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A Synthetic Peptide Corresponding to Human FSH β-Subunit 33-53 Binds to FSH Receptor, Stimulates Basal Estradiol Biosynthesis, and Is a Partial Antagonist of FSH†

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ABSTRACT: We have previously shown that hFSH-β 34-37 (KTCT) and 49-52 (TRDL) inhibit binding of ^{125}I-hFSH to FSH receptor in calf testis membranes and that hFSH-β 33-53, which encompasses these tetrapeptides, inhibits binding with increased potency. hFSH-β 33-53 rapidly dimerizes under conditions utilized in the receptor binding assay (pH 7.5) so that the binding inhibition reported earlier was due to the hFSH-β 33-53 dimer rather than the monomer. At pH 6.5, conversion to dimer does not occur, and binding inhibition could be unequivocally attributed to the monomer. Radioiodinated and alkylated hFSH-β 33-53 binds to the FSH receptor with a $K_d = (5.5 \pm 1.4) \times 10^{-5}$ M. The biological activity of hFSH-β 33-53 was assessed by its ability to affect the conversion of androstenedione to estradiol in rat Sertoli cells cultures. FSH-β 33-53 behaved as a partial antagonist of the FSH-induced estradiol synthesis. The required incubation medium, however, contains cystine as well as cysteine, which rapidly forms a hFSH-β Cys(51)-S-S-Cys derivative at the pH of the incubation, 7.4. When hFSH-β 33-53 was converted either to the hFSH-β Cys(51)-S-S-Cys or to a carboxymethylated derivative, inhibition of FSH-induced estradiol synthesis still was observed. This result demonstrates that the free R-SH group at Cys51 is not responsible for the inhibition. FSH-β 33-53 also significantly stimulated basal levels of estradiol synthesis, but not to maximal levels observed with FSH (partial agonist). Neither the carbohydrate content of hFSH-β nor the α subunit of FSH appears to be essential for signal transduction and expression of the hormone effect of FSH-β 33-53.

Follitropin (FSH), a pituitary glycoprotein hormone composed of two nonidentical subunits, plays an important role in ovulation in the female and spermatogenesis in the male. In an earlier study (Sluss et al., 1986), we reported inhibition of ^{125}I-hFSH binding to testicular FSH receptors by synthetic tetrapeptides corresponding to amino acids 34-37 (TRDL) and 49-52 (KTCT) of the β subunit of human follitropin (FSH). A subsequent report (Schneyer et al., 1988) concluded that the interaction of FSH with its receptor may involve multiple, discrete binding determinants, which included hFSH-β 34-37 (TRDL). In a preliminary report (Andersen et al., 1987), we noted that a synthetic peptide encompassing TRDL and KTCT, and representing hFSH-β 33-53 (H_2N-YTRDLVYKNPARPKIQKTCTF-$CONH_2$) (Pierce & Parsons, 1981), also inhibited ^{125}I-hFSH binding to receptor, at a potency significantly greater than that seen with either individual tetrapeptide. The presence of cystine at position 51 caused us to suspect the observed effect might be due, in part at least, to a dimer of the peptide. We report here the results of our studies on the receptor binding properties of monomer-dimer forms of hFSH-β 33-53, and on the ability of each form, as well as various cysteine-51 derivatives, to affect steroidogenesis in cultured rat Sertoli cells. Our results suggest hFSH-β 33-53 binds to the FSH receptor in a functional manner, affecting both basal and FSH-stimulated steroidogenesis. They also imply that neither carbohydrate content nor the FSH-α subunit is absolutely essential for FSH signal transduction.

Experimental Procedures

Materials. Highly purified human FSH (LER-1781-2, 4000 IU/mg) was radioiodinated and used as the radioligand in the radioreceptor assay. Purified ovine FSH (LER-1996-S, 8 NIH-FSH-S1 units/mg) was used in the Sertoli cell culture assay. Iodoacetamide, cysteine, and dithiothreitol (DTT) were obtained from Sigma (St. Louis, MO). All solvents and buffers utilized in the chromatographic procedures were HPLC-grade reagents.

