The Human Follitropin α-Subunit C Terminus Collaborates with a β-Subunit Cystine Noose and an α-Subunit Loop To Assemble a Receptor-Binding Domain Competent for Signal Transduction†

Carrie J. Arnold,‡,§ Cheng Liu,‡,§ Barbara Lindau-Shepard,|| Michele L. Losavio,|| Maria T. Patrascu,|| and James A. Dias*,‡,||

Department of Biomedical Sciences, School of Public Health, State University of New York at Albany, and Division of Genetic Disorders, Laboratory of Reproductive and Metabolic Disorders, Wadsworth Center, New York State Department of Health, Albany, New York 12201

Received July 28, 1997; Revised Manuscript Received October 30, 1997

ABSTRACT: FSH is a member of the pituitary/placental glycoprotein hormone family including luteinizing hormone, thyroid-stimulating hormone, and chorionic gonadotropin. These heterodimeric hormones share a common α-subunit and a highly homologous but distinct β-subunit. The determinant loop of the FSH β-subunit acts both as a specificity discriminator and as an essential receptor-binding site. The three-dimensional structure of hCG illustrates the proximity of the determinant loop to the carboxyl-terminal residues of the common α-subunit. Thus, site-directed mutagenesis was used to make high-resolution substitutions at this carboxyl-terminal locus. The effects of those substitutions were studied. Twelve single mutations and one composite mutation were made of the region of hFSHα 74−92, each residue substituted by alanine. Side chain replacement in this region of FSH proved to be detrimental to binding. hFSH with mutations of either αS85A, αT86A, αK91A, or αS92A only retained 10% or less of the hFSH receptor-binding activity, while compared to these, mutants αH79A, αY88A, and αY89A retained slightly more binding activity. The single mutant αF74A and composite mutant αV76A/E77A binding activity was reduced to half of that of wild-type (WT) hFSH. In contrast, mutations of either αK75A, αT80A, αH83A, or αH90A did not adversely affect receptor binding, demonstrating the specificity of observed effects. The hFSH and mutant hormones were tested in an in vitro bioassay for stimulation of progesterone production. Those mutations that did not affect receptor binding (αK75A, αT80A, αH83A, and αH90A) did not affect signal transduction, measured by progesterone responses. After comparison of wild-type and mutant hFSH activities determined in radioreceptor assays (ID_{50}) and in vitro bioassays (ED_{50}), it became evident that signal transduction correlated with receptor binding.

The human pituitary/placental glycoprotein hormones include follicle-stimulating hormone (hFSH), luteinizing hormone (hLH), thyroid-stimulating hormone (hTSH), and chorionic gonadotropin (hCG). Since these hormones all share a common identical α-subunit, understanding the structure−function relationships of the α-subunit will have broad impact on a better understanding of this family of hormones.

Previous immunochemical studies with human follicle-stimulating hormone (hFSH) showed that hFSHα primary sequence 73−92 was accessible to antipeptide antibodies against hFSHα-73−92 (*1*). The synthetic peptide hFSHα-73−92 also maps to the epitope of anti-hFSHα monoclonal antibody 10.4B6 (*2*). Since Fab′ fragments of 10.4B6 inhibit binding of radiolabeled hFSH to receptor, hFSHα-73−92 was implicated as a receptor-binding site (*2*). Two recently published crystal structures of hCG (*3*, *4*) allow for the assessment of these data. Although hFSH residues α89−92 could not be positioned in the hCG crystal structure, it is clear that they are surface-oriented. Residues α74−85 are near the long loop of the β-subunit and form an antiparallel β-sheet with α59−70 with a hairpin turn at α70−74. Indeed, mapping studies of five α-subunit monoclonal antibodies that could inhibit FSH binding to receptor demonstrated a juxtaposition of discontinuous sequences α16−36 and α66−92 in the epitope (*2*), in accord with assembly of these sequences in the crystal structure of hCG. All antibodies that inhibited FSH binding mapped to hFSHα 66−92, although the assembled epitopes differed.

The penultimate carboxyl-terminal residue K91 in the α-subunit of hCG is not essential for binding but is essential for signal transduction (*5*). Deletion of residues α88−92 (*6*) or α90−92 (*7*) of hCG resulted in a loss of hCG-binding activity. Two conservative mutations αY88F, αY89F demonstrated that these two tyrosine residues are important in receptor binding and activation (*6*). More detailed studies have shown that deletion of residues αK91 and αS92 of the α-subunit abolished FSH binding, but not hCG binding (*8*). Deletion of αS92 alone was without effect in the case of hCG and hFSH. In contrast, hTSH residue αS92 is essential for binding of hTSH to receptor (*9*).

† This work was supported by NIH Grant HD 18407.

‡ State University of New York at Albany.

§ Both authors contributed equally to this work, and therefore, each should be considered the first author.

|| New York State Department of Health.

