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Pharmacological Characterization of Soluble Human FSH Receptor Extracellular Domain

Facilitated Secretion by Coexpression with FSH

Panayiotis E. Stevis,[1] Darlene C. Deecher,[1] Francisco J. Lopez,[2] and Donald E. Frail

Molecular Biology and Peptide Pharmacology Divisions, Women's Health Research Institute, Wyeth-Ayerst Research, 145 King of Prussia Rd., Radnor, PA

Follicle-stimulating hormone (FSH) is a member of the glycoprotein hormone family that regulates gametogenesis and steroidogenesis. Glycoprotein hormones signal through a unique class of G-protein-coupled receptors (GPCRs) that have a long extracellular domain (ECD), which is the primary site for hormone binding. The hFSHR-ECD was expressed in insect cells as a C-terminal, epitope-tagged protein resulting in production of soluble active receptor in the intracellular compartment and in the secreted culture medium. Coexpression of hFSHR-ECD with FSHβ or FSHα/β increased the secretion of the truncated receptor. Pharmacological studies to assess ligand–receptor interactions without the transmembrane domains showed higher affinity values (K_Ds) for $^{[125I]}$hFSH using mammalian-expressed full-length receptor, secreted hFSHR-ECD, or secreted hFSHR-ECD coexpressed with FSHβ, whereas the K_D value for hFSHR-ECD coexpressed with FSHα/β subunits showed lower affinity. Competition of other glycoprotein hormones for secreted hFSHR-ECD coexpressed with FSHβ or mammalian full-length hFSHR resulted in similar binding profiles, indicating analogous pharmacology. Finally, we have demonstrated that a small molecule, suramin, which has been reported to interact with the mammalian full-length FSHR, competes for the binding of $^{[125I]}$hFSH by interacting directly at the hFSHR-ECD.

Key Words: Glycoprotein hormones; suramin; ectodomain; baculovirus; epitope tag.

Introduction

Follicle-stimulating hormone (FSH) is a heterodimeric glycoprotein that shares a common α-subunit with other members of the pituitary glycoprotein hormone family (luteinizing hormone [LH] and thyroid-stimulating hormone [TSH]) and has a hormone-specific β-subunit that confers hormone selectivity. FSH is involved in the regulation of gametogenesis and steroid production via interaction with a membrane-bound receptor. The FSH receptor (FSHR) is a glycoprotein consisting of an extracellular domain (ECD) in excess of 300 amino acids and seven transmembrane segments typical of G-protein-coupled receptors (GPCRs) *(1)*. Hormone binding recognition sites have been identified in the N-terminus of FSHR-ECD *(2)*, and it is presumed that the binding interaction between the FSHR-ECD and the hormone leads to conformational changes that ultimately result in receptor activation. The nature of this interaction, however, and the specific contact sites between the receptor and the hormone are not well defined.

The baculovirus expression system has been extensively utilized for the production of mammalian proteins, including those of the GPCR family *(3–9)*. These recombinant receptors were found to be biochemically and functionally similar to the native receptors. Furthermore, the full-length FSHR was successfully expressed in *Spodoptera frugiperda (Sf9) (10)* and *Trichoplusia ni* (His) *(11)* insect cells, and shown to be properly localized and functional. Attempts to produce secreted isolated ECD of a glycoprotein hormone from insect or mammalian cells have been hampered by the propensity of the protein to be trapped intracellularly usually in an aggregated form *(12)*. Therefore, the addition of detergents for extraction and refolding of the protein were necessary in order to demonstrate hormone receptor interactions, which, in many cases, did not correlate with those observed with the full-length receptor.

Previous data demonstrated that FSH could facilitate the reactivation of denatured unrelated proteins, such as rRNase *(13)*. Therefore, we hypothesized that coexpression

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Author to whom all correspondence and reprint requests should be addressed: Dr. Panayiotis E. Stevis, Women's Health Research Institute, Wyeth-Ayerst Research, 145 King of Prussia Road, Radnor, PA 19087. E-mail: StevisP@WAR.Wyeth.com

[1]Both authors contributed equally to this manuscript.

[2]Current address: Ligand Pharmaceuticals, Inc., 10255 Science Center Drive, San Diego, CA 92121.

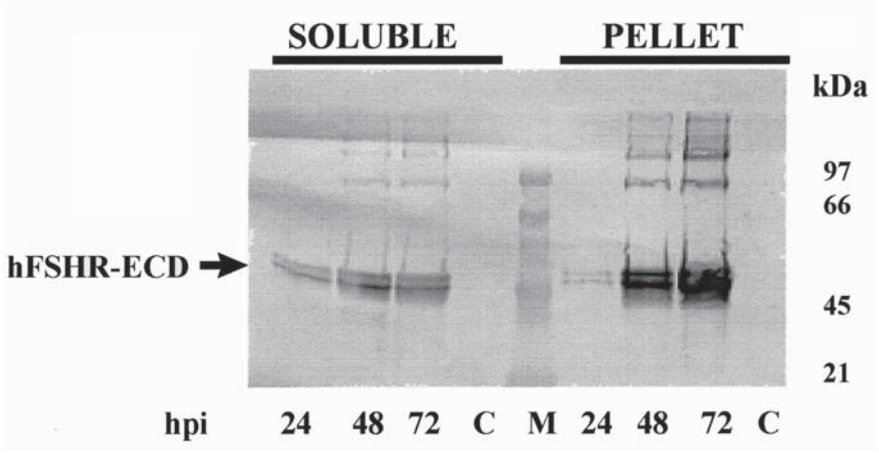

Fig. 1. Time-course of hFSHR-ECD expression levels of soluble intracellular and pellet (insoluble) material in insect cells. Hi5 cells were infected with hFSHR-ECD (MOI = 5) and then harvested at the indicated hours postinfection (hpi). C, protein samples derived from infection of insect cells with wild type virus; M, molecular-mass markers.

