Follitropin Conformational Stability Mediated by Loop 2β Effects Follitropin−Receptor Interaction[†]

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ABSTRACT: Follicle-stimulating hormone (FSH) is in the family of pituitary/placental glycoprotein hormones which also includes luteinizing hormone (LH), chorionic gonadotropin (hCG), and thyroid-stimulating hormone. These hormones are heterodimers composed of common α- and similar but unique β-subunits. The 21 amino acid loop between Y33 and F53 of the FSH β-subunit (L2β) can be switched into L2β of hCGβ without a loss of receptor binding, yet mutation of hFSHβ ^{37}LVY^{39} to ^{37}AAA^{39} was antecedent to a 20-fold reduction in receptor binding (based on ID_{50}). A mutation in the LHβ gene, which causes Q54 to be R, causes hypogonadism. This residue is conserved in the glycoprotein hormones and corresponds to Q48 in hFSHβ. Mutation of hFSHβ ^{48}QKTCT^{52} to ^{48}AAACA^{52} resulted in a failure of heterodimer formation. In the current study single mutations were made to pinpoint which of the seven hFSHβ residues in the ^{37}LVY^{39} to ^{37}AAA^{39} and the ^{48}QKTCT^{52} to ^{48}AAACA^{52} mutants were responsible for the observed phenotypes. A single mutation of T52 to alanine was sufficient to cause a reduction in expression of heterodimeric hormone. Single mutants Q48A, T50A, V38A, Y39A, and, to a lesser extent, T52A formed heterodimer. However, these hFSH mutants were markedly unstable at pH 2.0. Thus, acid dissociation can be used to reveal metastable forms of this protein. Mutant hFSHβ Q48A was also 8-fold less active than wild-type hFSH when assayed for binding to hFSH receptors. hFSHβ V38A and Y39A mutants affected receptor binding; however, neither mutation alone caused greater than a 2-fold decrease in receptor binding activity. In summary, these results identify single important residues in the long loop (between Y33 and F53) of the hFSH β-subunit which are required for proper subunit interactions that provide conformational stability which in turn is necessary for FSH−receptor interaction.

Follicle-stimulating hormone (FSH) plays an essential role in human reproduction. FSH along with luteinizing hormone (LH), thyroid-stimulating hormone (TSH), and chorionic gonadotropin (hCG) constitute the family at pituitary/placental glycoprotein hormones. These proteins are all heterodimeric composed of non-covalently associated α- and β-subunits. Within a species the α-subunits have an identical primary sequence, and in humans the α-subunit originates from a single gene (Fiddes & Goodman, 1981). However, the carbohydrate composition (Baenziger & Green, 1988) and antibody binding (Weiner et al., 1991) of the α-subunit differ in the different hormones. Thus, the unique β-subunits, in addition to conferring hormone specificity through primary sequence, also define the final α-subunit conformation. These hormones bind receptors which are coupled intracellularly to G-proteins and which activate the adenyl cyclase and possibly the inositol phosphate second-messenger pathways (Gudermann et al., 1992; Philip & Grollamn, 1986). The receptors, like their ligands, are homologous at the level of primary sequence (Dias, 1992a).

Previous studies have used chemical modification (Ryan et al., 1987), antibodies, peptides, and mutagenesis [reviewed in Dias (1992b)] in attempts to correlate structure with function in these hormones. The recently reported three-dimensional structure of hCG shows that both subunits contain a cystine knot motif and that the subunit interface is quite large (Wu et al., 1994; Lapthorn et al., 1994). However, the structure alone does not indicate which amino acids are functionally important in subunit interaction or receptor binding. Moreover, although the glycoprotein hormones are predicted to fold similarly, they are likely to differ significantly in amino acid residue type and placement at homologous positions.

A region of heterogeneity between the β-subunits corresponding to 33−53 of the FSH β-subunit (L2β) contains amino acids involved in subunit contact as well as receptor interaction. This region contains amino acids which are at the surface of the free β-subunit but which appear to be buried at the subunit interface when the α- and β-subunits associate to form heterodimer (Vakharia et al., 1991). A peptide corresponding to FSH 33−53 blocked binding of FSH to its receptor and stimulated steroidogenesis (Santa Coloma & Reichert, 1990; Santa Coloma et al., 1990), and similar results have been reported for the corresponding

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regions of hCG and TSH (Morbeck et al., 1993; Keutmann et al., 1987; Morris et al., 1990). The interpretation of those data has been rendered unclear since the hCG peptide blocks binding of FSH to its receptor (Keutmann et al., 1995) and substitution of hFSHβ residues 33−52 for hCG residues 39−58 in a chimeric hormone did not change the binding specificity of hCG (Campbell et al., 1991). One simple interpretation is that the 33−53 region contains amino acid residues that are important for receptor interaction but does not contain specificity determinants.

A long-held tenet in the field of heterodimeric gonadotropin hormones is that the β-subunit directs the folding of the α-subunit. Thus, just as properly assembled heterodimeric hormone is prerequisite for function, perturbation of tertiary structure might be predicted to alter the hormone−receptor interaction. We have been studying the 33−53 loop of hFSHβ using scanning alanine mutagenesis to study the role of amino acids in the context of the intact hormone. We determined that the ^{37}LVY^{39} region contains amino acids important to receptor binding. This is intriguing because previous structural studies indicate that these residues are buried at the subunit interface (Dericks Tan et al., 1984; Ericsson et al., 1984; Suganuma et al., 1989). Our mutagenesis study also showed that ^{48}QKTCT^{52} contains amino acids involved in subunit contact (Roth & Dias, 1995), but effects of this mutation on receptor binding could not be assessed because of abrogation of subunit association. In the current study mutations of single amino acids to alanine in the ^{37}LVY^{39} and ^{48}QKTCT^{52} regions were made to determine which residues are essential for subunit association and receptor binding activity.

