0013-7227/92/1312-0799$03.00/0
Endocrinology

Vol. 131, No. 2
Printed in U.S.A.

Expression of Recombinant Human Follicle-Stimulating Hormone Receptor: Species-Specific Ligand Binding, Signal Transduction, and Identification of Multiple Ovarian Messenger Ribonucleic Acid Transcripts*

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ABSTRACT

The ligand specificity and biochemical properties of the human (h) FSH receptor are poorly characterized due to the low abundance of these receptors and the limited availability of human tissues. Using a fragment of rat FSH receptor cDNA, we screened a human testicular cDNA library and obtained a FSH receptor cDNA covering the entire amino acid-coding region. After transfection of a human fetal kidney cell line (293) with the hFSH receptor cDNA, radioligand receptor analysis revealed the presence of high affinity (K_d, 1.7×10^{-9} M) FSH-binding sites on the plasma membrane. Both recombinant and wild-type hFSH displaced [^{125}I]hFSH binding, with ED_{50} values of 25 and 70 ng/ml, respectively, whereas hLH, hCG, and hTSH were ineffective. Although human, rat (r), and ovine FSH as well as equine CG competed for rat testicular FSH receptor binding, only hFSH and rFSH interacted effectively with the recombinant hFSH receptor, suggesting that species-specific ligand recognition exists between human and rodent receptors. After incubation of transfected cells with hFSH, but not recombinant hLH or hCG, a dose-dependent increase (ED_{50}, 10 ng/ml) in extracellular cAMP accumulation was observed, indicating a functional coupling of the expressed human receptor with the endogenous adenyl cyclase. In cells cotransfected with the FSH receptor expression plasmid and a luciferase reporter gene driven by the promoter of a cAMP-responsive gene, treatment with hFSH, but not hCG, resulted in a dose-dependent increase in luciferase activity. Northern blot analysis using a cRNA probe derived from the human receptor cDNA indicated the presence of multiple FSH receptor mRNA transcripts (7.0, 4.2, and 2.5 kilobases) in RNA prepared from human follicular phase ovary, but not from human corpus luteum or placenta. Additionally, two FSH-binding sites of 76 and 112 kilodaltons were detected in transfected 293 cells after ligand/receptor cross-linking and sodium dodecyl sulfate-polyacrylamide gel electorphoresis analysis. These results demonstrate the expression of functional hFSH receptor with unique ligand specificity and provide new data on the biochemical properties of the human receptor at the mRNA and protein levels. (*Endocrinology* **131:** 799–806, 1992)

FSH IS a member of the glycoprotein hormone family that also comprises LH, CG, and TSH (1, 2). Hormones of this family are dimers consisting of a common α-subunit and hormone-specific β-subunits joined together by noncovalent binding (3). As with the other glycoprotein hormones, FSH binding to target cells increases adenyl cyclase activity through interaction with membrane-associated G-proteins (4), thus classifying the FSH receptor to the G-protein-coupled receptor family (5–7). The hallmark of G-protein-coupled receptors is the presence of seven transmembrane-spanning segments that possess a homologous cluster of six or seven amino acid residues located on the carboxyl-terminal region of the third cytoplasmic loop. Recent molecular cloning of rat LH and FSH receptors have indicated that this conserved region, which has been implicated in G-protein coupling of the β-adrenergic receptor (8–10) and TSH receptor (11), is also present in the third cytoplasmic loop of gonadotropin receptors (12, 13).

Although the importance of FSH in testicular and ovarian development and reproductive function has been unequivocally established (for review, see Refs. 14–16), the limited availability of human gonadal tissues as well as the paucity of gonadal FSH-binding sites have precluded the study of human (h) FSH receptors. Recent studies from our laboratory have indicated that the ligand specificity of human *vs.* rat (r) LH receptors is dramatically different, suggesting that the properties of human gonadotropin receptors may differ from those of experimental animal models (17). To more clearly elucidate the properties of hFSH receptors, we report here the expression of a functional recombinant hFSH receptor with unique ligand specificity. Furthermore, the distribution of FSH receptor mRNA in human reproductive tissues and the biochemical properties of the expressed receptor are presented.

Received February 7, 1992.

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* This work was supported by NIH Grant HD-23273.

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Materials and Methods

Reagents and hormones

Restriction enzymes were obtained from Bethesda Research Laboratories (BRL; Gaithersburg, MD), Boehringer-Mannheim (Indianapolis, IN), and Stratagene (La Jolla, CA). A λgt11 human testicular cDNA

library was obtained from Clontech (Palo Alto, CA). The eukaryotic expression vector pCMX was a gift from Dr. K. Umesono of The Salk Institute (San Diego, CA). The recombinant human gonadotropin preparations were derived from serum-free conditioned medium of Chinese hamster ovary (CHO) cell lines transfected with human gonadotropin genes. The recombinant hLH contains a deletion of seven hydrophobic amino acids at the carboxyl-terminus and a substitution of Trp to Arg at position eight of the β-subunit for efficient dimerization and secretion from CHO cells (18). The recombinant hFSH has biological activity and chromatofocusing profiles similar to those of purified pituitary FSH (19). The concentrations of the recombinant gonadotropins were estimated in RIAs using purified pituitary preparations as standards (hLH, hLH I-3, 5,900 IU/mg; hFSH, hFSH I-3, 3,100 IU/mg by the hCG augmentation assay). hFSH (I-3), hCG (CR-127; 14,900 IU/mg), hLH (B1; 4,015 IU/mg), hTSH (B1; 15 IU/mg), rFSH (I-7; 4,714 IU/mg), and ovine (o) FSH (oFSH-17; 25 IU/mg) were obtained from the National Hormone and Pituitary Distribution Program (Bethesda, MD); recombinant hTSH was obtained from Genzyme (Cambridge, MA); equine (e) CG (PMSG; 2,530 IU/mg) was obtained from Calbiochem (La Jolla, CA); and porcine (p) FSH was the gift of Dr. H. Papkoff (University of California-San Francisco). Reagents required for the luciferase assay were purchased from Analytical Luminescence Laboratory (San Diego, CA).