of the cognate ligand with hFSHR-ECD may facilitate its secretion by promoting proper folding of the ECD. The availability of the FSHR-ECD would facilitate both the pharmacological characterization of the hormone binding site on the N-terminus in the absence of the transmembrane domains, and the interaction site of small molecule agonists or antagonists of the hFSHR.

In this article, we have successfully produced a secreted soluble form of the hFSHR-ECD. The pharmacological profile of the hFSHR-ECD was compared to that of the mammalian full-length recombinant hFSHR and found to be similar. In addition, we tested the hypothesis of whether coexpression of the hFSHR-ECD with its respective ligand modifies the processing of the receptor. Our data indicate that coexpression of the hFSHβ with the receptor increases secretion of the FSHR-ECD, and the pharmacological characteristics of this receptor are similar to those of the secreted hFSHR-ECD produced in the absence of hFSHβ. Increased secretion of receptor coexpressed with hFSHα/β was also demonstrated, although this material showed lower affinity for $^{[125I]}$hFSH. Finally, the truncated form of the receptor was used to establish the ECD, rather than the transmembrane domains of the hFSHR, as the site of action for a small molecule modulator of the hFSHR.

Results

Production of hFSHR-ECD in Insect Cells

Glycoprotein hormone GPCRs are unique in having a large extracellular domain that has been identified as the primary site for hormone–receptor interactions. We expressed the hFSHR-ECD in the baculovirus expression system and used it to further our understanding of FSH–receptor interactions. The baculovirus transfer vector pAC8 was used to provide a strong polyhedrin promoter in order to obtain high levels of expression of the hFSHR-ECD in insect cells. The native signal sequence of the hFSHR was retained in the construct to maintain the natural configuration. An epitope tag (CLEPYTACD), recognized by an antibody referred to as Ab179, was included in the hFSHR-ECD to aid in analysis of expression by Western blot and to provide a means of capturing the receptor for use in a solid-phase radioligand binding assay.

In order to establish optimal expression of hFSHR-ECD, Hi5 cells were infected with recombinant baculovirus containing the hFSHR-ECD construct and harvested at various times postinfection. This analysis indicated that maximal soluble protein in the intracellular fraction was produced at 48 h postinfection (Fig. 1). Longer infection times (>72 h postinfection) resulted in an increase in the amount of insoluble receptor. No immunoreactive proteins were detected with extracts derived from insect cells infected with a nonrecombinant control virus harvested at 72 h postinfection.

Characterization of Secreted hFSHR-ECD

The capture of the C-terminus epitope-tagged hFSHR-ECD was accomplished as described in the Materials and Methods section. Solid-phase radioligand binding assays performed using concentrated culture supernatants showed detectable amounts of specific binding, indicating that soluble hFSHR-ECD was secreted and active. Interestingly, competition experiments performed using the secreted hFSHR-ECD showed higher affinity values (IC_{50}) for hFSH than the full-length hFSHR (Table 1).

Enhanced Secretion of hFSHR-ECD by Coexpression of FSH Subunits

Additional experiments were designed to determine whether the expression level of secreted hFSHR-ECD could be enhanced. Previous work on the reactivation of rRNase in the presence of LH and FSH *(13)* suggests that coexpression of FSHα/β or the individual FSH subunits with its native receptor might enhance the secretion of active

Table 1
Estimation of Affinity Values for hFSH Using Equilibrium Binding Assays to Determine K_Ds and Competition Assays to Generate IC_{50} Values Using Various hFSHR Preparations[a]

Source hFSHR	K_D, p*M*, ± SE	IC_{50}, p*M*, ± SE
Full-length hFSHR (mammalian)	287 ± 141	411 ± 97.1[d,e]
hFSHR-ECD (secreted)	96 ± 8.5[b,c]	31 ± 10.1[f]
hFSHR-ECD + FSHβ (secreted)	131 ± 14.6[c]	11.4 ± 3.5[f]
hFSHR-ECD + FSHα/β (secreted)	687 ± 174	780 ± 109.6

[a]Data were generated from at least two independent experiments performed on different lots of receptor extract. Affinity estimations were determined using customized JMP programs. All K_D values were generated using a slope estimate of 1, as indicated from the initial evaluation of the slope value. Statistical significance was determined using a pairwise comparison of K_D or IC_{50} values by Z-test.
[b]$p < 0.05$ vs hFSHR-ECD + FSHβ (secreted).
[c]$p < 0.001$ vs hFSHR-ECD + FSHα/β (secreted).
[d]$p < 0.0001$ vs hFSHR-ECD (secreted) and hFSHR-ECD + FSHβ (secreted).
[e]$p < 0.05$ vs hFSHR-ECD + FSHα/β (secreted).
[f]$p < 0.0001$ vs hFSHR-ECD + FSHα/β (secreted).