S0006-2960(97)01816-3 CCC: $15.00
Published on Web 01/30/1998

A homology model for the FSH receptor has been proposed, and is based on the ribonuclease inhibitor structure (*10*). Docking of the FSH molecule would be permitted at one face of the FSHR model within the concave surface formed by predicted repeating β-sheets formed by a leucine-rich repeat motif. The three-dimensional structure of hCG illustrates that the inter-cysteine loop at β-subunit residues 93−100 is proximal to the C terminus of the α-subunit, suggesting that these sequences could collaborate in receptor binding by presenting this assembled structure to the concave β-sheets. Indeed, residues of the LH β-subunit inter-cysteine loop can be swapped in place of the FSH β residues, resulting in an FSH molecule that can bind the LH receptor (*11*). Residues β93DTV95, which form the C-terminal flank of this inter-cysteine loop, are essential for FSH binding to its receptor (*12*). In addition, α-subunit long-loop residues α49VQK51 are also essential for FSH binding to the FSH receptor (*13*). This portion of the α-subunit long loop forms an assembled structure with the β-subunit inter-cysteine loop. These observations led us to the objective of the present work, which was to test the hypothesis that the carboxyl-terminal residues of the human glycoprotein hormone common α-subunit (specifically residues α72−92 or L3α) which assemble with the long loop of α- and the inter-cysteine loop of the β-subunit are involved in human FSH receptor binding of human FSH.

MATERIALS AND METHODS

Mutagenesis. Thirteen mutants were made, each a single alanine substitution of a residue in the region of hFSHα 74FKVENHTACHCSTCYYHKS92, with the exceptions that cysteines and asparagine 78 were not changed, and α76VE77 was made as a double mutant. Oligonucleotide-mediated site-directed mutagenesis of human FSHα cDNA (621 bp) was done using pALTER (Promega, Madison, WI) as previously described (*13*). Oligonucleotides were prepared by the Wadsworth Center Molecular Genetics Core Facility. Single-stranded plasmid DNA generated with phage R408 was used as a template. Mutagenesis was performed by the extension of the mutagenic oligonucleotide in the presence of polymerase and T4 ligase. Simultaneous corrective mutation of a defective β-lactamase gene and subsequent selection with ampicillin gave high yields of mutations. The mismatch repair-deficient bacterium BMH71-18mutS was used for transformation and ampicillin selection. Desired mutant clones were identified by DNA sequencing. Once the sequence of each mutant cDNA was determined, the cDNA fragments were ligated into the Baculovirus transfer vector pVL1393 (Invitrogen, San Diego, CA). Mutations were shuttled into pVL1393 by digesting the pALTER construct with *Eco*RI and ligating the mutated cDNA fragment into *Eco*RI-digested pVL1393. The constructs were sequenced again before transfecting the recombinant transfer vector into Sf9 cells. Sequencing was performed by the Wadsworth Center Molecular Genetics Core Facility using a PRISM Ready Reacting Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.) and an ABI model 373A automated sequencer.

Expression of Recombinant Hormones in Insect Cells. Wild-type recombinant hFSHα and hFSHβ baculoviruses were prepared by X. Liu using a kit from Invitrogen as previously described (*11*). The cDNA for the α-subunit was provided by J. Fiddes (*14*) and was subcloned into the Baculovirus transfer vector pBLUEBAC2 (Invitrogen) (*11*). A mammalian expression construct for hFSHβ had previously been prepared from the hFSHβ gene, a gift from J. Larry Jameson, and was also subcloned into pBLUEBAC2.

Recombinant viruses were made by cotransfecting 2×10^6 Sf9 cells with 2 μg of recombinant transfer plasmid DNA [wild-type (WT) hFSHα or WT hFSHβ or mutant hFSHα] and 1 μg of baculovirus DNA (Pharmingen, San Diego, CA)-using the calcium phosphate precipitate method according to the manufacturer's protocol. After 4 h of incubation at 27 °C, the media were removed and replaced with TNM-FH [Grace's Medium and 10% fetal bovine serum (v/v)]. Media were collected 5 days postinfection and were plaque-purified. Single plaques were picked and amplified to prepare viral stocks and their titers determined.

Protein was expressed in Hi-5 cells (Invitrogen) in either roller bottles or 150 cm^2 flasks. Three expression experiments were tested in this study. Hi-5 cells were seeded in 150 cm^2 flasks at 2×10^7 cells per flask. Two days after seeding, the cells were co-infected with recombinant viruses containing the WT hFSHβ subunit and either WT or mutant hFSHα subunits, both at a multiplicity of infection (MOI) of 5. Hi-5 cells in roller bottles were seeded at a density of 1×10^8 cells/bottle and were infected 2 days after seeding with a MOI of 5 for both hFSHα and hFSHβ viruses. Media were collected 4 days postinfection and were concentrated 10-fold on either Amicon centriprep filters, 10 000 molecular weight (Amicon, Waltham, MA) as was the case with the 150 cm^2 flasks, or a stirred cell concentrator (Amicon), as with the roller bottles, before assay. A pool of WT hFSH was prepared and utilized as a reference preparation.