cDNA library screening

A fragment of rFSH receptor cDNA, corresponding to bases 621–1031 of the published sequence (13), was obtained by reverse transcription-polymerase chain reaction of RNA prepared from PMSG-primed rat ovaries (20). This cDNA fragment was radiolabeled by the random priming method (21) and used to screen the human testicular cDNA library (22). Eight positively hybridizing phage clones ranging in size from 1.8–2.2 kilobases (kb) were isolated, subcloned into the pBluescript II SK plasmid (Stratagene), and sequenced using the dideoxy chain termination method (23) with a DNA sequencing kit (U.S. Biochemical Corp., Cleveland, OH) and specific primers. Individual fragments obtained from two separate clones (H37 and G3) were prepared by *Bam*HI restriction enzyme digestion and ligated at nucleotide position 686 to yield the final hFSH receptor cDNA construct containing the entire amino acid-coding region. Clone H37 contains 75-basepairs (bp) of 5′-untranslated region plus 1562 bp of open reading frame, but lacks the 3′-end and polyadenylation signal. Clone G3 starts at 168 bp of the open reading frame and covers the entire amino acid-coding region to the termination codon plus 26 bp of the 3′-untranslated region. These clones contain a 1394-bp overlapping region with identical nucleotide sequence, the identity of which was further confirmed by sequence analysis of the remaining six clones. The DNA sequence of the final clone was determined on both strands and compared to the published sequence of a cloned, but not expressed, human ovarian FSH receptor cDNA (24). Sequence comparison indicated seven individual basepair substitutions between our and the reported clone, resulting in five different amino acids at the following positions: 112 (Thr to Asn), 197 (Ala to Glu), 198 (Val to Leu), 307 (Ala to Thr), and 680 (Ser to Asn).

Expression of full-length cDNA in eukaryotic cells

An *Eco*RI linker (Promega, Madison, WI) was inserted into the *Eco*RV restriction site of the pCMX expression vector (17) to generate a plasmid (designated pCME) containing an *Eco*RI cloning site. The cDNA construct coding for the entire hFSH receptor (−75 to 2085 bp plus 26 bp of the 3′-untranslated region) was then subcloned into the *Eco*RI site of the pCME vector and partially sequenced to determine the orientation of the cDNA insert. Exponentially growing 293 cells derived from human fetal kidney were transiently transfected in 5 ml Dulbecco's Modified Eagle's Medium (DMEM; supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate; Gibco, Santa Clara, CA) with the expression plasmid (designated pCME-hFSHR) using the calcium phosphate precipitation method (25) used routinely in our laboratory (17). Twenty-four hours after transfection, FSH receptor binding studies were performed by incubating cells (2×10^5/0.4 ml) with [^{125}I]hFSH at 22 C for 20 h or as indicated. Iodination of hFSH (I-3) was performed using the lactoperoxidase method (26). The specific activity and maximal binding of [^{125}I]hFSH, as determined by radioligand receptor assay using rat testicular membranes, were 99,000–110,000 cpm/ng and 7%, respectively. Nonspecific binding was determined by inclusion of a 1,000-fold excess of unlabeled ligand (Pergonal, Serono Laboratories, Randolph, MA). Similar procedures were used to analyze the ligand specificity of FSH-binding sites in testicular homogenates prepared from 15-day-old Sprague-Dawley rats (Johnson Labs, Bridgeview, IL). For cAMP analysis, transfected 293 cells (2×10^5/culture dish) were treated with various gonadotropins in the presence of 0.25 mM 3-isobutyl-1-methylxanthine (MIX; Sigma Chemical Co., St. Louis, MO) for 2 h at 37 C. After incubation, extracellular cAMP levels were determined by specific RIA, using [^{125}I]cAMP (ICN, Costa Mesa, CA) as the labeled ligand and a commercially available cAMP antiserum (ICN) (27). The intra- and interassay coefficients of variation were 6% and 10%, respectively.

Luciferase reporter gene

A 654-bp fragment of the 5′-flanking sequence of the rat tissue plasminogen activator (tPA) gene ligated to the luciferase gene plasmid p19LUC (28) was used (designated ptPA-LUC) (29). This portion of the tPA promoter region contains a cAMP-responsive element capable of mediating gonadotropin-stimulated tPA gene transcription (29). Exponentially growing 293 cells were transiently transfected in 5 ml DMEM with pCME-hFSHR and ptPA-LUC plasmids (at a ratio of 0.8:0.2; 7.5 μg total DNA). After transfection, cells were collected, counted, and dispensed into 12 × 75-mm culture tubes (5×10^4 cells/tube). The total volume was brought to 0.3 ml with DMEM containing 0.25 mM MIX with hFSH or hCG, and cells were incubated for 18 h at 37 C. After incubation, cells were lysed by the addition of 0.3 ml/tube 2 × lysis buffer [50 mM Tris-phosphate (pH 7.8), 4 mM dithiothrietol, 4 mM 1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid, 20% glycerol, and 2% Triton X-100] at 22 C for 15 min. For estimation of luciferase activity, 10 μl cell lysate mixture were combined with 100 μl assay reagent [20 mM tricine, 1.07 mM $(MgCO_3)_4$ $Mg(OH)_2$–$5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothrietol, 270 μM coenzyme-A, 470 μM luciferin, and 530 μM ATP], and light production was immediately measured for 10 sec in a luminometer (Monolight 2010, Analytical Luminescence Laboratory).