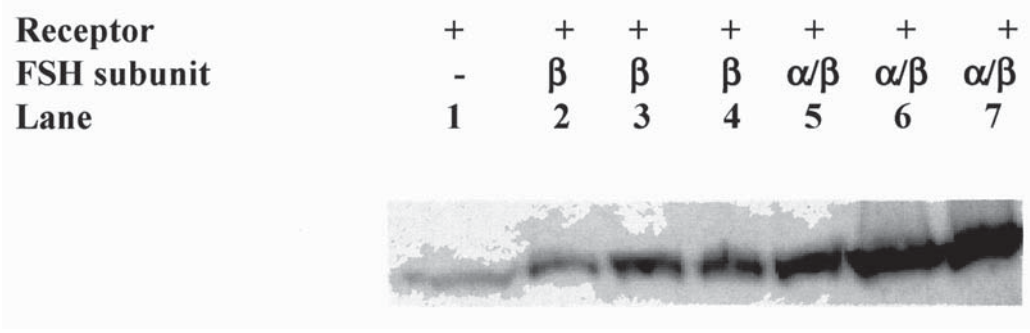

Fig. 2. Western blot of hFSHR-ECD using Ab179. Enhanced chemiluminescence Western blot detection system of culture supernatants derived from independent expression of hFSHR-ECD alone (lane 1), hFSHR-ECD with FSHβ (lanes 2–4) and hFSHR-ECD with FSHα/β (lanes 5–7).

hFSHR-ECD. His cells were infected with various combinations of virus containing the FSH subunits *(14)* and hFSHR-ECD. Equivalent volumes of culture supernatant were recovered at 48 h postinfection and analyzed by Western blot. Enhanced expression levels for the receptor were observed when hFSHR-ECD was coexpressed with FSHβ (Fig. 2, lanes 2–4) and FSHα/β (Fig. 2, lanes 5–7) relative to hFSHR-ECD alone (Fig. 2, lane 1). Equivalent amounts of the culture supernatants were also analyzed using the solid-phase radioligand binding assay. Coexpression of hFSHR-ECD with FSHβ or FSHα/β resulted in a statistically significant increase in $^{[125I]}$hFSH labeling (300 p*M*; 350,000) when compared to hFSHR-ECD alone (Fig. 3).

The affinity of hFSH for full-length hFSHR or secreted hFSHR-ECD coexpressed with FSHβ was high (p*M*) compared with the affinity values for other glycoproteins, which were low (n*M*) or were inactive at 1 μ*M* (Table 2). However, the IC_{50} values of hCG and hFSH were statistically significantly lower, indicating a higher affinity for hFSHR-ECD coexpressed with FSHβ than values noted for full-length hFSHR. Interestingly, hFSHR-ECD, which was derived by coexpression with FSHα/β, showed a lower affinity for FSH when compared to the other secreted receptor sources (Table 1).

Inhibition of FSH Binding to the hFSHR-ECD by Suramin

The ligand binding pocket for most GPCRs is contained within the transmembrane regions of the receptor *(15)*. In contrast, the ligand binding domain for the glycoprotein hormone receptors requires an interaction with the large ECD, and possibly a dual interaction or site of contact within the transmembrane regions of the receptor. Recently, a small molecule, suramin, a polysulfonated naphthylurea, has been shown to compete for the binding of $^{[125I]}$hFSH using full-length hFSHR receptor isolated from calf testis *(16)*. In order to determine if the binding site for this small molecule, suramin, resides in the hFSHR-ECD or requires the presence of the transmembrane regions, competition studies were performed using either the solid-phase cap-

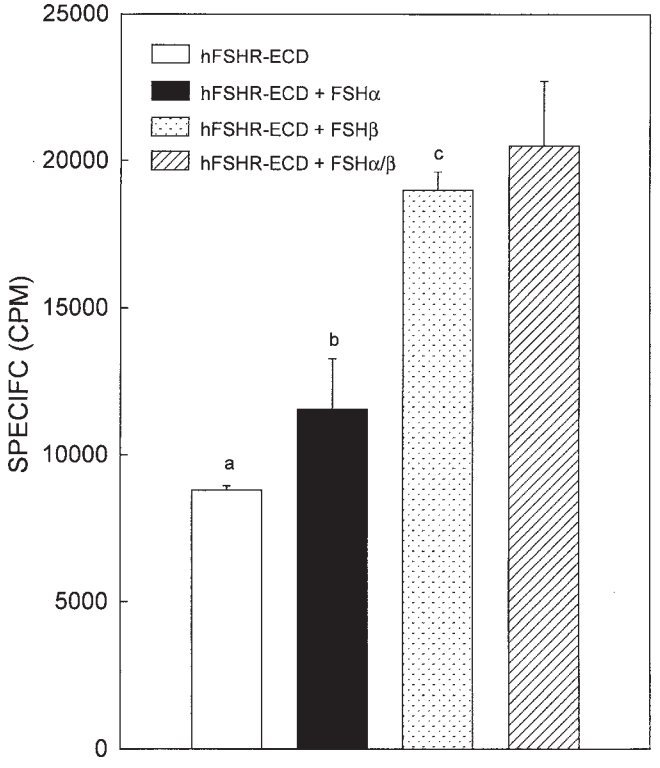

Fig. 3. Facilitated secretion of hFSHR-E CD by coexpression with FSH subunits. Specific binding (cpm) of [125I]hFSH (300 p*M*; 350,000 cpm) to secreted hFSHR-ECD, hFSHR-ECD coexpressed with FSHα, hFSHR-ECD coexpressed with FSHβ, or hFSHR-ECD coexpressed with FSHα/β using solid-phase radioligand binding assays. Statistically significance was determined using the least-significant difference test based on analysis of variance (ANOVA). **(A)** $p < 0.005$ vs hFSHR + FSHα, hFSHR + FSHβ and hFSHR + FSHα/β; **(B)** $p < 0.005$ vs hFSHR, hFSHR + FSHβ and hFSHR + FSHα/β; **(C)** $p < 0.005$ vs hFSHR and hFSHR + FSHα; hFSHR + FSHβ and hFSHR + FSHα/β are not statistically significantly different at level 0.05.

