not clear. We have determined, however, that this region of FSHR is not crucial for hormone binding and activation.

Based on the present results and the literature, a reasonable conclusion is that the hormone binding determinant of the FSHR is a discontinuous determinant, consisting of at least residues within the 9–30 region of the N-terminal and other hot spots of binding that remain to be identified, not including the 265–296 epitope.

Materials and Methods

Flow Cytometry Analysis with Antipeptide Antibody to FSHR

Preparation of antisera used in these studies has been previously described *(24)*. CHOR cells were obtained from ARES Advanced Technologies. Cells were washed twice with EDTA-phosphate-buffered saline (PBS) buffer (0.05 *M* EDTA-0.01 *M* sodium phosphate, 0.14 *M* NaCl, pH 7.0) and then incubated with buffer for approx 15 min. The cells were dislodged from the culture flasks, counted, and spun for 5 min at 1,000*g*. The buffer was aspirated and the cells were suspended in PBSA (PBS with 0.02% sodium azide) buffer. All assay tubes received 1×10^6 CHO or CHOR cells in 200 μL of HEPES buffer.

Control experiments confirmed that the antipeptide antibody X179 could bind to CHOR cells. Cells were added to 10 μL of nonimmune rabbit serum, immune serum, or secondary antibody alone and incubated at room temperature for 1 h on a shaker. FSH challenge tests were conducted to determine whether hFSH could inhibit binding of antipeptide antibodies to CHOR cells. In these experiments, hFSH (1 μg) was added to cells and incubated overnight in the cold. The next day, the cells were washed with 2 mL of PBSA buffer, mixed, and spun. Cells were resuspended in buffer, a 1:40 dilution of antiserum was added, and incubation was at room temperature for 1 h on a shaker. To assess the specificity of antisera binding, immunizing receptor peptides (10 μg) were added to a 1:40 dilution of nonimmune and immune sera and incubated overnight in the cold. The following day, cells were added (volume = 200 μL) and incubated for 1 h at room temperature on a shaker.

Following these incubations, all preparations were washed as just described. FITC conjugated antirabbit IgG (Tago Immunologicals, Camarillo, CA) was added and incubated for 1 h on ice. Cells in each tube were washed and centrifuged twice and the cells resuspended in 2 mL of buffer. Cell-surface immunofluorescence was measured using a FACS Scan flow cytometer (Becton Dickinson, Cockeysville, MD).

Radioreceptor Assay

hFSHR expressed in CHO cells *(25)* was measured by equilibrium displacement binding isotherm assay using purified pituitary hFSH prepared in our laboratory for radiolabeled and unlabeled ligand. hFSH was purified as previously described *(26)*, omitting zone electrophoresis and finishing with monoclonal antibody affinity chroma-

Table 3
Characterization of Signal-Transduction Properties of 10 rFSHR Mutants

Mutation	Basal cAMP (pmol)	hFSH (10 ng) stimulated cAMP (pmol)	Net increase in cAMP in cAMP produced (pmol) (%)
Wild type	0.23 ± 0.023	1.42 ± 0.10	1.19 (100)
M1[b]	0.22 ± 0.005	0.70 ± 0.081	0.48 (40.3)
Wild type	0.25 ± 0.013	1.20 ± 0.11	0.95 (100)
M2	0.25 ± 0.021	1.16 ± 0.10	0.91 (95.7)
M3	0.19 ± 0.0082	1.04 ± 0.015	0.85 (89.5)
M4	0.23 ± 0.010	1.06 ± 0.012	0.83 (87.4)
M5	0.26 ± 0.0042	1.31 ± 0.11	1.05 (110.5)
M6	0.27 ± 0.0009	0.89 ± 0.014	0.62 (65.3)
M7	0.25 ± 0.0020	0.91 ± 0.0075	0.66 (69.5)
M8	0.24 ± 0.038	0.68 ± 0.041	0.44 (46.3)
M9	0.22 ± 0.0031	0.94 ± 0.15	0.72 (75.8)
M10	0.28 ± 0.015	1.16 ± 0.13	0.88 (92.6)

[a]Concentrations of basal and hFSH-stimulated cAMP were measured by RAA. The net increase is equal to the difference between cAMP production by 10,000 Sf9 cells incubated with hFSH (10 ng) and basal cAMP production over a 1.5-h period. Numbers in parentheses are the percentage increase relative to wild-type rFSHR. Data are the average of duplication in an experiment (triplicate determination). At least two independent experiments have been done for each mutant.