Preparation of nucleic acid probe for hFSH receptor mRNA

A fragment of the hFSH receptor cDNA, corresponding to bases 744–1026, was isolated after digestion of the hFSH receptor cDNA with the *Hin*cII restriction enzyme. This fragment, which possesses less than 50% nucleotide sequence homology to the hLH receptor cDNA (17), was subcloned into the pGEM4z vector (Promega). The plasmid clone was linearized with the *Sal*I restriction enzyme and served as a template for the production of a cRNA probe using T7 RNA polymerase (BRL) and [α-^{32}P]CTP (3000 Ci/mmol; Amersham, Arlington Heights, IL), as previously described (30).

Northern blot analysis

Human ovarian and placental tissues were provided by Dr. T. Tanaka (Hokkaido University, Sapporo, Japan) and Dr. A. Murphy (University of California-San Diego), respectively. Total RNA was extracted from human tissues using the guanidinium thiocyanate-phenol-chloroform extraction procedure (31). Total RNA samples were enriched for poly(A) mRNA by a single round of oligo(dT) column chromatography (Pharmacia LKB Biotechnology, Piscataway, NJ) and electrophoresed through 1% agarose-2.2 M formaldehyde gels. Samples were blotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) by overnight capillary electrophoresis and covalently cross-linked using a UV cross-linker (Stratagene). Membranes were then prehybridized for 2–4 h at 65 C in the presence of 50% formamide under standard conditions, followed by hybridization with the radiolabeled hFSH receptor cRNA probe at the same temperature for 18–20 h (20, 32, 33). Membranes were washed in 2 × sodium chloride-sodium citrate (SSC)-0.1% sodium dodecyl sulfate (SDS) for 10 min at room temperature, followed by two or three consecutive 15- to 20-min washes in 0.1 × SSC-0.1% SDS at

65 C, and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY) for 1–5 days at −70 C.

Ligand receptor cross-linking

Cross-linking of [^{125}I]hFSH to binding sites in transfected 293 cells and rat testicular homogenates was performed using disuccinimidyl suberate (Pierce, Rockford, IL), as previously described (34). Briefly, 6×10^6 transfected 293 cells or homogenate from 10 immature rat testes were incubated with 8×10^5 cpm [^{125}I]hFSH in the absence or presence of a 1000-fold excess of unlabeled hormone at 22 C for 20 h in a total volume of 0.4 ml. After incubation, cells or homogenates were diluted with 1 ml wash solution (Dulbecco's PBS containing 0.1% BSA, 5 mM EDTA, and 5 mM *N*-ethylmaleimide), pelleted by centrifugation, washed, and recentrifuged. Pellets were then resuspended in 0.5 ml incubation buffer (D-PBS containing 10% dimethylsulfoxide), and disuccinimidyl suberate (freshly prepared in dimethylsulfoxide) was added to a final concentration of 1.5 mM. Cross-linking was carried out at 0 C for 30 min, and the reaction was stopped by the addition of 1 ml termination buffer [50 mM Tris-HCl (pH 7.5) and 100 mM NaCl]. The reaction tubes were centrifuged, and pellets were resuspended in 0.1 ml solubilization buffer [50 mM Tris-HCl (pH 7.5) and 1% Triton X-100]. Solubilization was performed by incubation at 0 C for 60 min, with mixing of samples every 15 min. The samples were centrifuged for 5 min at 13,000 × *g*, and the resultant supernatants were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using 8.5% polyacrylamide gels. The gels were then subjected to autoradiography at −70 C for 2–14 days.

Results

Expression and binding kinetics of hFSH receptors in eukaryotic cells

Cells derived from a human fetal kidney cell line (293) were transfected with the plasmid pCME-hFSHR. After a 24-h incubation, cells were analyzed by radioligand receptor binding (Fig. 1). A dose-dependent increase in specifically bound [^{125}I]hFSH was detected in transfected cells incubated with increasing concentrations of labeled FSH, whereas no specific binding was observed in nontransfected cells (data not shown). Analysis of receptor binding indicated a K_d value of 1.7×10^{-9} M.

To study the effects of incubation time and temperature on the kinetics of FSH receptor binding, transfected 293 cells were incubated with [^{125}I]hFSH at 4, 22, or 37 C for increasing lengths of time, after which specific binding was determined (Fig. 2). A time-dependent increase in the levels of specific FSH binding was observed in cells incubated at 22 and 37 C, whereas low levels of [^{125}I]hFSH binding were detected at 4 C. Maximal FSH binding was highest in cells incubated at 22 C, although the time required to reach maximal ligand binding was considerably longer in incubations conducted at 22 *vs.* 37 C (Fig. 2).