ture assay for hFSHR-ECD or the filtration radioligand binding assays for the mammalian full-length hFSHR. Suramin competed for [125I]hFSH binding using full-length hFSHR membrane preparations with an IC_{50} ± SE value of 62.4 ± 0.9 μ*M*. In parallel experiments, suramin competed for [125I]hFSH binding using captured secreted hFSHR-ECD coexpressed with FSHβ depicting a similar IC_{50} ± SE value of 54.5 ± 0.7 μ*M* (Fig. 4). The 95% confidence intervals for both IC_{50} values overlapped, suggesting no affinity differences between the two receptor forms. Therefore, these observations suggest that the binding site for suramin is contained within the ECD of the hFSHR, and the transmembrane region does not appear to be required for this binding interaction.

Discussion

The results of the present study provide evidence that the extracellular domain of the hFSHR contains ligand contact points of which both glycoproteins and small molecules, such as suramin, interact. This finding suggests that the transmembrane domains may play a secondary role in FSH binding. A solid-phase capture assay was used to characterize pharmacologically the hFSHR-ECD and compare the pharmacology of this receptor to the full-length hFSHR. Additionally, we have demonstrated that coexpression of the cognate ligand with the receptor enhances the secretion of the truncated form of the hFSHR.

Table 2
Selectivity Profile of Glycoprotein Hormones for Secreted hFSHR-ECD Coexpressed with FSHβ or Full-Length hFSHR in Competition Assays for [125I]hFSH

	Receptor source	
Glycoprotein	hFSHR-ECD + FSHb IC_{50}, n*M*, ± SE	Full-length hFSHR IC_{50}, n*M*, ± SE
hTSH	IA	IA
hCG	74 ± 10.82[b]	626 ± 115
hGH	IA	IA
hLH	97 ± 34.54	100 ± 39. 14
hFSH	0.011 ± 0.002[b]	0.411 ± 0.10
FSHα	IA	IA
FSHβ	IA	IA

[a]Values represent the means of IC_{50} (n*M*) values ± SE performed in at least two independent experiments. IA denotes inactive at 1 μ*M*. Statistical significance was determined using pairwise comparison of secreted vs full-length by Z-test.
[b]$p < 0.0001$ vs full length.

The sites involved in ligand binding in GPCRs typically involve amino acids present in several transmembrane helices *(17–19)*. The GPCRs for glycoprotein hormones are unique in having long extracellular domains, and these ECDs are thought to be an essential component of the ligand binding site *(20,21)*. However, the contribution of the transmembrane domains to the ligand binding site of glycoprotein hormones has not been completely addressed owing to the inability to express faithfully functional ECD in the absence of the transmembrane domains. For example, expression of the thyroid-stimulating hormone receptor-(TSHR) ECD in insect cells resulted in the production of large quantities of protein in the form of large aggregates that were not active in binding *(22)*. Refolding of the aggregates yielded a receptor that bound TSH, but with an order of magnitude lower affinity value than the value noted for the full-length receptor expressed in the same system. Further attempts to express TSHR-ECD using a different baculovirus transfer vector failed to yield reasonable amounts of TSHR-ECD, and the majority of the receptor remained trapped within the cell (293). Furthermore, expression of LH/CG (chorionic gonadotropin) hormone receptor-ECD in human embryonic kidney cells *(23)* was not secreted, and the protein was localized intracellularly *(24)*.

We have now successfully produced and characterized such material to distinguish the relative contributions of the