EXPERIMENTAL PROCEDURES

Mutagenesis and Protein Expression. Production of the scanning mutants (^{34}TRDL^{37} to ^{34}AAAA^{37}, ^{37}LVY^{39} to ^{37}AAA^{39} , ^{40}KDPA^{43} to ^{40}AAPA^{43}, ^{44}RPKI^{47} to ^{44}APAA^{47}, and ^{48}QKTCT^{52} to ^{48}AAACA^{52}) has been previously described (Roth & Dias, 1995). For mutation of single amino acids, oligonucleotide-mediated site-directed mutagenesis of the β-subunit cDNA of hFSH was carried out using the Altered Sites mutagenesis kit (Promega, Madison, WI) as described previously (Roth et al., 1993). The oligonucleotides used for each mutation were

L37A 5'...CTACACCAGAGATGCCGTGTATAAGGACC...3'

V38A 5'...CACCAGAGATCTGGCCTATAAGGACCCAG...3'

Y39A 5'...CAGAGATCTGGTGGCTAAGGACCCAGCA...3'

Q48A 5'...CAGGCCCAAAATCGCCAAAACATGTACCTT...3'

K49A 5'...GCCCAAAATCCAGGCAACATGTACCTTCAA...3'

T50A 5'...AATCCAGAAAGCATGTACCTT...5'

T52A 5'...GAAAACATGTGCCTTCAAGGA...5'

The *Eco*RI/*Pst*I fragments of each mutant cDNA were ligated into the baculovirus transfer vector pVL1393 (Invitrogen, San Diego, CA). The fidelity of each mutation was confirmed by dideoxy-sequencing (Tabor & Richardson, 1987; Sanger et al., 1977).

The baculovirus-infected insect-cell expression system (Invitrogen, San Diego, CA) was used for expression of recombinant proteins. Production of viruses carrying wild-type α- or β-subunit cDNAs has been previously described (Dias et al., 1994). Recombinant viruses for expression of mutant β-subunits were produced by co-transfection of Sf9 cells with pVL1393 carrying mutated FSHβ-cDNA and Baculogold (Pharmingen, San Diego, CA) linearized baculovirus DNA as described previously (Lindau-Shepard et al., 1994). Recombinant viruses carrying either the Q48A, K49A, T50A, or T52A mutations were plaque purified. Recombinant viruses carrying either the L37A, V38A or Y39A mutations were expanded from the initial transfection without plaque purification.

Recombinant wild-type and mutant heterodimeric hFSH was expressed in High Five cells (Invitrogen). Cells were maintained in spinner flasks in Exell-400 medium (JRH Biosciences, Lenexa, KS) with 2% fetal bovine serum (FBS). For heterodimer production cells were seeded in 175 mm^2 flasks or roller bottles in Exell-400 medium without serum at a density of 1.4×10^7 per 175 mm^2 flask and 1×10^8 cells per roller bottle. The medium was removed from the flasks 2 days later, and cells were co-infected with virus carrying wild-type α-cDNA and virus carrying the wild-type or mutant FSHβ-cDNA in 5 mL of medium with gentle rocking. After 1 h, 20 mL of fresh Exell-400 was added. Infection in roller bottles was achieved by simply adding virus carrying wild-type α-cDNA and virus carrying the wild-type or mutant FSHβ-cDNA directly to the roller bottle. Levels of α- and β-virus used gave the highest expression level determined as described previously (Roth & Dias, 1995). Medium containing recombinant protein was collected on the third or fourth day after infection and PMSF (to 1 mM) and sodium azide (to 0.02%) were added to inhibit protease activity and bacterial growth, respectively. Media were tested for expression of heterodimer and stored at 4 °C or frozen at −20 °C until needed.

FSH Enzyme-Linked Immunosorbant Assay (ELISA). The ELISA-based capture (sandwich) assay was performed as described previously (Roth et al., 1993) with modifications as described in Roth and Dias (1995). Dosages of wild-type and mutant recombinant hormones were determined by comparison with standard preparations of hFSH and hFSHβ-subunit purified from frozen human pituitaries, as described previously (Weiner & Dias, 1992).

Protein Purification. Wild-type hFSHβ, QKTCT/AAACA hFSHβ, and each of the scanning mutant heterodimers were purified using immunoaffinity chromatography as described previously (Dias et al., 1994). Eluted protein was concentrated on Amicon Centriprep concentrators (3000 or 10 000 MW cutoff) and then dialyzed against 20 mM Tris, pH 7.0 (scanning mutant heterodimers), or 10 mM potassium phosphate buffer, pH 7.0 (free β-subunits). Purified protein was subjected to N-terminal sequencing (without reduction and alkylation) and amino acid analysis.

Circular Dichroism (CD) of hFSH β-Subunits. CD measurements were performed on a JASCO J-720 spectropolarimeter. Experiments were performed at room temperature with a path length of 0.5 mm. Samples were diluted in 0.02 M potassium phosphate buffer to a final concentration of 0.2 mg/mL. Protein concentration was determined by amino acid analysis. A base line was obtained by the same protocols used for samples using a buffer blank, and this spectrum was subtracted from the sample spectra. Sixteen scans were averaged to improve the signal to noise ratio,

and a Savitzky−Golay filter was used to further improve this ratio. The instrument was calibrated with 0.06% (w/v) ammonium camphorsulfonate.

FSH Radioreceptor Assay (RRA). The receptor binding activity of each mutant heterodimer was determined by RRA using human FSH receptor as described previously (Lindau-Shepard et al., 1994). An ELISA was used to quantitate heterodimer in samples before and after each RRA to control for any loss of heterodimer due to storage or during overnight incubation at room temperature under assay conditions. The affinity constants (K_a) for each mutant were calculated using the program LIGAND (Munson & Rodbard, 1980). The half-maximal inhibitory dose (ID_{50}) for each mutant was determined using the NIHRIA program (Yanagishita & Rodbard, 1978).