Interaction of hFSH receptor with recombinant and pituitary/urinary-derived human gonadotropins and TSH

To test the binding specificity of the expressed hFSH receptor for human gonadotropins, transfected 293 cells were incubated with [^{125}I]hFSH in the absence or presence of increasing doses of unlabeled urinary-derived hCG, or hFSH, hLH, or hTSH of both pituitary and recombinant origin. Displacement of labeled FSH from its binding sites was expressed relative to the total amount of specifically bound [^{125}I]hFSH (Fig. 3). Both recombinant and pituitary FSH preparations competed with [^{125}I]hFSH for FSH receptors expressed in 293 cells (ED_{50}: recombinant, 25 ng/ml; pituitary-derived, 70 ng/ml), whereas human pituitary LH and

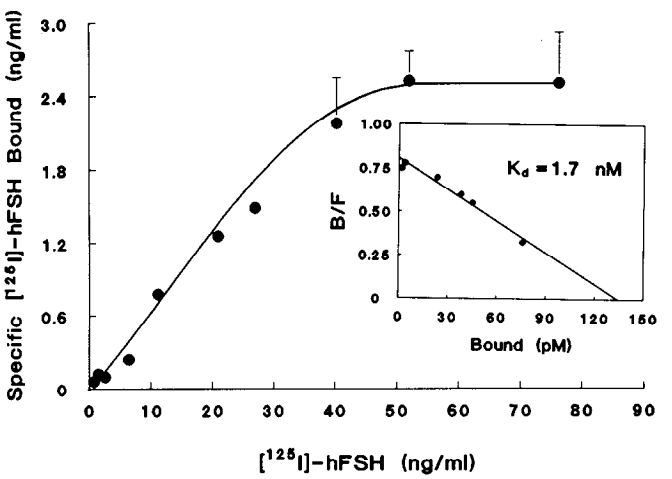

FIG. 1. Binding of [^{125}I]hFSH to hFSH receptors expressed in eukaryotic cells. 293 cells were transfected with the hFSH receptor plasmid pCME-hFSHR, and binding of radiolabeled hFSH was determined 24 h later. Cells (2×10^5/tube × 0.4 ml) were incubated with increasing concentrations of [^{125}I]hFSH in the absence or presence of a 1000-fold excess of unlabeled ligand. Levels of specifically bound [^{125}I]hFSH are shown together with the derived Scatchard plot (*inset*; B/F, bound to free ratio). Data are the mean ± SEM of triplicate determinations from a representative experiment.

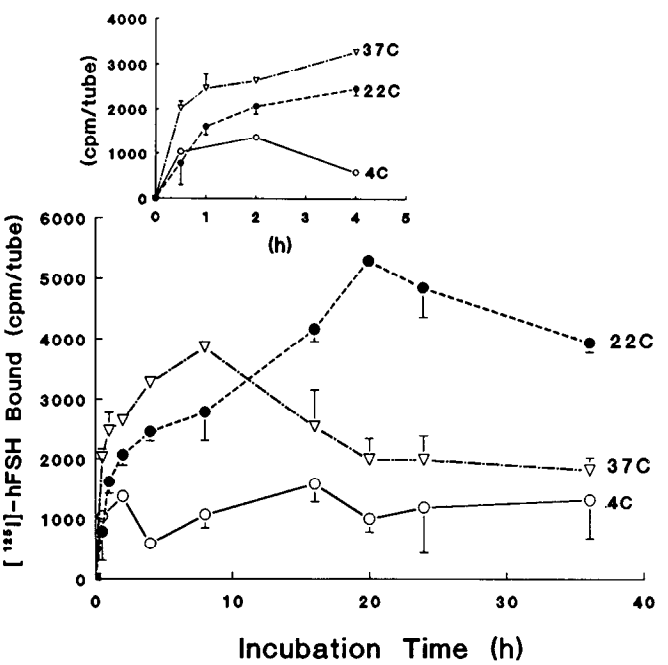

FIG. 2. Effects of incubation time and temperature on rate and extent of [^{125}I]hFSH binding to 293 cells. Transfected 293 cells (2×10^5/tube) expressing hFSH receptors were incubated with radiolabeled hFSH in the absence or presence of a 1000-fold excess of ligand at 4, 22, or 37 C for increasing lengths of time, after which levels of specifically bound [^{125}I]hFSH were calculated (mean ± SEM of triplicate determinations from a representative experiment). An enlarged figure for the first 4 h of incubation is presented in the *inset*.

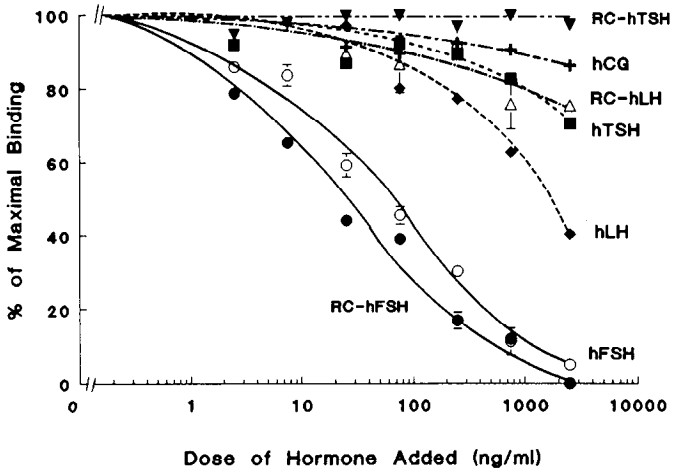

FIG. 3. Interaction of recombinant (RC) and pituitary/urinary-derived human gonadotropins and TSH with hFSH receptors expressed in transfected 293 cells. Displacement of [^{125}I]hFSH binding to hFSH receptors by hFSH, hLH, hCG, or hTSH was determined in radioligand receptor assays (mean ± SEM of three replicate experiments).