Enzyme-Linked Immunosorbent (ELISA) Capture Assay. To determine the concentration of WT hFSH and mutants, an ELISA capture assay was performed as described previously (*15*). Each expression experiment was quantitated by two to seven ELISA assays. Briefly, Immulon-1 plates (Dynatech laboratories, Chantilly, VA) were coated with 0.5 μg/100 μL per well of protein-A-purified hFSHβ specific monoclonal antibody 46.3H6.B7 in coating buffer (0.05 M Tris at pH 9.5) overnight at 4 °C. The wells were washed with buffer (PBS, 0.05% Tween 20, and 0.02% NaN_3) and then blocked with 200 μL of 5% nonfat dried milk in coating buffer for 4−8 h at room temperature. Samples, diluted in binding buffer (PBS, 0.05% Tween, 127 nM EDTA, 0.23% IgG-free BSA, and 0.02% NaN_3), were then added at 100 μL/well and were left overnight at 4 °C. The following day, plates were washed and 100 μL/well of polyclonal anti-hFSHα subunit detection antibody W921, diluted 1:1000 in binding buffer, was added. Following a 2 h incubation period at room temperature, the plates were washed and 100 μL of a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-rabbit antibody (Fisher Scientific, Pittsburgh, PA) in binding buffer was added to each well. After 2 h at room temperature, the plates were washed and the substrate *p*-nitrophenyl phosphate, diluted in diethanolamine buffer (Bio-Rad, Hercules, CA), was added (100 μL/well). Absorbance at 405 nm was determined by using an EL-340 microplate reader (Bio-Tek Instruments, Winooski, VT). Data were collected every 15 min for up to 2 h. When the highest OD reading exceeded 2.2, data collection was terminated. The doses of wild-type and mutant recombinant hormones

were determined by comparison with standard preparations of hFSH purified from frozen human pituitaries (*1*).

Radioreceptor Assay (RRA). Each mutant hFSH was tested for binding to human FSH receptor in a radioreceptor assay. Each expression experiment was tested two to four times. The concentration of WT hFSH and mutants were determined by a capture ELISA (see above). Deoxyuridine-resistant Chinese hamster ovary cells (CHO)-DUKX cells stably transfected with hFSH receptor obtained from Ares Advanced Technologies (Randolf, MA) were used as a source of human FSH receptor. Radiolabeled hFSH (25 μCi/μg) was prepared using pituitary hFH purified in this laboratory as previously described (*2*). Confluent cultures were rinsed twice and then were left for 15 min in 0.01 M PBS/0.05 M EDTA (PBS/EDTA) buffer, then dislodged from flasks, and taken up in PBS/EDTA. Cells were counted and then pelleted at 3000*g* in a clinical centrifuge. Cells were resuspended in RRA buffer [0.05 M Tris (pH 7.5) and 25 mM $MgCl_2$] at a concentration of 1.0×10^7 cells/mL. Each aliquot was frozen at −70 °C, and they were thawed and diluted in RRA buffer for assay.

For each RRA, tracer, [^{125}I]hFSH (150 000 cpm/100 μL), diluted in RRA buffer with 1% BSA, was incubated with 200 μL of increasing concentrations of sample and 100 μL of hFSH receptor (5×10^5 cells) overnight at room temperature with shaking. The reaction was terminated by adding 1 mL of ice-cold 0.05 M Tris (pH 7.5). Bound hFSH was separated from free hFSH by centrifugation for 1 h at 3000*g* at 4 °C. The liquid was aspirated, and the pellet was counted on a γ-counter. Data were processed using the computer program NIHRIA (*16*).

In Vitro FSH Bioassay. To determine the signal-transduction potential of WT hFSH and mutants, an in vitro bioassay was used (*17*). The FSH-responsive Y-1 cells (Ares Advanced Technologies, Randolf, MA) were seeded in 48-well plates at 2.5×10^5 cells/well in 0.5 mL of Eagle's Minimum Essential Medium supplemented with 5% fetal bovine serum and 80 μg/mL G418 (Geneticin, Gibco). Two days after seeding the cells, the medium was replaced with fresh medium. On the third day after plating, the cells were rinsed twice with Y-1 assay medium (Eagle's medium with 0.1% BSA and 1% L-glutamine), and then recombinant WT or mutant hFSH was added to the wells. An additional ELISA capture assay was used to determine the concentration of hFSH concurrent with the bioassay. After incubation for 20−24 h, media were collected into 10 × 75 mm glass tubes, heated at 100 °C for 10 min, and spun at 3000*g* for 30 min. Each supernatant was decanted into 1.5 mL Eppendorf tubes and was frozen at −20 °C.