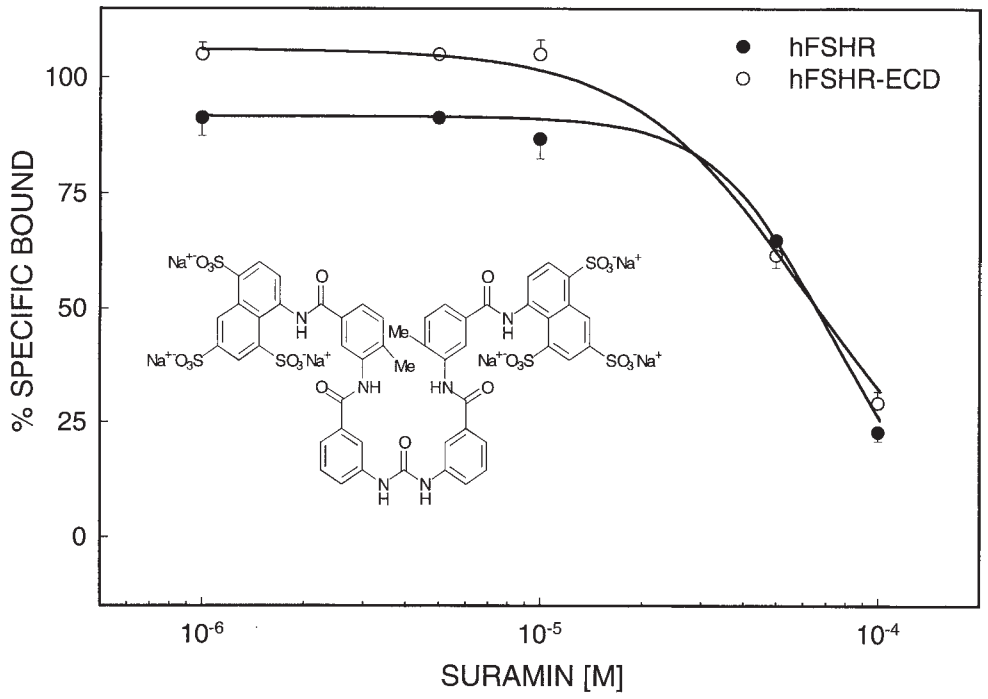

Fig. 4. Competition of suramin for [125I]hFSH using extracts from mammalian full-length hFSHR or secreted hFSHR-ECD coexpressed with FSHβ. Values depicted in the graph are the means ± SE of triplicate determinations of % specific bound at each concentration of suramin tested. Control binding (100%) was measured in the presence of buffer only. Competition curves using secreted hFSHR-ECD coexpressed with FSHβ (○) or hFSHR (full length) (●) yielded IC_{50} ± SE (95% confidence limits) values of 61.0 ± 3.1 (55–68) and 70.0 ± 3.3 (64–78) μ*M*, respectively. IC_{50} values and 95% confidence intervals were generated using a customized JMP program. Competitions were repeated twice with different receptor extracts.

ECD and transmembrane domains to the ligand binding site. Human FSHR-ECD containing a carboxy-terminal epitope tag was expressed in Hi5 insect cells and was localized in the intracellular compartment as both soluble and insoluble protein, as shown by Western blot analysis. Forty-eight hours postinfection, maximal levels of soluble intracellular hFSHR-ECD were yielded. The majority of the hFSHR-ECD produced in insect cells was localized intracellularly when the receptor was expressed alone.

The intracellular hFSHR-ECD soluble fraction was used initially to develop a solid-phase radioligand binding assay, which was subsequently used to characterize the secreted form of the receptor. The detection of secreted hFSHR-ECD in the culture supernatant of recombinant baculovirus-infected insect cells was facilitated by the purification, enrichment, and proper spatial orientation provided by the capture assay format. The purification and enrichment served to remove proteins that contribute to background while concentrating the tagged hFSHR-ECD. Capture of the receptor via the C-terminal tag served to place the receptor in a nonsterically hindered orientation, comparable to its position in the membrane, thereby allowing full access to the ligand. The assay does not assess the contribution of any low-affinity binding sites present in the extracellular loops and transmembrane regions of the receptor *(25,26)*.

Secretion of hFSHR-ECD into the culture medium was enhanced by coexpression with FSHβ or FSHα/β. This enhancement of secreted ECD was demonstrated by Western blot analysis of the proteins; in the culture supernatants as well as by radioligand binding methods. Pharmacologically, the binding characteristics of the secreted hFSHR-ECD are similar to those noted for the full-length hFSHR used in this study. Expression of the full-length rat FSHR using the baculovirus insect cell expression system depicts K_D values of 31 p*M*, which are similar to the values obtained using membrane preparations from native rat testicular receptors of 55 p*M (11)*. Additionally, the affinity values for full-length hFSHR expressed in *Sf9* cells are 170 p*M*, which are comparable to the values shown using calf testis membranes *(10)*. Therefore, our K_D values obtained for the secreted hFSHR-ECD are comparable to values reported by others for both the native and baculovirus-produced full-length FSHR *(10,11)*. The affinity values of hFSH for full-length hFSHR or secreted hFSHR-ECD coexpressed with FSHβ were high (p*M*) compared with the affinity values for other glycoproteins, which were low (n*M*) or were inactive at 1 μ*M* (Table 2). However, the IC_{50} values of hCG and hFSH were lower in a statistically significant manner, indicating a higher affinity for hFSHR-ECD coexpressed with FSHβ than values noted for full-length hFSHR. This could reflect an inhibitory influence of the transmembrane region owing to spatial accessibility of the ligand, or could reflect differences between mammalian and insect-based expression systems. It should be noted that the activity shown for

hCG in this article does not reflect the activity noted by others. The hCG competing for $^{[125I]}$hFSH using full-length hFSHR expressed in *Sf9* cells was inactive, and only equine CG competed for $^{[125I]}$hFSH binding *(10)*. It is possible that the quality of these glycoproteins differs depending on means of production and purification, and that differences noted between our study and others is owing to different glycoprotein material used in these studies. It is also possible that the affinity values of glycoproteins that have been reported to crossreact with the FSHR (LH, CG) increase owing to the accessibility of binding contacts on the ECD.