TSH were effective only at high doses. In contrast, negligible interaction of hCG or recombinant hLH or hTSH with the expressed FSH receptors was observed (Fig. 3).

Ligand specificity of human vs. rat FSH receptors

To assess the ligand specificity of the hFSH receptor, 293 cells expressing human receptors were incubated with [^{125}I] hFSH in the absence or presence of increasing doses of eCG (PMSG) or FSH from human, rat, ovine, and porcine origin. Displacement of [^{125}I]hFSH by unlabeled hormone in the human receptor was compared to that of testicular homogenates from 15-day-old rats (Fig. 4). hFSH and rFSH preparations were effective in binding to the recombinant hFSH receptor (ED_{50}: hFSH, 70 ng/ml; rFSH, 125 ng/ml), whereas only minimal interaction of oFSH, pFSH, or eCG with hFSH receptors was observed (Fig. 4A). In contrast, FSH from rat, human, and ovine origin as well as eCG effectively competed with radiolabeled FSH for binding sites in rat testicular homogenates (hFSH = rFSH > oFSH > eCG); however, pFSH was effective only at high doses (Fig. 4B).

Gonadotropin stimulation of cAMP production and tPA promoter-luciferase reporter gene by transfected 293 cells expressing hFSH receptors

The functional capacity of the recombinant hFSH receptor was tested based on gonadotropin stimulation of cAMP production by transfected 293 cells. Treatment of cells with human pituitary FSH caused a dose-dependent increase in cAMP formation (ED_{50}, 10 ng/ml), with a maximal 13-fold increase observed in response to 100 ng/ml FSH (Fig. 5A). Human pituitary LH also increased cAMP production at doses of 100 (4-fold) and 1000 (6.6-fold) ng/ml, whereas neither hCG nor recombinant hLH altered cAMP levels compared to control values (Fig. 5A).

Our earlier data demonstrated the stimulation of rat tPA

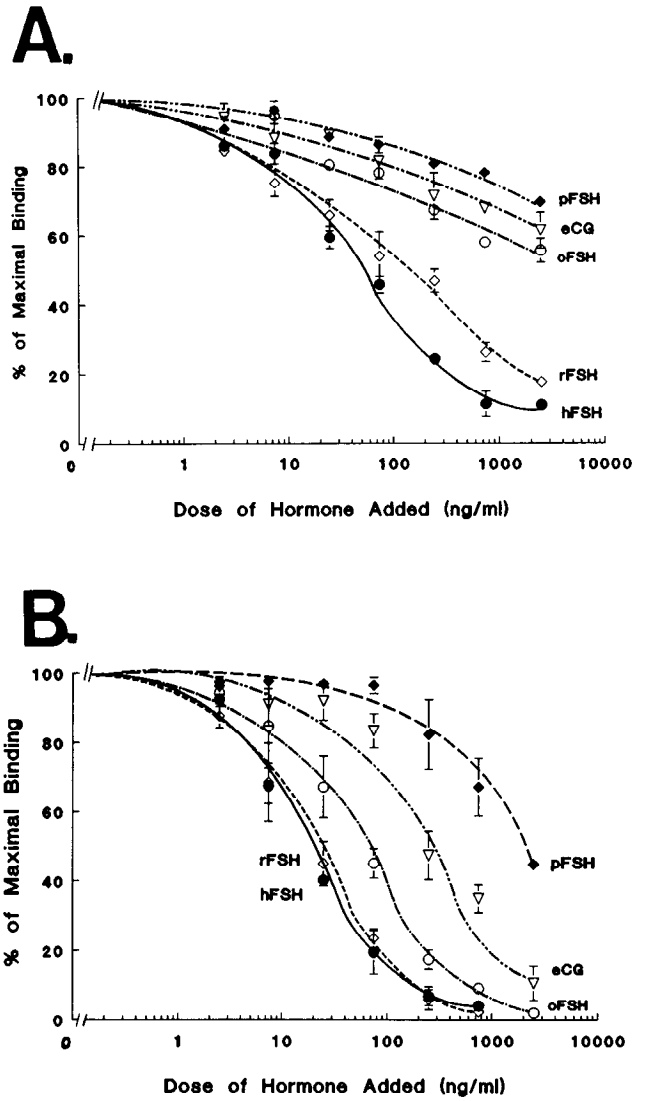

FIG. 4. Displacement of [^{125}I]hFSH binding to human and rat FSH receptors by human, rat, ovine, equine, and porcine gonadotropins. Transfected 293 cells expressing hFSH receptors (A) or rat testicular homogenates (B) were incubated with [^{125}I]hFSH in the absence or presence of hFSH, rFSH, oFSH, or pFSH as well as eCG. Displacement curves are presented as a percentage of maximal binding at each dose of unlabeled hormone (mean ± SEM of three replicate experiments).

gene expression in ovarian granulosa cells (32). In granulosa cells transfected with a luciferase reporter gene driven by a cAMP-responsive region of the rat tPA gene promoter, treatment with FSH increases luciferase expression (29). Using this tPA-luciferase reporter plasmid, we evaluated the ability of human gonadotropins to induce luciferase activity in 293 cells transfected with plasmids for both hFSH receptor and the reporter constructs. Treatment of cells with increasing doses of FSH, but not hCG, caused a dose-dependent increase in luciferase activity, with an estimated ED_{50} of 8 ng/ml and a maximal 2-fold increase at 100 ng/ml (Fig. 5B). These findings demonstrate a functional linkage of recombinant hFSH receptors to the tPA gene.