The amount of progesterone in each sample was measured by RIA. Media samples were diluted in PBS-G (0.1% gelatin dissolved in PBS with 0.02% NaN_3), and aliquots of 100 μL were added to 10 × 75 mm glass tubes with 100 μL of sheep anti-progesterone antiserum (GDN 337, provided by G. Niswender, Colorado State University, Fort Collins, CO), diluted 1:2500 in PBS-G, 100 μL of [^{3}H]progesterone, 25 000 dpm (Dupont NEN, Boston, MA) in PBS-G, and 200 μL of PBS-G. Tubes were incubated overnight at 4 °C. Bound progesterone was separated from free progesterone by adding 0.5 mL of dextran-coated charcoal to the samples, and then incubating at 4 °C for 10 min, followed by centrifugation at 3000*g* for 10 min. Each supernatant was decanted into 20 mL scintillation vials, and 12 mL of scintillation fluid (Aquasol, Dupont NEN) was added. The contents of vials were mixed to give a clear emulsion and were then counted in a LKB/Wallac Beta Counter (Gaithersburg, MD). Data were processed using the NIHRIA (*16*) and ALLFIT (*18*) programs.

Homology Modeling of the hFSH Molecule. Homology modeling of the hFSH molecule was carried out using the software suite LOOK (Molecular Applications Group, Palo Alto, CA). The hCG three-dimensional structure coordinates were used (*4*).

RESULTS

Expression of Wild-Type (WT) and Mutant Heterodimeric hFSH. Insect cells were co-infected with virus carrying WT hFSHβ cDNA and virus carrying the WT or mutant hFSHα cDNA. Expression levels of WT or mutant hormones secreted from Hi-5 cells were determined in an ELISA capture assay on the concentrated media as described previously (*14*). All mutant hFSHα subunits combined with hFSHβ to form heterodimer. For each of the C-terminal mutations, the expression levels varied from 0.01 to 8.2 μg/10^6 cells. Heterodimeric mutant αS92A was consistently expressed at levels lower than those of WT hFSH and other mutants, yet total α-subunit levels measured in a competition ELISA (*14*) were similar to that of the wild type (data not shown).

Receptor-Binding Activity of WT and Mutant Heterodimeric hFSH. Receptor-binding activity of WT and mutant heterodimeric hFSH was tested in an RRA using CHO cells stably transfected with recombinant human FSH receptor (*17*). Each expression experiment was tested in an RRA several times. A representative illustration of all of the mutants made shows that each mutant could compete with [^{125}I]hFSH for human receptors (Figure 1A−C). WT recombinant hFSH was twice as potent as pituitary FSH in this assay (data not shown), probably owing to the absence of sialic acid on its carbohydrate chains (*14*). Some mutations had a large effect on binding activity. For example, the largest effect on receptor binding was seen with mutants αS85A, αT86A, αK91A, and αS92A, which retained less than 10% of WT activity. Mutations αH79A, αY88A, and αY89A had an intermediate effect and retained 15−20% of WT receptor-binding activity. Mutants αT80A and αF74A and composite mutant αV76A/E77A had almost identical (56 and 68%, respectively) WT hFSH receptor-binding activity. Mutants αH90A, αK75A, and αH83A were slightly more potent than WT hFSH and had 125, 146, and 220% of WT receptor-binding activity, respectively.

Stimulation of Progesterone Production by WT and Mutant Heterodimeric hFSH. Stimulation of progesterone production by WT and mutant heterodimeric hFSH was measured with an in vitro bioassay using Y-1 cells stably transfected with recombinant hFSH receptor. Figure 2 is a representative illustration of the receptor-activating potential of each mutant. We noted that the Y-1 cells produce less maximal progesterone with frequency of passage (compare panels A and B of Figure 2 with panels C−E of Figure 2). This loss in steroidogenic capacity was only minimally restored by using 1 mM *p*-nitrophenyl β-D-xyloside (*19*). Those mutations that did not adversely affect receptor binding (αF74A, αK75A,