Peptide Synthesis, Purification, and Characterization. The synthetic peptide corresponding to amino acids 33-53 of hFSH-β subunit was prepared by Dr. Jean Rivier, Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, San Diego, CA, under Contract No1-HD-7-2907 from the Contraceptive Development Branch, Center for Population

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Research, NICHHD. Synthesis was by the solid-phase method (Merrifield, 1963) using the *t*-BOC protection scheme. The hFSH-β 33–53 peptide was purified by us utilizing preparative reverse-phase liquid chromatography on octadecyl-silica (Waters Delta-Pak C-18, 100-Å pore diameter) using a linear acetonitrile gradient in 0.1% TFA from 5% to 100% CH_3CN, at 30 °C. Homogeneity was verified by analytical HPLC. Amino acid composition of the peptide was determined using the Waters Pico-Tag system (Bidlingmeyer et al., 1984) and was in accord with predicted values.

Preparation of Radiolabeled FSH. Radiolabeled hFSH was prepared as we described previously (Schneyer et al., 1986). This method involves lactoperoxidase-catalyzed iodination of highly purified human FSH. Polyacrylamide gel electrophoresis (PAGE) was utilized to purify the hormone following radioiodination (Schneyer et al., 1986). The resulting ^{125}I-hFSH had a specific activity of 25 μCi/μg and a minimum of 35% bindability to excess receptors.

Radioligand Receptor Assay (RRA). Preparation of calf testis membranes containing FSH receptors and the RRA were performed essentially as we described elsewhere (Schneyer et al., 1986) with the following modifications: 3 mg wet weight of membrane preparation, 2.5 ng of radioligand, and samples or buffer (HEPES, 50 mM; sucrose, 250 mM; and $MgCl_2$, 5 mM, adjusted to pH 7.5 or 6.5 to a total volume of 0.25 mL) were incubated for 18 h at 20 °C. After incubation, hormone bound to membrane receptor was separated from unbound hormone by centrifugation (30000*g*, 15 min). Nonspecific binding (NSB) was defined as radioligand bound in the presence of a 300-fold excess of unlabeled hFSH.

Conversion of hFSH-β 33–53 Dimer to Monomer by Reduction. The hFSH-β 33–53 peptide contains a cysteine residue at position 51, leading to rapid dimerization during storage and under various experimental conditions as described under Results. In order to convert hFSH-β 33–53 dimer to monomer, the dimer (15 mg) was dissolved in 1 mL of buffer A (HEPES, 50 mM; Sucrose, 0.1 M; and $MgCl_2$, 5 mM, made to pH 8.5 with triethylamine) and reduced in the presence of a 100-fold molar excess of dithiothreitol (DTT) by incubation for 30 min at 25 °C. Conversion of dimer to monomer, which was 100% under the conditions described, could be monitored by taking advantage of their differential elution times upon analytical reverse-phase HPLC. The reduced peptide was immediately subjected to HPLC to remove excess DTT. The resulting fractions were evaporated under a nitrogen stream, extracted with ether (to remove any TFA remaining from HPLC), suspended in acetone, dried as before under nitrogen, and stored at −20 °C until needed. Under these conditions, the reduced peptide remained stable as a monomer for several days. Alternatively, the reduced peptide was immediately alkylated after reduction.

Peptide Alkylation with Iodoacetamide. After reduction with DTT as described above, the peptide was protected from light and incubated for 30 min at 4 °C with a 2-fold molar excess (relative to DTT) of iodoacetamide, followed by incubation for 4 h at 25 °C. The alkylated peptide was purified by reverse-phase HPLC, as described above.