FSH in Vitro Bioassay. The FSH in vitro bioassay was performed as described previously (Lindau-Shepard et al., 1994; Dias et al., 1994) with modifications as described in Roth and Dias (1995). As with the RRA, an ELISA was used to assess heterodimer levels in samples diluted in bioassay media immediately before each cell treatment to control for any loss of heterodimer due to storage.

cAMP Assay. Chinese hamster ovary (CHO) cells expressing the human FSH receptor were maintained in α-MEM medium supplemented with penicillin/streptomycin, 10% FBS, and 0.02 μM methotrexate. For the cAMP assay, cells were seeded in 12 well plates at a density of 2.5×10^5 cells/well. Cells were rinsed once with 3 mL of warm growth medium (48 h later and then incubated with 300 μL of treatment medium (α-MEM containing 0.1 mM 3-isobutyl-1-methylxanthine used to inhibit phosphodiesterase activity) for 15 min at 37 °C. Doses of wild-type or mutant rechFSH were made up in treatment medium, and 100 μL was added to each well which still contained the original 300 μL of treatment medium. After 30 min, plates were immediately frozen at −90 °C. Cells were lysed with four freeze/thaw cycles. The lysate was then transferred to 12 × 75 mm polystyrene tubes (Sarstedt, Germany), 300 μL of ethanol was added, and tubes were vortexed and centrifuged at 2300*g*. The supernatants were decanted into 1.5 mL microfuge tubes and stored at 20 °C.

cAMP levels were measured in a double-antibody radioimmunoassay (RIA) (Swift & Dias, 1987). The diluent for all assay components was 50 mM sodium acetate buffer, pH 6.2. Reference dilutions of cAMP or lysate diluted to 100 μL were added to 400 μL of buffer, 100 μL of a 1:5000 dilution of anti-cAMP antibody (Dr. F. Labrie, Laval University, Quebec, Canada), 100 μL of [^{125}I]cAMP (30 000 cpm/100 μL) and 200 μL of a 1:80 dilution of sheep anti-rabbit secondary antibody produced by the laboratory. Tubes were shaken briefly to mix and left overnight at 4 °C. The following day 1 mL of ice-cold 50 mM sodium acetate buffer was added to each tube, and the tubes were centrifuged at 2300*g* at 4 °C for 1 h. The liquid was aspirated, and the tubes were counted in a LKB/Wallac γ counter (Gaithersburg, MD).

Heterodimer Stability in Low pH. To determine the sensitivity of heterodimeric FSH and mutants to low pH, media containing wild-type or mutant heterodimers were diluted 100-fold in 0.1 M sodium acetate, pH 2 or 7. Samples were vortexed to mix and left on ice for 30 min, and then samples at pH 2 were neutralized by adding 300 μL of 2 M Tris base. Additional buffer (pH 7.0) was added to the samples to equalize the volumes. Samples were the mixed 1:1 with ELISA binding buffer (Roth et al., 1993), and 100 μL of each was tested in the ELISA capture assay.

Table 1: Levels of Wild-Type and Mutant recFSH Secreted in Three Different Trials by High Five Cells

protein	heterodimer (μg/10^6 cells)
wild-type	3.3−9.6
scanning mutation[a]	
^{34}TRDL^{37} to ^{34}AAAA^{37}	1.9−2.6
^{37}LVY^{39} to ^{37}AAA^{39}	2.9−3.0
^{40}KDPA^{43} to ^{40}AAPA^{43}	3.7−3.8
^{44}RPKI^{47} to ^{44}APAA^{47}	1.8−3.8
single mutations[b]	
L37A	4.0−5.4
V38A	5.4−7.2
Y39A	5.2−7.4
Q48A	2.2−3.6
K49A	2.9−8.4
T50A	4.1−5.6
T52A	0.3−2.2
single virus isolates of T52	
1	0.5
2	0.31
3	0.18
4	0.25
5	0.31

[a] Some of the data which contribute to the range were from Roth and Dias (1995). [b] All data derived from the present study.

RESULTS

Expression of Single Alanine Mutants. High Five insect cells were coinfected with baculovirus carrying wild-type or mutant β-subunit cDNAs and virus carrying wild-type α-subunit cDNA. Expression levels of each form of heterodimeric hFSH determined in the ELISA capture assay are listed in Table 1. Expression levels of each scanning mutant are shown for comparison. Each of the single mutants in the ^{37}LVY^{39} region was expressed at levels similar to wild-type rechFSH. Each of the single mutants in the ^{48}QKTCT^{52} region was expressed as heterodimer. However, the T52A mutation caused a reduction in the level of heterodimer, which ranged from 4- to 12-fold over a series of experiments compared to expression of wild-type rechFSH. To confirm this finding virus containing the T52A mutation expanded from five single viral isolates was used to express T52A heterodimeric hormone (results shown in Table 1). Compared to wild-type rechFSH expressed in the same experiment (4.2 μg/10^6 cells) the T52A mutant viral clones showed a decrease in heterodimer production which ranged from 8- to 23-fold.

Structural Characterization of Wild-Type and QKTCT/AAACA β-Subunits. N-terminal sequencing showed that the β-subunit purified from insect cells alone or as heterodimer was missing the N-terminal N1 and S2 expected from the hFSH cDNA sequence used for expression. The cause of this deficiency has not yet been determined but may result from alternate signal sequence cleavage or protease activity in the insect-cell expression system. This terminus did not affect the biological activity of insect-cell FSH.