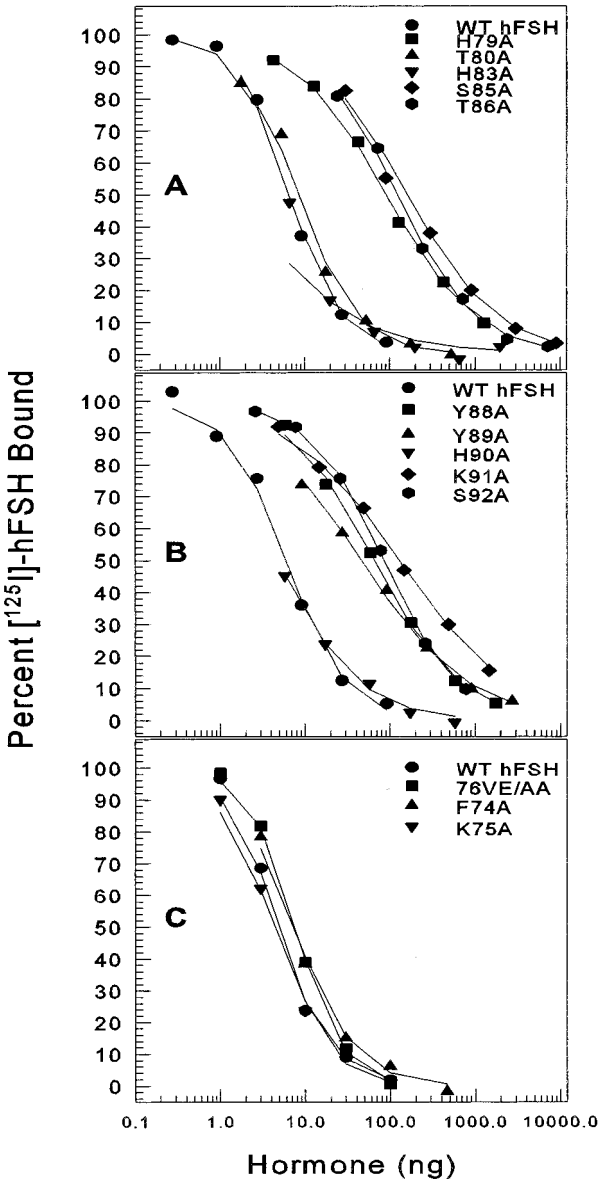

FIGURE 1: Characterization of recombinant mutant or WT hFSH in a competitive binding radioligand receptor assay for hFSH. Receptor-binding activity of WT and mutant heterodimeric hFSH was tested in an RRA using CHO cells stably expressing recombinant hFSH receptor. The radioligand was [^{125}I]hFSH. This is a representative graph: (A) hFSH with C-terminal mutations αH79A, αT80A, αS85A, αT86A, and αH83A and WT; (B) mutants αY88A, αY89A, αH90A, αK91A, and αS92A and WT; (C) double mutant αV76A/E77A and mutants αK75A and αF74A. The doses of recombinant hormones were determined by capture ELISA using pituitary hFSH as the reference preparation. Note that all of the α C-terminal mutations displace the radioligand, albeit with different potencies.

αV76A/E77A, αT80A, αH83A, and αH90A) did not affect progesterone responses when compared to WT hFSH (Figure 2A−E). When all mutants were compared at similar doses, it appeared that the remaining seven mutants stimulate progesterone production minimally. This would lead to the conclusion that these mutants were defective in signal transduction. However, when further testing was done with higher doses of these mutant hormones, we found that all mutants stimulate progesterone albeit with different potencies. As expected, those mutants which showed a decrease in receptor binding had an effect on progesterone production (αH79A, αS85A, αT86A, αY88A, αY89A, αK91A, and αS92A) and retained 3−10% of the bioactivity of WT hFSH. Mutants αF74A, αK75A, αV76A/E77A, αT80A, αH83A, and αH90A had virtually no adverse effect on stimulation of progesterone.

Correlation between Receptor Binding and Steroidogenesis. The levels of each heterodimer required to displace 50% of radiolabeled hFSH (ID_{50}) were compared with amounts of heterodimer required to stimulate 50% of the maximal progesterone production (ED_{50}) and are represented in Figure 3. It is evident that the biological activity is correlated with the receptor-binding activity in each case. This finding suggests that the role of the α-subunit C terminus in FSH differs from the role of the C terminus in hCG and TSH, in which case signal transduction is disproportionately affected.

DISCUSSION

This study extends previous studies where enzymatic truncation of αK91-αS92 significantly reduced bioactivity of LH, hCG, and TSH (*20*, *21*), while chemical modification of the hCGα COOH-terminal region also affected its hormonal activity (*22*). Deletion of hCGα residues 89−92 reduced both receptor binding and steroidogenic activity (*6*). Here we show that only residues that do not interact with the α-subunit itself, and with side chains oriented to solvent, are essential for receptor binding of FSH. The exception, H79, probably affects receptor binding by inducing a subtle conformational change in the α-subunit, as its side chain is buried in the interior of the molecule. We discovered that two additional residues, S85 and T86, are also essential for binding, while S85 delineates the boundary of the extension of L3α, which participates in receptor binding. All other residues tested in the L3α sequence 74−92 had no effect on binding and apparently do not destabilize the conformation of the molecule despite substantive contacts within L3α between its β-sheets 3 and 4. For example, F74 makes hydrophobic contacts with L2β residues V44 and L45 in hCG (*3*). Changing this residue to alanine had no effect on receptor-binding activity. In contrast, changing homologous FSH L2β residues decreases receptor binding by destabilizing the molecule (*23*). The benign result of an αF74A mutation does not rule out the importance of αF74 in this stabilization, since the side chain of alanine may fulfill the requirement for hydrophobic interaction. Another interpretation is that αF17 and αF18 are the key players in this role. It is difficult to evaluate the role of αF17 since its side chain placement differs in the two structures. In one structure, it forms part of a hydrophobic core (*3*).