The increase in $^{[125I]}$hFSH labeling noted when hFSHR-ECD was coexpressed with FSHβ or FSHα/β compared to receptor alone suggests that FSHβ subunit or FSHα/β may be facilitating the folding and/or secretion of the hFSHR-ECD. The secreted hFSHR-ECD and hFSHR-ECD coexpressed with FSHβ showed similar affinities for $^{[125I]}$hFSH, whereas the hFSHR-ECD coexpressed with FSHα/β showed a lower affinity. The lower affinity may be owing to receptor occupancy, differences in glycosylation, or a change in conformation resulting from an interaction with FSH during coexpression. The exact mechanism for the facilitated secretion of hFSHR-ECD by the FSH subunits remains unknown. Presumably, FSH, which is expressed efficiently as a functional heterodimer in insect cells *(14)*, may be serving as a scaffold for the proper folding of the hFSHR-ECD within the endoplasmic reticulum or Golgi apparatus. This interaction may divert the receptor from the aggregated state, which would preclude its secretion. A more active role for FSH in the proper folding of the receptor may be suggested from the proposed thioredoxin-like catalytic activity of the FSHβ subunit *(13)*.

The availability of the secreted hFSHR-ECD and the capture assay provide an ideal opportunity to study ligand–receptor interactions at the ectodomain and assess the effect of various peptides and chemical compounds on ligand binding. Since the baculovirus-produced hFSHR-ECD appears to have similar pharmacological characteristics to the mammalian full-length hFSHR, our system provides an ideal opportunity to study ligand–receptor interactions at the ectodomain and evaluate the role of the transmembrane domains in ligand binding. The proper secretion and spatial arrangement of the receptor will provide a less complex system to assess and determine whether multiple contact sites for ligand interactions exist for the FSHR. As a demonstration of this application, suramin, a small-molecule FSH ligand, was utilized. Suramin has been reported to inhibit the binding of $^{[125I]}$hFSH directly using the full-length FSHR prepared from calf testis with a reported IC_{50} value of 10 μ*M* *(16)*. These reports suggest that suramin may be acting directly at the FSHR. Therefore, we wanted to determine whether small molecules, such as suramin, would also interact with the ECD. Preliminary experiments to assess the specificity and interaction of suramin demonstrated that surmain showed binding interactions with FSH and LH for their respective receptors, but showed no interaction for other GPCRs, such as galanin (D. C. D., unpublished observation). Additionally, we determined that suramin was a competitive inhibitor for FSHR using kinetic and competitive equilibrium binding assays for the full-length hFSHR (D. C. D., unpublished observation). Therefore, suramin was used to compete for $^{[125I]}$hFSH binding using the secreted hFSHR-ECD coexpressed with FSHβ, and we showed for the first time a direct interaction of suramin with the ECD. Thus, we expanded previous studies *(16)* by demonstrating that suramin directly interacts at the ECD of the hFSHR, and unlike the majority of GPCRs, the transmembrane domains of the hFSHR do not appear to be essential for suramin's action.

Our findings also point out that the strategy of ligand-assisted receptor domain expression could have applicability to other receptor–ligand combinations. The enhanced secretion of hFSHR-ECD observed with the cognate ligand may also have physiological implications in systems here receptor and ligand are coexpressed *(27)*. Furthermore, the reduced K_D value observed for $^{[125I]}$hFSH for hFSHR-ECD coexpressed with FSHα/β may reflect a mechanism for receptor desensitization, unique to the glycoprotein hormone receptor family. This desensitization may involve an induced conformational change following exposure to ligand. These points are the focus of further studies aimed at understanding the role of the ECD in hormone action and receptor function.

Materials and Methods

Materials

Affinity-purified human pituitary FSH as well as the other glycoprotein hormones (hLH, hTSH, hCG) were purchased from Patrick Sluss, Reproductive Endocrine Unit, Massachusetts General Hospital (Charlestown, MA). Stock solutions of proteins were made at 100 μ*M* in FSH binding buffer (10 m*M* Tris HCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 0.1% bovine albumin, 0.025% NaN_3), aliquoted, and stored at –80°C until the day of the assay. Stock solutions were serially diluted in FSH binding buffer on day of the assay. Iodinated hFSH was purchased from NEN™ Life Sciences Products (Boston, MA).

Construction of Baculovirus Transfer Vectors

An epitope-tagged hFSHR-ECD cloned in a mammalian expression vector was kindly provided by K. Koller, Affymax Research Institute (Palo Alto, CA). Using a combination of restriction digests and polymerase chain reactions (PCRs), the epitope-tagged hFSHR-ECD was recreated in the transfer vector pAC8 (Clontech, Palo Alto, CA). The hFSHR-ECD corresponds to amino acids 1–349 of the mature receptor and was designed to include the native signal sequence. The epitope tag, CLEPYTACD, is

recognized by an antibody referred to as Ab179, kindly supplied by Affymax Research Institute.

The cDNAs for FSHα and FSHβ were isolated from human pituitary polyA+ RNA (Clontech) as described previously *(14)*. The cDNA fragments were cloned into the baculovirus transfer vector pBluBacIII (Invitrogen, San Diego, CA) and used for the generation of high-titer viral stocks (HTVS) for the expression of recombinant FSH.