We have reported previously that the mutation of ^{48}QKTCT^{52} to ^{48}AAACA^{52} results in a β-subunit which is expressed with the same secretion dynamics (Roth et al., 1993) and immunoreactivity (Roth & Dias, 1995; Roth et

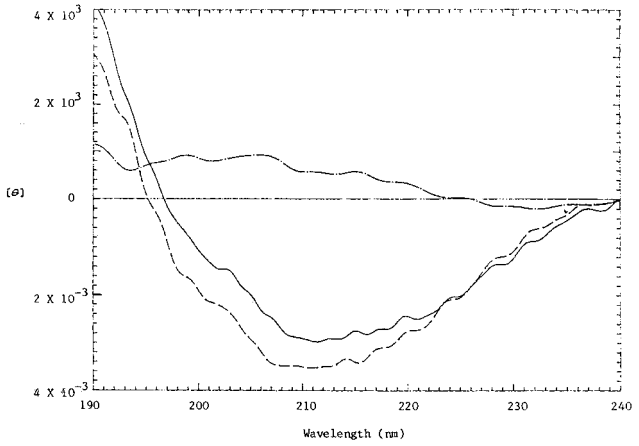

FIGURE 1: CD spectra of wild-type and mutant β-subunits. The spectra were taken at 23 °C, 0.5 mm path, in 0.02 M potassium phosphate buffer. (—) Wild-type β-subunit; (- - -) QKTCT/AAACA mutant β-subunit; (- · -) difference spectrum generated by subtracting the QKTCT/AAACA spectrum from the wild-type β-subunit spectrum.

al., 1993) as wild-type β-subunit but which fails to combine with α-subunit to form heterodimeric hormone. However, as reported above, only the T52A mutation caused a significant decrease of heterodimer formation. In order to assess the conformation of this mutant β-subunit both the ^{48}QKTCT^{52}/^{48}AAACA^{52} mutant and the wild-type β-subunits were purified and examined by CD. The results are shown in Figure 1. The mean residue molecular weights (peptide MW/number of residues) used to determine the molar CD units were 114.43 and 112.83 for wild-type and mutant β-subunit, respectively. Concentrations were determined by amino acid analysis, and spectra were taken at several concentrations. The spectra indicate that these β-subunits have very similar but not identical conformations.

Receptor Binding Activity of Single-Alanine Mutants. The receptor binding activity for each of the single-alanine mutant heterodimers was determined in an RRA assay using CHO cells expressing the human FSH receptor (FSHR) as a receptor source. All of the mutant heterodimers could displace [^{125}I]hFSH from receptors (Figure 2). The dissociation constants (K_d) for each mutant and for the scanning mutants are listed in Table 2 and shown graphically in Figure 3 for ease of comparison. The mutation of ^{37}LVY^{39} to ^{37}AAA^{39} causes a 14- to 16-fold reduction in receptor binding activity (based on K_d). The L37A mutation had no effect on receptor binding while the V38A and Y39A caused a 2.4- and 2.8-fold decrease in receptor binding, respectively. Thus it appears that it is the combined effect of the mutation of V38 and Y39 that leads to the much larger effect on binding seen with the ^{37}LVY^{39} to ^{37}AAA^{39} mutation.

The effect of the composite ^{48}QKTCT^{52} to ^{48}AAACA^{52} mutation on receptor binding could not be assessed because this mutant does not form heterodimer. However, since each single amino acid substitution mutant formed heterodimer, it was possible to assess each for receptor binding activity. The mutation of Q48A caused a decreased receptor binding activity of the heterodimer over 7-fold (Figure 3). None of the other single mutations in this region had a significant effect on receptor binding.

Activation of the Signal Transduction Pathway by Single-Alanine Mutants. Each of the single-mutant heterodimers was tested to determine their ability to stimulate progesterone

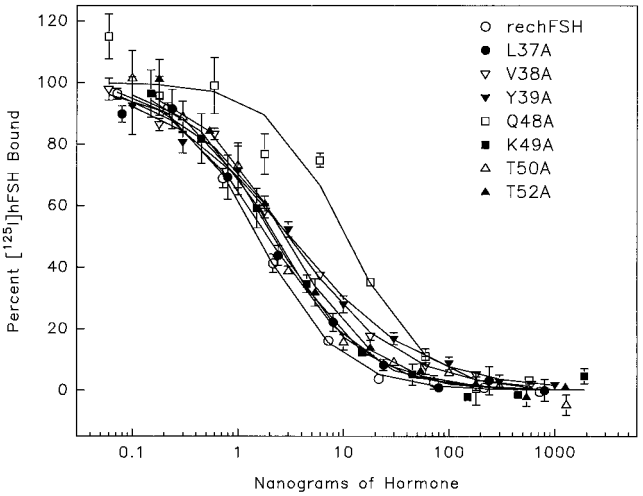

FIGURE 2: Binding of mutant and wild-type heterodimeric FSH to human FSH receptors. A representative RRA. Media samples were incubated overnight with [^{125}I]hFSH isolated from pituitaries and membranes from CHO cells expressing the human FSH receptor as described in Experimental Procedures. Sample doses were based on quantitation in the ELISA capture assay.

Table 2: Binding of Wild-Type and Mutant recFSH to Human FSH Receptors from a Representative Experiment

protein	K_d ($\times 10^{-10}$)
wild-type	3.01 ± 0.72
scanning mutation[a]	
TRDL to AAAA	10.4 ± 1.98
LVY to AAA	48.0 ± 6.72
KDPA to AAPA	6.27 ± 2.32
RPKI to APAA	17.2 ± 2.60
single mutation[b]	
L37A	3.55 ± 0.64
V38A	7.18 ± 1.22
Y39A	8.55 ± 1.80
Q48A	22.80 ± 5.47
K49A	3.93 ± 1.03
T50A	3.80 ± 1.22
T52A	5.36 ± 0.96

[a] Values calculated from RRA data published previously (Roth & Dias, 1995). [b] Values calculated from RRA in the present study.

production in an *in vitro* bioassay using Y-1 cells expressing the human FSHR (Kelton et al., 1992). All of the single-mutant heterodimers could trigger a full progesterone response in these cells (Figure 4). The ED_{50} of the Q48A mutant was higher than wild-type hFSH, consistent with its decreased receptor binding activity.