A recent mutagenesis study showed that deletion of αS92 had no effect on mutant hCG or FSH, but deletion of both αK91 and αS92 affected receptor binding (*8*). It has since been demonstrated that this is indispensable for hTSH binding and activity (*9*).

Mutations αS85A, αT86A, αK91A, and αS92A in the common α-subunit demonstrated a greater than 10-fold decrease in receptor binding. Mutations αH79A, αY88A, and αY89A led to a 5−8-fold decrease in receptor binding. Mutations αT80A, which removes a consensus glycosylation sequon, αH83A, and αH90A had no effect on hFSH binding to the hFSH receptor. In fact, αH83A displayed a slight

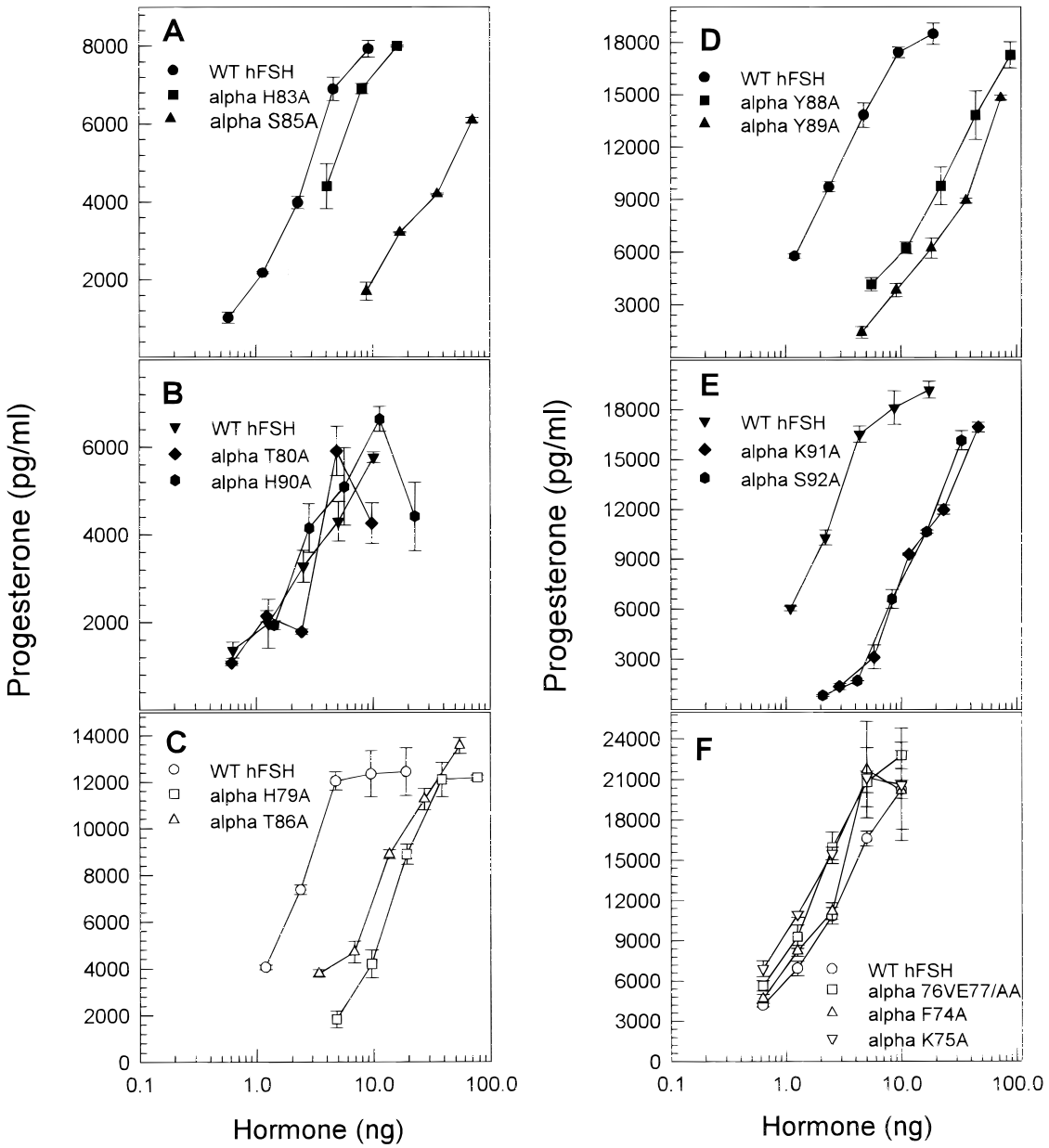

FIGURE 2: Activity of WT and mutant heterodimeric hFSH in an in vitro bioassay. Stimulation of progesterone production by WT and mutant heterodimeric hFSH was measured with an in vitro bioassay using Y-1 cells stably transfected with recombinant hFSH receptor. Y-1 cells were incubated for 24 h with doses of WT from stock pool or mutant hFSH. Since the receptor assay results obtained with three different experiments were consistent, only medium from the third and final expression experiment was used for the in vitro bioassay. Treatments were performed in triplicate, and this assay was repeated four times. This is a representative graph. Panels A−F show dose−response relationships of hFSH or various α C-terminal mutations in an in vitro bioassay. Doses of hFSH were determined by capture ELISA (see above). Media were collected and assayed for progesterone by radioimmunoassay. Bars represent standard errors. Those mutations that did not affect receptor binding (αF74A, αK75A, αV76A/E77A, αT80A, αH83A, and αH90A) also produced progesterone in a manner similar to that of WT hFSH. The remaining seven hFSH mutants all affected receptor binding and were found to stimulate progesterone at higher doses of hFSH, albeit with different potencies.