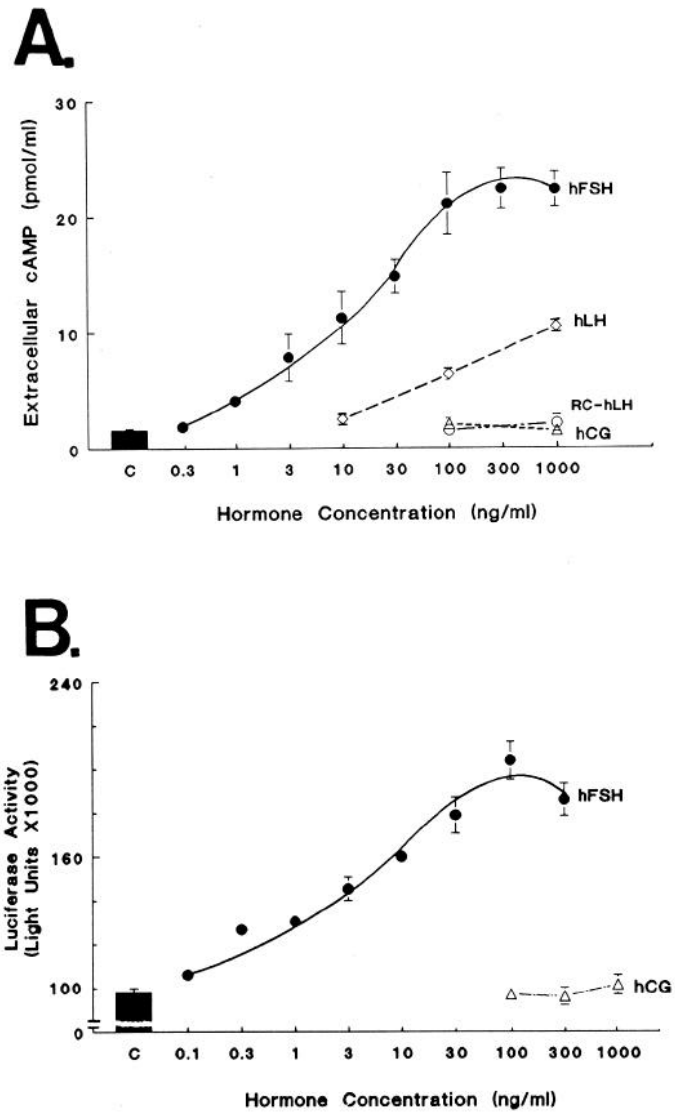

FIG. 5. Gonadotropin stimulation of cAMP production (A) and luciferase activity (B) by 293 cells expressing hFSH receptors. A, Extracellular cAMP accumulation was measured after incubation of transfected 293 cells (2×10^5/culture dish) for 2 h at 37 C with 0.25 mM MIX in the absence or presence of hFSH, hLH, or hCG. Data are the mean ± SEM of six cultures from three replicate experiments (RC, recombinant). B, Dose-dependent stimulation of luciferase activity by hFSH, but not hCG, in 293 cells cotransfected with the hFSH receptor plasmid and a tPA promoter-luciferase reporter gene construct (note the break in the y-axis). Data are the mean ± SEM of triplicate determinations from a representative experiment.

Northern blot analysis of FSH receptor mRNAs in human reproductive tissues

To study the expression of hFSH receptor mRNA, RNA was extracted from human reproductive tissues and analyzed by Northern blot, using a radiolabeled cRNA probe corresponding to the extracellular region of cDNA from our cloned hFSH receptor (Fig. 6). Analysis of poly(A)-enriched mRNA prepared from human follicular phase ovary indicated the existence of three mRNA transcripts (7.0, 4.2, and 2.5 kb) that were not detected in an equivalent amount of mRNA prepared from corpus luteum (day 21 of the menstrual cycle) or placenta (week 19 of pregnancy; Fig. 6).

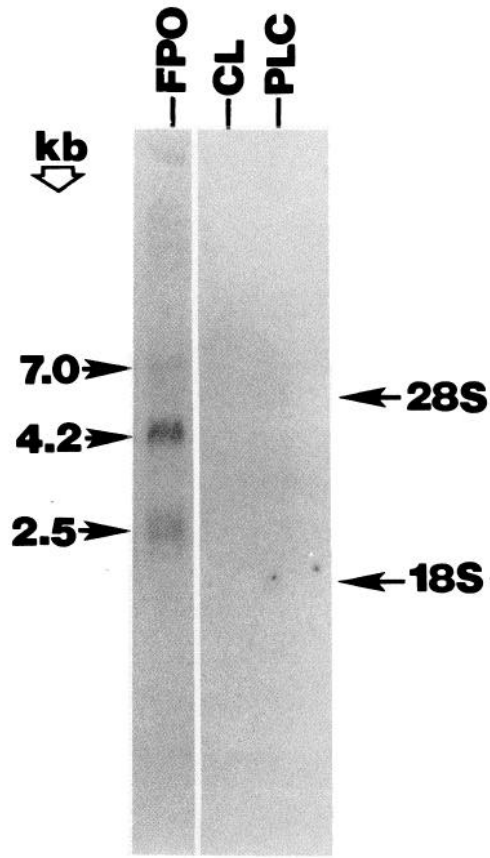

FIG. 6. Northern blot analysis of hFSH receptor mRNAs in human reproductive tissues. Poly(A)-enriched RNA samples prepared from human follicular phase ovary (FPO), 21-day-old corpora lutea (CL), or 19-week-old placenta (PLC) (2 μg/lane) were resolved through denaturing agarose gels, transferred to nitrocellulose filters, and hybridized to a ^{32}P-labeled hFSH receptor cRNA probe. Filters were washed and exposed to photographic films for 5 days at −70 C. Migration distances of the 28S and 18S ribosomal RNAs of a parallel total RNA sample from human ovary are indicated.