Stimulation of cAMP by LVY/AAA and RPKI/APAA Mutant Heterodimers. We previously reported that mutation of ^{37}LVY^{39} to ^{37}AAA^{39} and of ^{44}RPKI^{47} to ^{44}APAA^{47} in hFSH failed to produce a maximum response in the *in vitro* bioassay (Roth & Dias, 1995). In order to determine if this was the result of a reduced ability to stimulate cAMP we assessed the ability of these mutants to stimulate cAMP production in CHO cells expressing the human FSHR. Both of these mutants stimulated maximal cAMP levels comparable to wild-type (Figure 5). As expected, the ^{37}LVY^{39} to ^{37}AAA^{39} mutant required higher doses to stimulate this response, reflecting its lower receptor binding ability (Figure 3).

Sensitivity of Mutant Heterodimers to Low pH. While purifying each of the scanning mutants by immunoaffinity chromatography, we discovered that the ^{37}LVY^{39} to ^{37}AAA^{39}

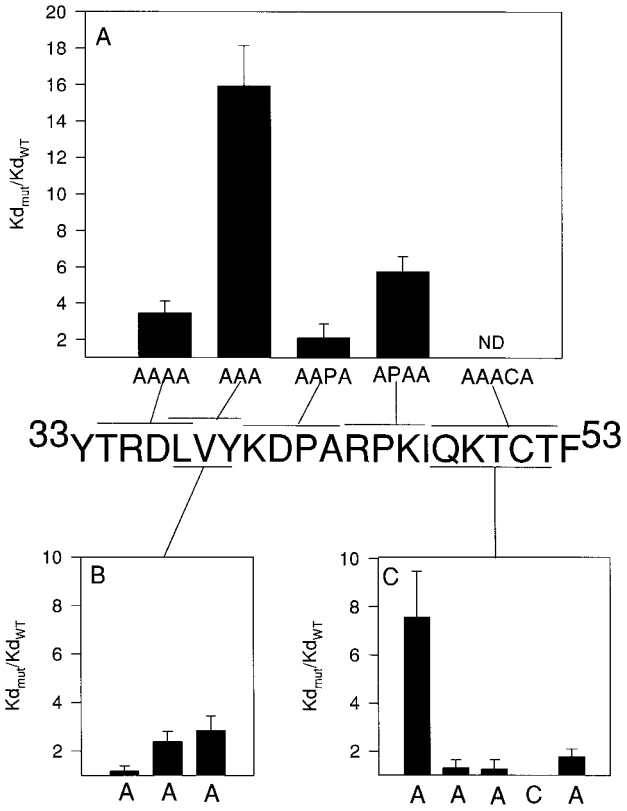

FIGURE 3: Dissociation constants (K_d) for heterodimeric FSH mutants relative to wild-type rechFSH. Values were generated by dividing the K_d for each mutant ($K_{d_{mut}}$) by the K_d for wild-type heterodimeric ($K_{d_{WT}}$). (A) Change in K_d for scanning mutations. ND = not determined because this mutant did not form heterodimer. (B) Change in K_d for single mutations in the ^{37}LVY^{39} region. (C) Change in K_d for single mutations in the ^{48}QKTCT^{52} region. "C" = cysteine 51: no mutation was made at this residue, thus no data are shown.

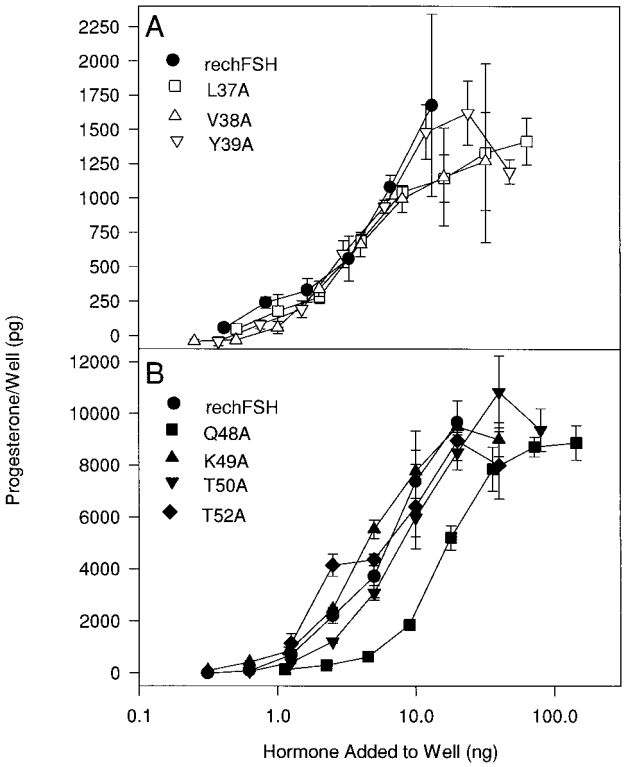

FIGURE 4: Activation of signal transduction by wild-type and mutant heterodimeric FSH. Y-1 cells expressing the human FSH receptor were incubated with mutant or wild-type heterodimers for 24 h. Sample doses were based on quantitation in the ELISA capture assay. Secreted progesterone produced in response to treatment with single mutants in the (A)^{37}LVY^{39} region and the (B)^{48}QKTCT^{52} region was measured by RIAs as described in Experimental Procedures. Each experiment was repeated two times. In these representative RIAs, points represent the average of three wells treated per dose and progesterone in each well was determined in triplicate. Bars represent the standard deviation for the three wells. The difference in total progestrone produced in the two experiments shown is due to a reduction in progesterone production with increased passage number in the Y-1 cells.