Preparation of Radioiodinated Synthetic Peptides. The procedure used for radioiodination of the synthetic peptides was similar to that utilized for radioiodination of hFSH, with the exception that a total of 100 μg of peptide was utilized and the separation of free iodine from iodinated peptide was performed by gel filtration through Sephadex G-25. The specific activity of the alkylated and radioiodinated peptide was 13.4 μCi/μg.

Scatchard Analysis of Binding Data. Increasing amounts of alkylated and radioiodinated synthetic hFSH-β 33–53 (from 25 to 125 μg) were incubated with membrane receptors (3 mg) in a final volume of 0.5 mL (see RRA procedure) at pH 6.5. Nonspecific binding was determined at each concentration using excess intact hFSH. Incubation was for 18 h at 20 °C, after which the bound and unbound ligands were separated by centrifugation at 30000*g* for 15 min. Scatchard analysis (Scatchard, 1949) was done by using the LIGAND-PC program version 3.0 (Munson & Rodbard, 1988).

Cysteinyl Derivative. Ten milligrams of peptide was dissolved in buffer A, pH 6.5. A 2-fold molar excess of cysteine and a 2-fold molar excess of cystine were added to the solution, and the pH was adjusted to 7.5 with triethylamine. The solution was stirred for 16 h at 25 °C. Conversion of the peptide to its cysteinyl derivative was monitored by taking advantage of differential elution times upon reverse-phase HPLC. The derivative was purified by reverse-phase HPLC as indicated above.

Measurement of Forms of hFSH-β 33–53 under Various Assay Conditions. The stability of hFSH-β 33–53 under conditions of RRA and in vitro bioassay (see below) was assessed by incubating the peptide (0.025 and 0.1 mM) in a final volume of 2 mL of either RRA or cell culture medium. The incubation conditions were those utilized for the respective assays, with aliquots removed at various times for assessment of conversion to other forms through use of analytical reverse-phase HPLC.

In Vitro Bioassay for FSH Activity. Assessment of FSH agonist or antagonist activity was based on the ability of peptides to affect either the basal or the hormone-stimulated conversion of androstenedione to estradiol by cultures of Sertoli cells taken from immature rat testis. The method utilized was essentially that described by us elsewhere (Sluss et al., 1987), slightly modified for multi-well plating (Grasso & Reichert, 1989). Medium was changed 48 h after isolation of Sertoli cells and initial incubation. After an additional 24 h, oFSH or test peptides were added to the cells, and incubation was allowed to proceed for another 24 h. The final incubation medium contained methylisobutylxanthine (MIX) (0.1 mM) and androstenedione (0.04 mM) as substrate for aromatase. Measurement of estradiol was done by RIA as previously described (Schneyer et al., 1986, 1988) with modifications (Grasso & Reichert, 1989) using antiserum generously made available by Dr. Gordon Niswender, Department of Physiology and Biophysics, Colorado State University, Fort Collins, CO.

Results

pH-Related Conversion of hFSH-β 33–53 from Monomer to Dimer Forms and Inhibition of ^{125}I-FSH Binding. In initial studies (Andersen et al., 1987), we reported that hFSH-β 33–53 inhibited binding of ^{125}I-hFSH to testis membrane receptors with a potency considerably greater than observed with the tetrapeptides hFSH-β 34–37. On the basis of elution times from reverse-phase HPLC (Figure 1A–D), it appeared that the purified hFSH-β 33–53 was rapidly converted to another form when incubated under conditions utilized for the FSH RRA. The latter is conducted by incubating peptide with membrane receptor for 20 h at pH 7.5. Because of the presence of a free R–SH group in hFSH-β 33–53, at Cys51, it was presumed this conversion was to the corresponding cystine dimer. Reduction by dithiothreitol of the pH 7.5 generated component confirmed that this was the case. Under the conditions of the assay, there is, at pH 7.5, a rapid conversion of the monomer to dimer form (Figure 2) that is essentially complete after only a few minutes at a peptide