increase in receptor-binding activity. The displacement curves in radioreceptor assays were parallel to each other, which suggested that, despite significant changes in binding affinity, single alanine mutations do not disrupt the integrity of the receptor-binding interface.

We observed that doses of loss of function mutant hormones that were comparable to that of wild-type FSH showed comparatively little progesterone production. However, by increasing the mass of loss of function mutants, we could derive full dose−response curves. The bioactivity of mutant hormones correlated well with the reduction of their receptor-binding activity.

A majority of the residues in the COOH terminus of the common α-subunit are accessible on the surface of hCG (*3*, *4*) and the FSH model (Figure 4). The side chains of the residues shown in this study which affect binding, with the exception of αH79, are exposed in the proposed receptor-binding interface. Residues αS85, αT86, and αY89 are exposed in the hCG crystal structure, suggesting that these side chains are involved in direct contact with the receptor. The side chain of αY88 is placed between the disulfide *β*87−94 and α59−87 residues in the Lapthorn structure (*3*), making it likely that this mutant could exert its effect by affecting the conformation of nearby residues. However, in

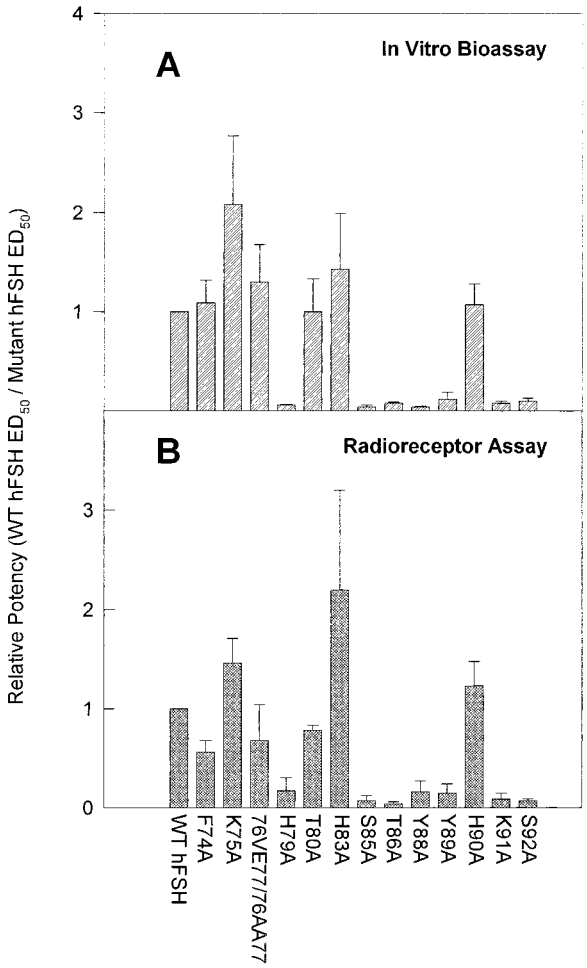

FIGURE 3: Correlation between signal transduction with receptor binding. The mean relative potency value from the progesterone assay was compared with the mean relative potency value from the RRA for each mutation. As an example, mutant αT86A, which was less potent in the RRA, is proportionally less potent in the progesterone assay. This mutation has decreased receptor-binding ability but does not have an added effect on progesterone production.