Insect Cell Conditions

The insect cell lines *Spodoptera frugiperda (Sf9)* and *Trichoplusia ni* (Hi5) were obtained from Invitrogen Corporation (San Diego, CA) and maintained in EXCEL-400 medium (JRH Biosciences, Lenexa, KS). Recombinant baculovirus for hFSHR-ECD was produced by cotransfection of *Sf9* cells (2×10^6 cells in T25 flask) with 500 ng of Baculogold viral DNA (Pharmingen, San Diego, CA) and 2.5 μg of transfer vector, using the calcium phosphate transfection protocol recommended by the supplier. The transfection supernatant was harvested after 3 d and subjected to one round of plaque purification. Individual plaques that were positive for expression of recombinant protein based on Western analysis with Ab179 were subjected to two rounds of amplification to generate an HTVS. All expression experiments were performed in Hi5 cells, which were maintained in EXCEL 400 serum-free medium. An expression time-course for the hFSHR-ECD was performed with Hi5 cells seeded at a density of 2×10^7 cells/T_{150} flask and infected at a multiplicity of infection (MOI) of 5. Generation of recombinant viruses for the FSH subunits was previously described *(14)*, and for these experiments, an MOI of 2 was used for the respective baculovirus stocks. Infected cells and culture supernatants were harvested at regular intervals and analyzed by Western blot with Ab179 and radioligand binding assays.

SDS-PAGE and Western Blots

For the analysis of hFSHR-ECD, protein extracts were resolved using denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Novex, San Diego, CA). For Western analysis, proteins were transferred to nitrocellulose (Bio-Rad, Hercules, CA), and the filters were subsequently incubated in TBST buffer (0.05 *M* Tris-HCl, pH 8.0, 0.138 *M* NaCl, 0.0027 *M* KCl, 0.05% Tween 20) containing 10% nonfat dry milk. Immunoreactive proteins were detected by incubation of primary antibody in TBST buffer containing 10% nonfat dry milk. The monoclonal antibody (MAb) Ab179 (1:2000 dilution) was used to detect the epitope tagged hFSHR-ECD. The secondary antibody, goat antimouse coupled to horseradish peroxidase, was purchased from Bio-Rad Laboratories and was used at 1:10,000 dilution. Signal development of Western blots was achieved by the enhanced chemiluminescence (ECL™) system (Amersham Life Sciences, Arlington Heights, IL).

Preparation of Receptor for Radioligand Binding Assay

Secreted

Clarified culture supernatants were obtained by centrifugation and dialyzed against 0.1× phosphate-buffered saline (PBS) (2 × 4 L) using dialysis tubing with a molecular-weight exclusion limit of 12–14 kDa (Gibco-BRL, Gaithersburg, MD). The clarified supernatants were frozen, lyophilized, and stored at –70°C. Prior to assay, the material was resuspended in dH_2O (1/10 initial volume) and further diluted in 1× PBS as needed.

Full-Length hFSHR

A Chinese hamster ovary cell line (K_1) expressing the mammalian full-length hFSHR was kindly provided by K. Koller (Affymax Research Institute). The preparation of receptor for radioligand binding was performed as described previously *(14)*.

Radioligand Binding Assay

For the solid-phase capture assay, Immulon-4 96-well microtiter plates (Dynatech, Chantilly, VA) were coated with 100 μL/well of 25 μg/mL streptavidin (Pierce, Rockford IL) in 0.05 *M* carbonate buffer (pH 9.6) and incubated for 1 h (37°C). The plates were then rinsed four times with 1× PBS (pH 7.4), tamped to dryness, and 100 μL/well of biotinylated Ab179 (10 μg/mL in PBS) was added. The antibody (Ab179) was incubated in the plates for 1 h at 37°C. The plates were rinsed with 1× PBS, and then 200 μL/well of 1% bovine serum albumin (BSA) in 1× PBS was added to block nonspecific sites. These plates were incubated with blocking agent for 1 h at 37°C followed by two rinses (1× PBS) and tamped to dryness. Diluted extracts of hFSHR-ECD were added to the pre coated plates and incubated for 2 h at 4°C to allow for capture of the ECD of the receptor. Microtiter plates containing captured hFSHR-ECD were rinsed twice with 200 μL of FSH buffer (10 m*M* Tris-HCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 0.1% BSA, pH 7.2), and 100 μL of FSH buffer was added to each well. Wells representing total $^{[125I]}$hFSH bound received 50 μL of buffer, and wells determining nonspecific binding received 50 μL of 1 μ*M* hFSH. Initiation of the binding reaction was started by adding 50 μL of various concentrations of $^{[125I]}$hFSH made in FSH binding buffer. Typically for competition assays, 100–300 p*M* (100,000– 400,000 cpm) $^{[125I]}$hFSH (SA = 2900–3300 Ci/m*M*) were used, and for equilibrium binding assays, 20–2000 p*M* (25,000–2,500,000 cpm). The reaction was incubated on an orbital shaker for 3 h (25°C), and thereafter, stopped by rinsing the plates with 1.2 mL of ice-cold wash buffer (50 m*M* Tris-HCl, 10 m*M* $MgCl_2$, 0.5 m*M* ethylenediamine tetraacetic acid [EDTA], pH 7.2) to remove free radioligand. Bound radioactivity was determined by incubating the plates with 100 μL/well of 0.2 *M* HCl for 15 min to release the bound $^{[125I]}$FSH. The 100-μL sample of HCl was collected and counted for 1 min

using a γ-counter (ICN Biomedical, Costa Mesa, CA) to determine the amount of radioactivity bound. Radioligand binding assays for the human full-length receptor were performed as described previously *(14)*.