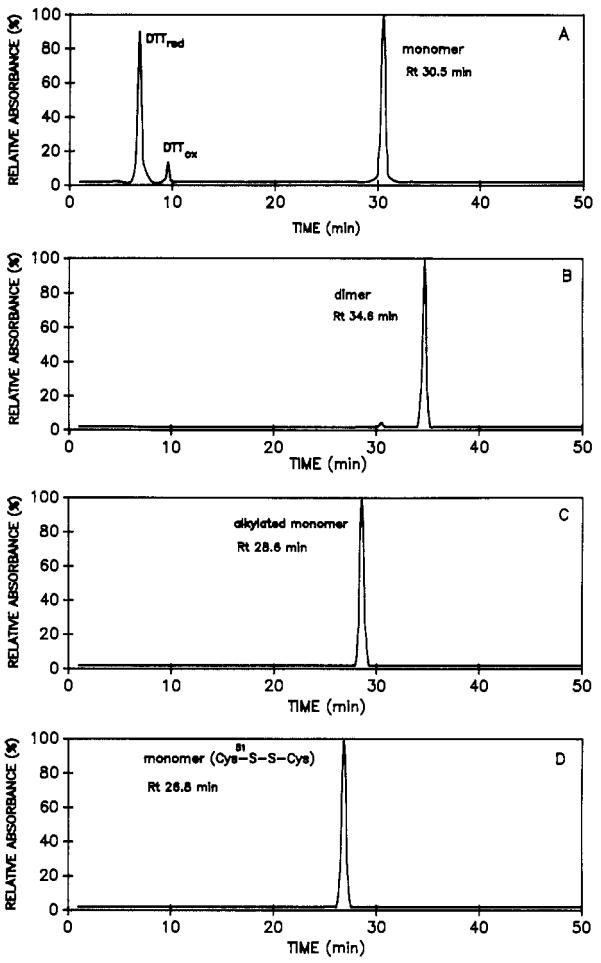

FIGURE 1: Analytical HPLC profiles of monomer, dimer, alkylated, and cysteinylated peptide after preparative HPLC purification: hFSH-β 33–53 peptide and DTT (100× molar excess, pH 8.5) were added to keep the peptide as monomer (room temperature, 30.5 min) (A); monomer converted to dimer at pH 7.5 (room temperature, 34.6 min) (B); carboxymethylated peptide (room temperature, 28.6 min) (C); cysteinylated peptide (room temperature, 26.8 min) (D).

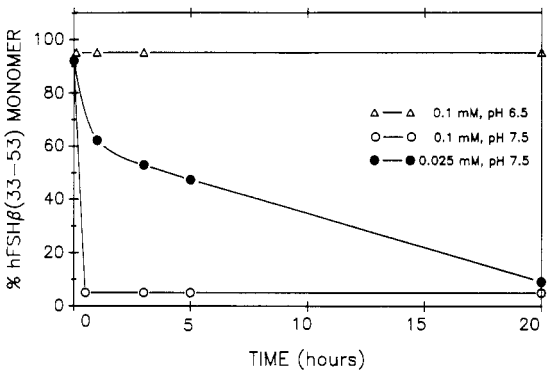

FIGURE 2: Time course of the hFSH-β 33–53 monomer to dimer conversion in RRA buffer: hFSH-β 33–53 monomer was incubated at pH 6.5 or 7.5 in RRA buffer (HEPES, 50 mM, sucrose, 250 mM, and $MgCl_2$, 5 mM). At different times, aliquots were removed and analyzed by HPLC. The percentages of remaining monomer are indicated [mean ± standard error (SE), n = 2]. At pH 6.5, the hFSH-β 33–53 monomer was stable for 20 h.

concentration of 0.1 mM, and more slowly at a more dilute concentration of peptide (0.025 mM). The hFSH-β 33–53 monomer could be regenerated from the dimer form by reduction with DTT (Figure 1A) and stabilized in the monomeric form by alkylation with iodoacetamide (Figure 1D). It seems clear, however, that ^{125}I-hFSH binding inhibition initially reported by us (Andersen et al., 1987) was due to the