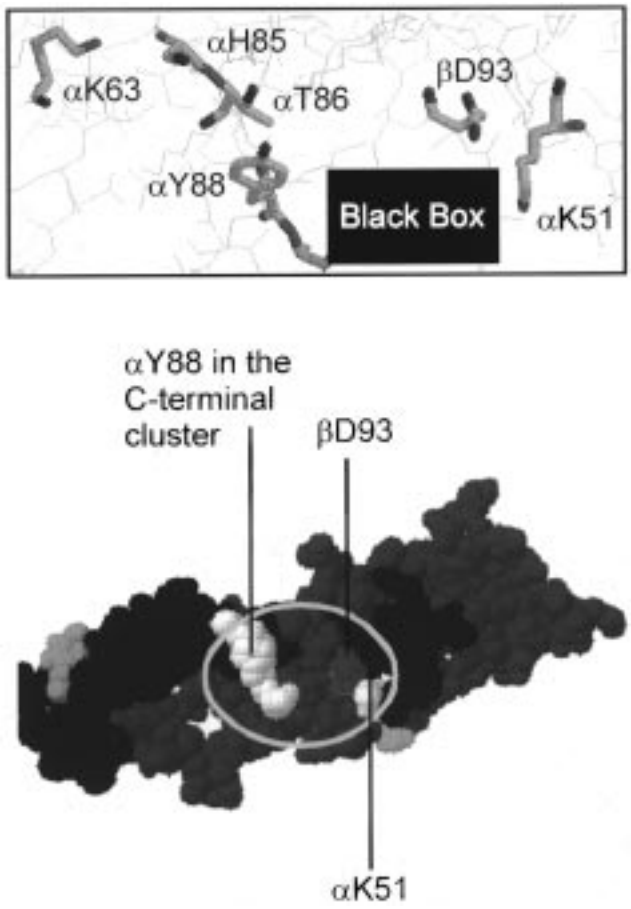

FIGURE 4: (Top) CPK presentation of a high-affinity receptor-binding site identified in hFSH. Illustrated are C-terminal residues that affect receptor binding in a model of FSH based on hCG crystal structure coordinates. Also illustrated are αK51 and *β*D93 (equivalent to hCG *β*D99). The accompanying homology model of hFSH based on the three-dimensional structure of hCG (*4*) shows the putative assembled receptor-binding site of the hFSH molecule circled in yellow. α-Subunit residues identified in the current study as being critical for FSH binding to receptor are colored in white. Residues in mutations identified in previous work to be essential for FSH binding to receptor are colored either in gray (*β*-subunit determinant-loop residue *β*D93) or in white (α-subunit long-loop residue αK51).

the Wu structure (*4*), this side chain is oriented into solvent, making it a good candidate for a receptor-binding site interaction. It was surprising that neither histidine αH83 nor αH90 is important to hFSH receptor binding and/or activation, since αH90 was implicated as being critical for cAMP production in hCG, but not for binding of hCG to LH receptor (*8*).

There are three half-cystines in the carboxyl-terminal sequence α72−92 that participate in formation of a cystine knot in the α-subunit, constraining the first half of this region in hCG and likely in FSH as well. A difference in structure is most likely to exist only in the last five residues, 88YYHKS92, but it is unlikely that the last three residues are more ordered in FSH than in hCG, although this cannot be ruled out. Interestingly, the expression of αS92A FSH was consistently lower compared with that of wild-type and other mutants in the same region, despite the expression of αS92A α-subunit being comparable to that of the wild-type free α-subunit (data not shown). This leads us to speculate that this residue may form a contact with another residue in hFSH*β* which in some way stabilizes subunit association. Although exact structural data are lacking for 90HKS92, the close proximity of the C terminus to the α33−58 long loop and the *β*93−100 determinant loop makes it likely for this kind of interaction to occur.

These results indicate that the C-terminal region in the glycoprotein hormone common α-subunit is an important receptor-binding site for hFSH binding to the FSH receptor, in contrast to the reports for hCG indicating that this region is a low-affinity site with a disproportionate effect on signal transduction. A distinct conformation of this region that involves interactions with the *β*-subunit is suggested, and may explain the difference in receptor binding of FSH and hCG. The residues in this region that contribute significantly to the total receptor-binding energy are juxtaposed to other high-affinity binding sites in space (Figure 4). The inset illustrates this, and also demonstrates a lack of knowledge (black box) about residues α89−92. The homology model of hFSH illustrates how the long loop of the α-subunit and the determinant loop of the *β*-subunit could collaborate with the carboxyl terminus of the α-subunit to form a putative receptor-binding site (Figure 4). This reinforces the notion

that the receptor-binding site of hFSH is composed of multiple noncontiguous sequences from both subunits assembled as a conformationally interdependent binding interface.

ACKNOWLEDGMENT

The authors acknowledge the scientific support services provided by the Wadsworth Center Molecular Genetics Core Facility. Mr. Ivan Auger of the Computational Biology and Statistical Services Core prepared the FSH homology model. The Wadsworth Center Tissue Culture Facility at the David Axelrod Institute was instrumental in the propagation and maintenance of the insect cell lines, and in accommodating our laboratory when the Wadsworth Center at the Empire State Plaza was closed following a fire.

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BI971816O