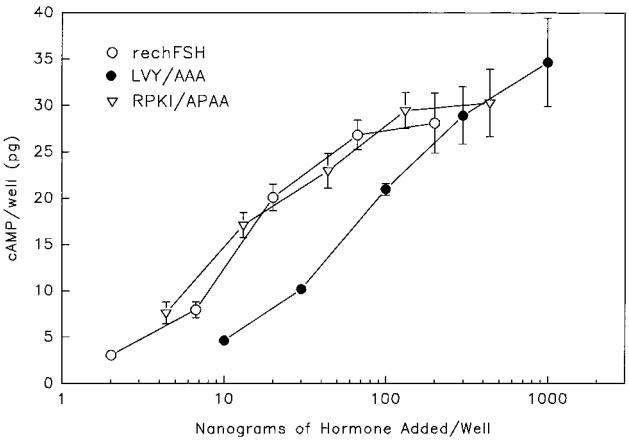

FIGURE 5: cAMP produced in response to treatment with wild-type or mutant heterodimeric FSH. CHO cells expressing the hFSH receptor were incubated with mutant or wild-type heterodimers for 30 min. Sample doses were based on quantitation in the FSH ELISA capture assay. Total cAMP was measured by RIA as described in Experimental Procedures. This experiment was repeated two times. In this representative RIA, points represent the average of three wells treated per dose and the cAMP in each well was determined in triplicate. Bars represent the standard deviation for the three wells.

mutant heterodimer almost completely dissociates when eluted with acidic buffer (pH 2.0). This was unexpected because pituitary FSH requires treatment with 6 M guanidine at low pH for complete dissociation. The pH sensitivity of this mutant might indicate that it lacks important α-subunit contacts. Intrigued by this finding, we tested each of the single-mutant heterodimers for their sensitivity to low pH. The results are shown in Figure 6. The L37A mutant does not dissociate when exposed to low pH. However, the V38A and Y39A heterodimers show almost complete dissociation, indicating that these two amino acids are responsible for the pH sensitivity of the ^{37}LVY^{39} to ^{37}AAA^{39} mutant. In the ^{48}QKTCT^{52} region the Q48A and T52A mutants are the most sensitive, showing almost complete dissociation. However, the T52A mutation has a greater effect on stability because the Q48A mutant is less sensitive to lowered pH at higher protein concentrations (data not shown). Although the T50A mutant shows a 30% dissociation at low pH, no dissociation was seen at higher protein concentrations (data not shown). The K49A mutation is not sensitive to low-pH treatment. In fact L37A and K49A mutants are somewhat more stable at pH 2 is than wild-type rechFSH.

DISCUSSION

The results of the current study serve to refine and extend previous studies aimed at defining the role of amino acids in the L2β in FSH function. In regard to subunit contact our previous studies with monoclonal antibodies indicated that parts of this region are at the surface of the β-subunit

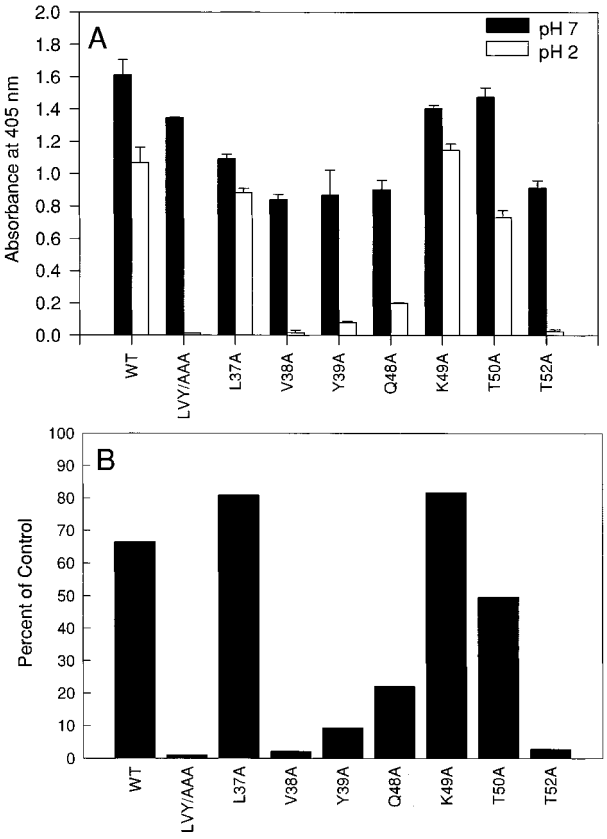

FIGURE 6: Sensitivity of wild-type and mutant heterodimers to low pH. Wild-type or mutant heterodimers were diluted 10-fold in buffer at pH 2 or 7 as described in the Experimental Procedures. After 1 h on ice, samples were neutralized and heterodimer remaining was measured in the ELISA capture assay. (A) Raw absorbance data from ELISA plate. (B) Data from A represented as percent of heterodimer remaining after treatment at pH 2 relative to pH 7. This experiment was repeated two times, and each sample was measured in triplicate. Bars in A represent the standard deviation for three wells in the ELISA.

hFSH	^{31}YCYTRDLVYKDPARP.KI.QKTCTF^{53}
hCG	^{37}--P-MTR-LQGVLPA.LP.-VV-NY^{59}
hLH	^{37}--P-MMR-LQAVLP-.LP.-VVC-Y^{59}
hTSH	^{30}--M---ING-LFLPKYALS-DV--Y^{55}