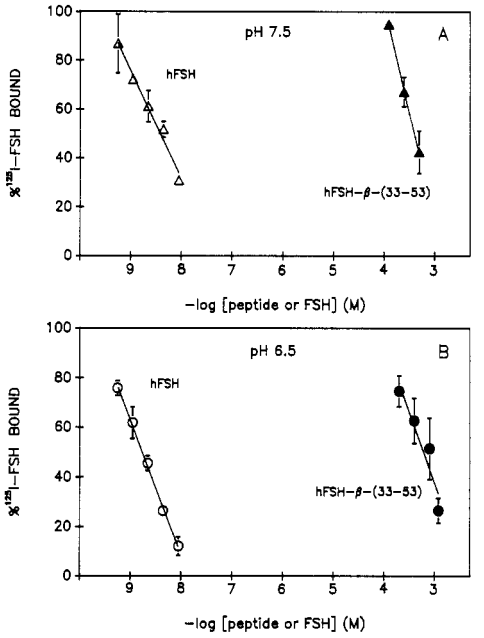

FIGURE 3: ^{125}I-FSH binding inhibition by hFSH-β 33–53 at pH 6.5 and 7.5: binding inhibition at pH 7.5 produced by competition with hFSH or hFSH-β 33–53 peptide (dimer) (A); binding inhibition at pH 6.5 (peptide as monomer) (B). Similar results were obtained at each pH (ED_{50} = 0.4 ± 0.1 mM at pH 7.5; ED_{50} = 0.7 ± 0.2 mM at pH 6.5), suggesting that the dimer and monomer inhibitory potencies are similar [mean ± standard error (SE), n = 2].

action of the peptide dimer. hFSH-β 33–53 was stable in the monomeric form at pH 6.5 (Figure 2). The FSH RRA assay was performed at pH 6.5 instead of 7.5 to determine the effect of the peptide monomer on ^{125}I-hFSH binding. Inhibition of binding of ^{125}I-hFSH to membrane receptor by unlabeled hFSH was similar between pH 6.5 and 7.5 (EC_{50} = 80 ng/mL at pH 6.5, ED_{50} = 120 ng/mL at pH 7.5). Inhibitions of ^{125}I-hFSH binding to membrane receptor by hFSH-β 33–53 monomer (at pH 6.5) and dimer (at pH 7.5) were also comparable (ED_{50} = 0.7 mM and ED_{50} = 0.4 mM, respectively) (Figure 3A,B). Thus, apparently both monomeric and dimeric forms of FSH-β 33–53 are capable of inhibiting the specific binding of FSH to its receptor, and with comparable potency. Presumably, the dimer is sufficiently small so that it may fit into the hormone binding site on the membrane without complications due to steric hindrance. Another implication of these observations is confirmation of the results obtained with derivatives of hFSH-β-Cys51, that the free sulfhydryl group at Cys51 is not responsible for the interaction of peptide with receptor (because the dimer also inhibited the FSH binding).

Binding of hFSH-β 33–53 to FSH Membrane Receptor. The interaction of hFSH-β 33–53 with receptors on calf testis membranes was studied utilizing Scatchard analysis (Scatchard, 1949). hFSH-β 33–53 was radioiodinated, and binding to FSH receptor enriched membrane was measured in the presence and absence of FSH. The usual procedure for radioiodination is performed at pH 7.5. As shown previously (vida supra), at this pH there is a rapid conversion of hFSH-β 33–53 to the dimer form, which iodinates poorly (Figure 4). To overcome this problem, hFSH-β 33–53 was maintained in the monomer form by carboxymethylation of Cys51. The carboxymethylated hFSH-β 33–53 iodinates readily (Figure 4), and the iodinated derivative was separated from free ^{125}I by Sephadex G-25 gel filtration. Scatchard analysis (Figure 5), utilizing increasing amounts of radiolabeled peptide, indicated a single class of binding sites with a K_d = (5.5 ± 1.4)