Cross-linking of [^{125}I]hFSH to FSH-binding sites in transfected cells and immature rat testes

To estimate the molecular size of the recombinant hFSH receptor, [^{125}I]hFSH was cross-linked to FSH-binding sites in transfected 293 cells using disuccinimidyl suberate, followed by SDS-PAGE analysis (Fig. 7). A predominant autoradiographic band of protein, with an estimated molecular mass of 109 kilodaltons (kDa; 76 kDa for the binding protein after correction for mass attributed to the ligand) was detected in transfected cells expressing hFSH receptors, whereas a 1000-fold excess of ligand completely blocked [^{125}I]hFSH binding (Fig. 7). A less abundant protein band of approximately 145 kDa (112 kDa for the binding protein) was also present. These findings were comparable to those observed for FSH

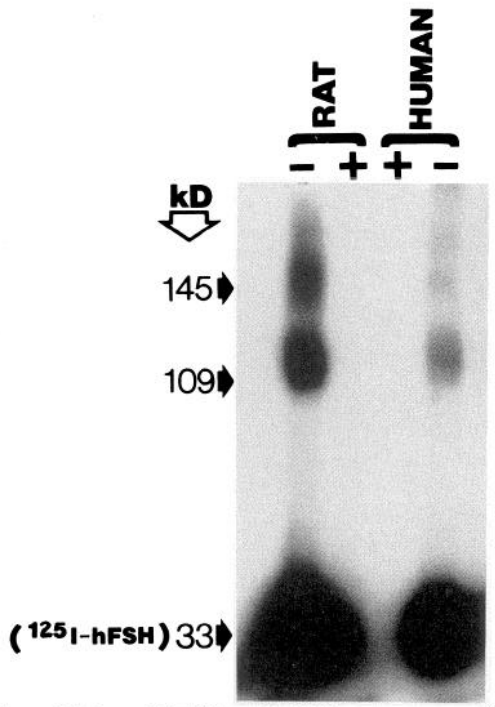

FIG. 7. Cross-linking of hFSH- and rFSH-receptors to [^{125}I]hFSH. Radiolabeled hFSH was cross-linked to FSH receptors in transfected 293 cells expressing hFSH receptors (HUMAN) or rat testicular homogenates (RAT) using disuccinimidyl suberate. Proteins were separated through 8.5% polyacrylamide gels and analyzed by autoradiography (14 days at −70 C). The sizes of the proteins were estimated by comparison to migration distances of known protein mol wt standards. Symbols indicate the absence (−) or presence (+) of a 1000-fold excess of ligand (Pergonal) during the binding incubation period before the cross-linking reaction.

receptors found in testicular homogenates of immature rats under the same experimental conditions (Fig. 7).

Discussion

We report here the ligand specificity and biochemical properties of the recombinant hFSH receptor. Our data indicate that hFSH, but not LH or CG, competes with [^{125}I] hFSH for binding to the recombinant FSH receptor. In addition, hFSH stimulates cAMP production in 293 cells expressing the human receptor, whereas recombinant hLH and hCG are without effect. As a result of stimulation of endogenous cAMP levels by FSH, a tPA promoter-driven luciferase reporter gene was also activated. Similar to the species-specific ligand binding recently reported for the hLH receptor (17), the recombinant hFSH receptor interacts with hFSH and rFSH, but only minimally with eCG or FSH of ovine and porcine origins. We also report here the first identification of FSH receptor mRNA in human ovaries as well as the determination of the molecular size of the recombinant hFSH receptor through ligand cross-linking analysis.

Comparison of the present human testicular FSH receptor cDNA with an ovarian cDNA clone recently reported (24) indicated seven individual basepair substitutions throughout the receptor sequence, resulting in five amino acid changes. Although we are uncertain of the basis for the observed disparity between our and the reported clone, our FSH receptor cDNA could be expressed, whereas no expression data were reported for the ovarian clone. Additionally, four of the five variant amino acids are identical between our hFSH and the reported rFSH receptor sequence (13). The ability of 293 cells transfected with our cDNA to express high affinity FSH receptors coupled to the endogenous adenyl cyclase and a luciferase reporter gene indicates that our clone encodes for a functional protein.

The availability of a cell line that expresses recombinant hFSH receptors has provided an unlimited source of the human receptor and enabled us to perform analysis of the ligand specificity of the human receptor. Radioligand receptor assays demonstrated that the hFSH receptor does not interact with hCG or recombinant hLH or hTSH at physiological or supraphysiological concentrations. However, highly purified human pituitary LH and TSH did cross-react with recombinant FSH receptors, suggesting that the pituitary hormone preparations contain minor FSH contamination. Furthermore, unlike the ability of rat testicular FSH receptors to recognize gonadotropin preparations from diverse species (Ref. 35 and the present study), the human receptor interacted preferentially with human and rat FSH. These findings coupled with a similar species-specific ligand binding of hLH receptors (17) indicate significant evolutionary changes in both human gonadotropin receptors. Alternatively, we cannot rule out subtle differences in posttranslational processing of the receptor in 293 cells *vs.* gonadal cells that may influence its binding characteristics. However, the ligand specificity of hLH-binding sites expressed in 293 cells (17) is identical to that of native LH receptors present in human corpus luteum (36), suggesting that the properties of these recombinant proteins are similar to those of endogenous gonadal receptors.