FIGURE 7: Sequence of the large inter-cysteine loop in the human glycoprotein hormone β-subunits (Wu et al., 1994). A dash (—) represents identity with the FSH sequence while a dot (·) represents a space insertion to preserve the positions of half-cysteines.

but are less accessible in heterodimeric hormone (Vakharia et al., 1991). Also, a peptide corresponding to FSHβ 41—55 binds to the α-subunit (Santa Coloma & Reichert, 1991). In a discription of the hCG crystal structure Wu et al., (1994) stated that the residues corresponding to FSHβ 33—40 and 48—53 are not exposed at the surface of the heterodimer. Figure 7 shows the sequence of the glycoprotein hormone β-subunits in this region. Consonant with the 3-D structure of hCG, mutation of ^{34}TRDL^{37} to ^{34}AAAA^{37} causes a reduction of heterodimer formation when expressed in CHO cells (Roth et al., 1993). Mutation of ^{48}QKTCT^{52} to ^{48}AAACA^{52} causes a failure in heterodimer formation regardless of the expression system used (Roth & Dias, 1995; Roth et al., 1993). In the current study we found that although mutants L38A, V39A, Q48A, T50A, and T52A form heterodimers, these mutants are sensitive to dissociation at low pH. The results suggest that these residues are important for subunit interaction. Moreover, mutation T52A resulted in lowered levels of heterodimer expression. Thus our results correlate well with previous studies which indicate that the flanking regions of 33—53 are involved in subunit contact and identify residues important to subunit interaction.

In our previous studies, mutation of ^{48}QKTCT^{52} to ^{48}AAACA^{52} caused a failure of heterodimer formation (Roth & Dias, 1995; Roth et al., 1993). Previous studies indicate that this mutant is not grossly misfolded based on its ability to bind a conformation sensitive monoclonal antibody (Roth et al., 1993). Our CD results indicate a slight structural difference in the wild-type and QKTCT to AAACA mutant β-subunits which may lead to failure of heterodimer formation. In the current study, when the single mutations were made none of the mutant β-subunits failed to make heterodimer. In the reported hCG crystal structure, Q54 (FSHβ Q48) forms bonds across the loop and this bonded pattern is predicted to stabilize the molecule. Additionally, C57 (FSHβ C51) forms part of the cysteine knot, and residues 56—58 (FSHβ 50—52) are in a β-strand involved in a 7-membered β-barrel (Wu et al., 1994). Finally, V56 (FSHβ T50) makes nonbonded contacts with the disulfide bridge between hCGβ 93—100, which mutational studies have shown is critical to association with the α-subunit (Keutmann et al., 1987). Thus the hCG residues corresponding to those in the FSHβ ^{48}QKTCT^{52} region at which single mutations to alanine affect heterodimer stability (FSHβ Q48, T50, and T52 = hCGβ Q54, V56, and V58) have been identified as residues potentially important for subunit interaction. These results taken together indicate that the failure of the ^{48}QKTCT^{52} to ^{48}AAACA^{52} mutant to form heterodimer was due to the cumulative effect of the multiple mutation on structure as well as subunit interaction.

The single mutants in the QKTCT region provide crucial information about which single amino acids in this region are important to subunit interaction. Thus, the reduced expression level of the T52A heterodimer as well as its pH sensitivity indicates that T52 is important to subunit association. Also, the pH sensitivity of the Q48A and T50A mutants indicates that it is the combined effect of mutating these three amino acids which causes failure of the ^{48}QKTCT^{52} to ^{48}AAACA^{52} mutant to form heterodimer. The pH instability of these mutants could result from the loss of an important hydrogen bond and/or from a change in the microenvironment of surrounding amino acids resulting in a change in their pK_as. The pK_as of residues within folded proteins can vary greatly from the pK_as calculated for free amino acids and are a function of the microenvironment surrounding the side chain (Goldenberg, 1992; Alber, 1989). The pK_a of histidine is especially sensitive to microenvironmental changes, and in FSHβ there is a histidine between residues C83 and C85 (hCGβ C88 and C90), which are part of the cysteine knot motif. Since the cystine knots of both subunits are closely associated, a change of the pK_a of this histidine may affect subunit association.

Immunochemical studies have suggested that L2β is near a receptor binding site. Epitope mapping of an immunoneutralizing antibody showed that part of the epitope was in the 33—53 region (Vakharia et al., 1990). A peptide at 100 mM corresponding to the entire 33—53 region has been reported to block receptor binding and stimulate steroidogenesis (Andersen et al., 1987). Smaller peptides had various effects. Peptide 31—45 blocks FSH receptor binding but does not affect steroidogenesis, while peptide 41—55 does

not block receptor binding (Santa Coloma & Reichert, 1990). The tetrapeptides ^{34}TRDL^{37} and ^{49}KTCT^{52} at millimolar concentrations have been reported by themselves to block receptor binding (Sluss et al., 1986). Finally, the corresponding region in the other glycoprotein hormones has been implicated as being involved in receptor binding (Ryan et al., 1988). Studies aimed at determining regions which control specificity of receptor binding have yielded results which seem at odds with the synthetic peptide results. Using chimeric hormones, Campbell et al., (1991) showed that substituting the FSHβ 33−53 region into hCGβ did not change the receptor binding characteristics of the resulting hormone. Although the reciprocal experiment substituting the hCG residues into FSH has not been reported, results of a recent study showed that synthetic peptides corresponding to the FSHβ 33−53 region or the corresponding region on hCGβ both have the ability to block FSH as well as hCG receptor binding (Keutmann et al., 1995).