Statistical Analysis

A customized JMP (SAS Institute, Cary, NC) application was written using a four-parameter logistic model to determine IC_{50} values. A square-root transformation and Huber weighting were performed on cpm. Total bound cpm and nonspecific bound cpm were used in the analysis as the maximum and minimum of the competition curves, respectively. For compounds repeated over several days, the IC_{50}s were weighted by their respective standard errors (SE) to obtain an average IC_{50} and a confidence interval using a customized JMP (SAS Institute) application. A three-parameter logistic model with parameters K_D, B_{max}, and slope was fitted to evaluate the two-site saturation models. If the slope estimate indicated a one-site model (slope not significantly different than 1), the slope was locked to 1, and the analysis was rerun to provide a linear Rosenthal plot. In contrast, if the slope differed significantly from 1, a curvilinear plot was generated, and the two-site saturation model was run to determine the binding parameters of each binding site. Using this procedure, we were able to demonstrate that a one-site model was suited for our data. Statistical significance between IC_{50}s and K_Ds was determined using a pair-wise Z-test. The customized JMP applications were developed by Biometrics Research (Wyeth-Ayerst, Princeton, NJ). Statistical significance for the amount of FSHR-ECD produced was determined using the least-significant-difference test after a one-way analysis of variance (ANOVA). A p value of 0.05 was defined as the minimum criterion to establish statistically significant differences.

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References

1. Sprengel, R., Braun, T., Nikolics, K., Segaloff D. L., and Seeburg P. H. (1990). *Mol. Endocrinol.* **4,** 525–530.
2. Dattatreyamurty, B. and Reichert, L., Jr. (1993). *Mol. Cell. Endocrinol.* **93,** 39–46.
3. Mills, A., Allet, B., Bernard, A., Chabert, C., Brandt, E., Cavegn, C., et al. (1993). *FEBS Lett.* **320,** 130–134.
4. Mouillac, B., Caron, M., Bonin, H., Dennis, M. and Bouvier, M. (1992). *J. Biol. Chem.* **267,** 21,733–21,737.
5. Ng, G. Y., George, S. R., Zastawny, R. L., Caron, M., Bouvier, M., Dennis, M., and O'Dowd, B. F. (1993). *Biochemistry* **32,** 11,727–11,733.
6. Parker, E. M., Grisel, D. A., Iben, L. G., Nowak, H. P., Mahle, C. D., Yocca, F. D., et al. (1994). *Eur. J. Pharmacol.* **268,** 43–53.
7. Richardson, R. M. and Hosey, M. M. (1992). *J. Biol. Chem.* **267,** 22,249–22,255.
8. Schreurs, J., Yamamoto, R., Lyons, J., Munemitsu, S., Conroy, L., Clark, R., et al. (1995). *J. Neurochem.* **64,** 1622–1631.
9. Woodcock, C., Graber, S. G., Rooney, B. C., and Strange, P. G. (1995). *Biochem. Soc. Trans.* **23,** 93S.
10. Christophe, S., Robert, P., Maugain, S., Bellet, D., Koman, A., and Bidart, J. M. (1993). *Biochem. Biophys. Res. Commun.* **196,** 402–408.
11. Liu, X., DePasquale, J. A., Griswold, M. D., and Dias, J. A. (1994). *Endocrinology* **135,** 682–691.
12. Huang, G. C., Page, M. J., Nicholson, L. B., Collison, K. S., McGregor, A. M., and Banga, J. P. (1993). *Mol. Endocrinol.* **10,** 127–142.
13. Boniface, J. J. and Reichert, L. E., Jr. (1990). *Science* **247,** 61–64.
14. Arey, B. J., Stevis, P. E., Deecher, D. C., Shen, E. S., Frail, D. E., Negro-Vilar, A., et al. (1997). *Mol. Endocrinol.* **11,** 517–526.
15. Watson, S. and Arkinstall, S. (1994). *The G-Protein Linked Receptor Factsbook.* Academic: San Diego, CA. p. 427.
16. Daugherty, R. L., Cockett, A. T., Schoen, S. R., and Sluss, P. M. (1992). *J. Urol.* **147,** 727–732.
17. Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G., and Lefkowitz R. J. (1988). *Science* **240,** 1310–1316.
18. Strader, C. D., Candelore, M. R., Hill, W. S., Dixon, R. A., and Sigal, I. S. (1989). *J. Biol. Chem.* **264,** 16,470–16,477.
19. Strader, C. D., Sigal, I. S., and Dixon, R. A. (1989). *FASEB* **3,** 1825–1832.
20. Tsai-Morris, C. H., Buczko, E., Wang, W., and Dufau, M. L. (1990). *J. Biol. Chem.* **265,** 19,385–19,388.
21. Xie, Y. B., Wang, H., and Segaloff, D. L. (1990). *J. Biol. Chem.* **265,** 21,411–21,414.
22. Seetharamaiah, G. S., Kurosky, A., Desai, R. K., Dallas, J. S., and Prabhakar, B. S. (1994). *Endocrinology* **134,** 549–554.
23. Chazenbalk, G. D. and Rapoport, B. (1995). *J. Biol. Chem.* **270,** 1543–1549.
24. Thomas, D. M. and Segaloff, D. L. (1994). *Endocrinology* **135,** 1902–1912.
25. Ji, I. and Ji, T. H. (1991). *J. Biol. Chem.* **266,** 13,076–13,079.
26. Ji, I. and Ji, T. H. (1995). *J. Biol. Chem.* **270,** 15,970–15,973.
27. Lei, Z. M., Toth, P., Rao, C. V., and Pridham, D. (1993). *J. Clin. Endo. Metab.* **77,** 863–872.