Saturation binding and Scatchard analysis demonstrated that the recombinant FSH-binding site has an estimated K_d of 1.7 nM, comparable to that reported for FSH receptors in human testes (37). In addition, the effects of incubation time and temperature on the rate and extent of [^{125}I]hFSH binding by recombinant hFSH receptors were similar to those reported for rat testicular FSH (35) and LH (38) receptors. Recombinant hFSH receptors occupied by gonadotropin are also capable of interacting with the endogenous G-proteins of the 293 cells to increase cAMP formation, thus providing a useful model to study FSH-activated signal transduction. Additionally, the use of a luciferase reporter gene driven by the cAMP-responsive promoter of the rat tPA gene (29) has indicated that transfected cells expressing hFSH receptors respond to FSH with increased luciferase activity. These findings provide evidence that FSH-induced signal transduction in cells expressing recombinant hFSH receptors is coupled to the activation of genes that are regulated by gonadotropins under physiological conditions within gonadal cells (32). However, the relatively small magnitude of the FSH response using the luciferase system (compared to cAMP) suggests a potential limitation of this assay in its present form. The reasons for this observation are unclear, but may

be related to the lack of gonadal cell-specific transcription factors in 293 cells that limit the activation of the luciferase reporter gene construct. Nonetheless, future modifications of the present cell model and the cAMP-driven luciferase gene reporter system should provide a useful and sensitive bioassay for hFSH.

The availability of hFSH receptor cDNA enabled us to study FSH receptor mRNA transcripts within human reproductive tissues. Northern blot analysis revealed the existence of several FSH receptor mRNAs within human follicular phase ovary, consistent with those reported for multiple FSH receptor mRNA transcripts in rat gonadal tissues (20, 39, 40). However, RNA prepared from human corpora lutea did not contain detectable levels of this message, suggesting that ovarian FSH receptor mRNA levels undergo up- and down-regulation during the human menstrual cycle in a manner similar to that reported for experimental animal models (20, 41).

Based on the deduced amino acid sequence of the hFSH receptor cDNA, the calculated molecular mass of the mature protein is approximately 75.6 kDa, consistent with the size estimation of recombinant FSH receptor based on ligand cross-linking and SDS-PAGE and comparable to that observed for FSH receptors in immature rat testes. Of interest was the finding of a less abundant 112-kDa FSH-binding site in both transfected 293 cells and rat testes, presumably resulting from posttranslational glycosylations of the protein. Alternatively, the smaller FSH-binding site may arise from proteolytic cleavage of the larger 112-kDa protein, as has been suggested by previous studies on hCG-binding sites in rat gonadal tissues (42).

Previous studies using nonreducing PAGE followed by ligand blotting have estimated the size of the FSH receptor in bovine and rat testicular membranes to be approximately 240 kDa (43, 44), possibly resulting from receptor aggregation and dimer formation. Moreover, photoaffinity labeling of the pFSH receptor revealed the presence of a major cross-linked complex of 104 kDa, which could be reduced with dithiothrietol into two smaller complexes of 75 and 61 kDa (45). Although the reasons for the discrepancy between our and earlier findings are unclear, they may result from varying methodologies (*e.g.* ligand-receptor cross-linking *vs.* ligand blotting) or species differences. Our data, however, suggest that binding of FSH does not require prior dimerization of its receptor on the plasma membrane, although it is possible that receptor aggregation may be important for receptor stability and/or signal transduction (46).

Expression of the hFSH receptor provides unlimited material for future studies of clinical interest. Additionally, the ability to measure cAMP production and luciferase reporter gene activity in a cell line expressing hFSH receptors should allow the establishment of a sensitive bioassay for human gonadotropins and for screening new FSH agonists and antagonists. Because earlier reports have suggested the presence of circulating antibodies against FSH receptor in patients with premature ovarian failure (47–49), the etiology of pathophysiological conditions associated with gonadotropin receptor dysfunction may also be more clearly elucidated.

Note Added in Proof

Amplification of human ovarian and testicular mRNA by reverse transcription polymerase chain reaction with oligonucleotide primers specific for human FSH receptor cDNA sequences, followed by direct sequencing of resultant PCR products after T7 gene 6 exonuclease treatment (50), indicated 100% sequence identity of this human FSH receptor cDNA with our clone obtained by cDNA library screening. Furthermore, independent cloning of human FSH receptor cDNA by R. Dijkema and R. de Leeuw (Organon, Oss, the Netherlands) also indicated 100% sequence identity with our cDNA clone (personal communication). Accession no. M95489.

Acknowledgments

The authors thank Dr. T. Ny (University of Umea, Umea, Sweden) for the rat tPA promoter-luciferase reporter gene; Drs. M. Oikawa and P. S. LaPolt for polymerase chain reaction analysis of the rFSH receptor cDNA; Dr. I. Boime (Washington University, St. Louis, MO) for provision of CHO cells secreting human LH and FSH; Dr. K. Umesono (The Salk Institute, San Diego, CA) for the pCMX expression vector and 293 cell line; Dr. T. Tanaka (Hokkaido University, Sapporo, Japan) for human ovarian tissues; Dr. A. Murphy (University of California-San Diego) for human placental tissue; Dr. H. Papkoff (University of California-San Francisco) for the gift of pFSH; and the National Hormone and Pituitary Distribution Program (Bethesda, MD) for hFSH, hLH, hCG, hTSH, rFSH, and oFSH.

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