The current study shows that V38, Y39, and Q48 are essential for high-fidelity FSH−receptor interaction. It is intriguing that, despite the lack of similarity in this region between FSH and hCG, of only three identical amino acids both V38 and Q48 affect FSH receptor binding. In this regard it is important to note that a spontaneous mutation in hLH causing a change of Q54R (Q48 in FSH) and LH from this individual did not bind LH receptors (Weiss et al., 1992). The present study confirms the importance of this residue in gonadotropin activity, primarily by sustaining appropriate conformational stability. Thus, if as in hCG, Q48 in FSH makes side chain to backbone bonds across this loop, direct contact between the side chain and the receptor is unlikely. However, the alkyl chain of Q48 could make hydrophobic contacts with the receptor, as was reported for a lysine in the co-crystal of hGH human growth hormone and its receptor (Clackson & Wells, 1995).

Our previous study using scanning alanine mutagenesis showed that mutation of ^{37}LVY^{39} to ^{37}AAA^{39} caused a 20-fold reduction in receptor binding activity (Roth & Dias, 1995). That result was somewhat surprising because this region of the loop had not been previously implicated in receptor binding and the hCG crystal structure indicated these amino acids might be buried at the subunit interface (Lapthorn et al., 1994). In our work single mutations at each of these amino acids showed that L37 does not affect receptor binding but V38 and Y39 both result in a 2−3-fold decrease in receptor binding activity. In the hCG crystal structure the residue corresponding to L37 (hCGβ R43) extends away from the α-subunit interface into the center of the loop [see Figure 4e in Wu et al. (1994)] while V44 and L45 (FSH, V38, and Y39) make hydrophobic contacts with phenylalanine residues 17 and 18 in one and 74 in the other of two α-subunit loops (Wu et al., 1994; Lapthorn et al., 1994). Those contacts could be important in holding the α-loops in a particular orientation. These α-loops form an epitope for a monoclonal antibody which blocks binding of FSH to its receptor, and the conformation of this epitope appears dependent on association with hFSHβ (Weiner et al., 1991). Also, all other mammalian α-subunits have a tyrosine at position 17. Chemical modification of this tyrosine is possible, indicating that this amino acid is exposed at the surface of the heterodimer and could form part of a receptor binding site at the interface of the two subunits (Strickland et al., 1984). These α- and β-residues could form a hydrophobic patch important for receptor binding. In a proposed model of hCG bound to its receptor, the region containing the tops of these loops (β 33−53 and the two α-loops) is predicted to contact the transmembrane portion of the receptor (Jiang et al., 1995). The implication of these studies is that a change in the hydrophobicity of residues at these positions could be the cause of the disruption of receptor interaction. Alanine has a shorter side chain and as a free amino acid is less hydrophobic than L, V, or Y, as shown in Table 3. Thus a mutation of any one of these residues would affect hydrophobic contacts. In hCGβ R43 (FSHβ L37) is projecting away from this hydrophobic patch. The calculated $\Delta\Delta G$ of receptor binding indicates that the change in hydrophobicity made by this mutation does not affect FSH structure important for receptor interaction. However, the $\Delta\Delta G$ of receptor binding for the V38A and Y39A correlates well with the change in hydrophobicity of the side chain. These values indicate that mutation of V38 and Y39 together in the ^{37}LVY^{39} to ^{37}AAA^{39} mutant have a cooperative effect which results in the much larger change in receptor binding seen with the triple mutant. Finally, the mutation of ^{34}TRDL^{37} to ^{34}AAAA^{37} had a smaller effect on receptor binding than the ^{37}LVY^{39} to ^{37}AAA^{39} mutation dictating that mutation of L37 does not contribute significantly to the large change in receptor binding seen with the ^{37}LVY^{39} to ^{37}AAA^{39} mutant. Taken together these results indicate that mutation of ^{37}LVY^{39} to ^{37}AAA^{39} affects receptor binding by changing the structure and/or hydrophobicity of an important receptor binding site.

Table 3: Hydrophobicities and Free Energies for Mutated Residues

residue (R)	ΔG (kcal/mol) H_2O−organic[a]	ΔΔ*G* (kcal/mol) R-A[b]	mutation	ΔΔ*G* (kcal/mol) Receptor Binding[c]
L	−1.5	−1.0	L/A	0.6
V	−1.8	−1.3	V/A	1.3
Y	−2.3	−1.8	Y/A	1.6
A	−0.5			
			LVY/AAA	8.8
			TRDL/AAAA	1.9

[a] Hydrophobicity expressed as the free energy gained from transferring residue from aqueous to organic environment (Nozaki & Tanford, 1971). [b] Change in hydrophobicity by mutating residue to alanine. [c] Change in free energy of receptor binding compaired to wild-type rechFSH. This value is derived from the formula $\Delta\Delta G = +Rt \ln(K_{d_{mut}}/K_{d_{WT}})$ (Clackson & Wells, 1995).

The overall goal of our recent studies has been to examine the role the long loop (residues 33−53) of the FSHβ subunit in hormone function. Several of the single mutations affected subunit interaction as well as receptor binding, showing that subunit interaction is critical for full receptor binding potential in the FSH heterodimer. The finding that one mutation (T52A) affected subunit interaction without affecting receptor binding provides strong evidence that it is the specific interaction of the other amino acids (V38, Y39, Q48, T50) with the α-subunit which is important, rather than merely heterodimer stability for example conformational stability. These results have important clinical implications. Design of a more active stable therapeutic hFSH could exploit manipulations at L37 and/or K49, both of which tended to stabilize heterodimeric hFSH. Substitution of phenylalanine for V38 may likewise enhance conformational stability. A general principle emerged, which is simply that receptor binding activity may be affected by subtle confor-

mational changes. Accordingly, the crystal structure of such mutants will likely reveal important potential receptor contacts which shift ever so slightly and compromise receptor binding activity but not immunoreactivity. Such considerations need to be kept in mind when studying spontaneous mutations in the gonadotropins. In this regard, the present results offer predictive outcomes of certain spontaneous mutations.

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