[b]The assays of M1 were done separately, and the positive control (wild type) assayed is included for comparison.

tography. For competitive displacement, fixed amounts of ^{125}I-hFSH (150,000 cpm) (specific activity = 70.0 μCi/μg) were used for binding to 500,000 CHO cells. Specificity of binding was determined by comparing the counts bound to cells when incubated in the absence or presence of 1 μg of unlabeled pure hFSH. Cells were washed twice and then left for approx 15 min in 5 mL of EDTA-PBS buffer. Cells were collected, centrifuged at 1,000*g* for 5 min, and counted. Cells were then homogenized in a Kontes glass homogenizer and suspended in HEPES buffer (0.05 *M* Hepes, 0.05 *M* $MgCl_2$, 0.1% bovine serum albumin). The RRA was carried out in a 400-μL reaction volume. Radioligand, plus increasing concentrations of hFSH (0 μg–1 μg), were incubated for 1 h prior to the addition of cell homogenates. When antireceptor antisera were used in the RRA, 10 μL of immune serum was incubated with 500,000 cells at 4°C overnight. Radioligand with or without 1 μg of unlabeled hFSH was added and incubated at room temperature on a shaker overnight. Following the second incubation, 2 mL of ice-cold HEPES buffer was added and the tubes were centrifuged. The supernatants were aspirated and pellets counted in a gamma counter (Tracor Analytic, Austin, TX).

Alanine Scanning Mutagenesis of hFSHR

The rFSHR cDNA was inserted into the mutagenesis vector pSELECT, as previously described (14). A *Kpn*I site was introduced between the stop codon of the rFSHR reading frame and the vector *Bam*HI site to facilitate mutagenesis and for subcloning purposes. Ten mutagenesis oligonucleotides (length: 42 or 43 bases) were designed to mutate three different consecutive amino acids to three alanines except that P273 and C275 remained intact (Table 2). One mutagenesis oligonucleotide was made to mutate GGTACC, the *Kpn*I site, to GGAACC, making mutagenized plasmids resistant to *Kpn*I digestion (*see* below). All oligonucleotides were synthesized at the Wadsworth Center Molecular Genetics Core facility. In vitro mutagenesis was performed with the Altered Sites In Vitro Mutagenesis System kit (Promega, Madison, WI), according to the manufacturer's instructions except that the oligonucleotide to restore ampicillin resistance was replaced by the oligonucleotide to mutate the *Kpn*I site. Plasmid DNA from mass cultures of repair-defective BMH 71-18 mutS bacteria was transformed with mutagenized plasmids, digested with *Kpn*I, and transformed into JM 109. Mutagenesis of targeted sequences in plasmids was confirmed using dideoxy sequencing *(27)*. *Eco*RI-*Bsu*36I fragments from these constructs were then ligated into the baculovirus transfer vector pVL-1392 rFSHR similarly digested. pVL-1392 rFSHR contains rFSHR sequences with the polyhedron sequences deleted (unpublished data). Transfer of a small (1-kbp) "mutation cassette" to the pVL-derived vector minimized the introduction of

extraneous mutations into the other rFSHR sequences. DNA sequencing verification of the mutation cassette on the pVL-1392 vector was also performed. Expression of the recombinant mutant rFSHRs in insect cells was performed as described previously *(14)*.

Assessment of rFSHR and Mutant Expression and Function

Sf9 cells (2×10^7) were infected with the wild-type rFSHR virus or the mutant viruses (10 MOI) and incubated for 3 d. Follitropin-binding activity of each receptor type on Sf9 cells was determined as follows. For competitive displacement, fixed amounts of ^{125}I-hFSH (150,000 cpm, 70.0 μCi/μg) were added to 100,000 cells. Displacement curves were generated with increasing concentrations of unlabeled hFSH (0–500 ng) purified in our laboratory. Other conditions and procedures were as previously described *(14)*.

Functional coupling to adenylate cyclase of each receptor type in Sf9 cells was determined by measurement of cAMP following a challenging dose of FSH ($^1/_2v_{max}$). Measurement of cAMP in insect cells has been described previously *(14)*. Protein production of each receptor type was assessed by Western blots of Sf9 cell lysates as described previously *(14)*.

Acknowledgments

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