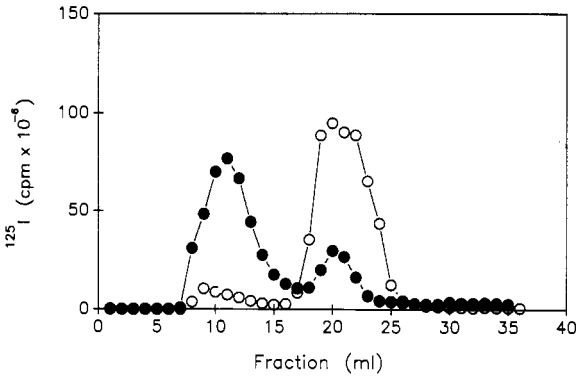

FIGURE 4: Sephadex G-25 chromatography of radioiodinated hFSH-β 33–53 peptide: the peptide (○) and its carboxymethylated derivative (●) were radioiodinated as indicated under Experimental Procedures. Radioiodinated peptides were separated from free iodine by gel filtration. The underivatized peptide was poorly iodinated and could not be used for receptor binding studies. The alkylated peptide was used for Scatchard analysis.

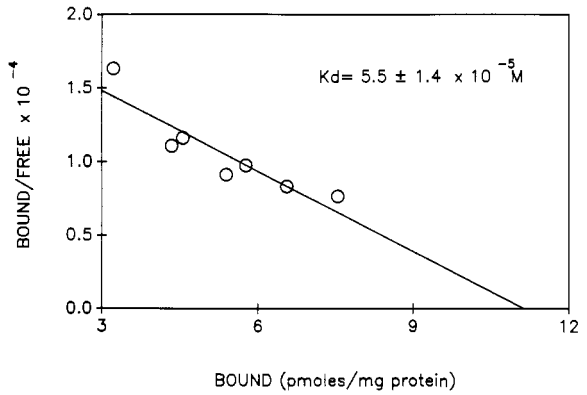

FIGURE 5: Scatchard plot of ^{125}I-hFSH-β 33–53 binding to calf testis membrane: because of the low affinity of peptide for receptor, it was necessary to add unlabeled peptide to the radioiodinated and alkylated peptide (1.6 μg/ng of tracer) to create a favorable condition for binding. Nonspecific binding was determined at each concentration (50–250 μg/mL) using excess of intact hFSH. The binding affinity constant [K_d = (5.5 ± 1.4) × 10^{-5}] (mean ± standard error, n = 2) was calculated by using the program LIGAND version 3.0 (Munson & Rodbard, 1988).

× 10^{-5} M. We have developed evidence earlier (Schneyer et al., 1988) for the presence of multiple points of interaction between hormone and receptor, so that the decreased affinity of hFSH-β 33–53 compared with the intact FSH molecule (K_d = 10^{-10} M) was not unexpected. A similar value of K_d has been estimated for synthetic hCG peptides (Keutmann et al., 1988).

Effect of hFSH-β 33–53 on Estradiol Synthesis in Cultured Rat Sertoli Cells. We next examined the effect of hFSH-β 33–53 on the conversion of androstenedione to estradiol in cultures of Sertoli cells taken from testis of immature rats. The effect of increasing concentrations of ovine FSH on estradiol synthesis in this system is shown in Figure 6. A maximum response was obtained with 20 ng/mL oFSH, and the response to 10 ng/mL FSH was consistently submaximal. hFSH-β 33–53, at 40 μM, significantly (p < 0.01, Student's t test) inhibited the response to 10 ng of oFSH (Figure 7). Higher concentrations of peptide (80 μM) did not further decrease the effect of FSH. This suggested that the peptide may be functioning as a partial agonist of FSH action, possibly by occupying FSH binding sites, but stimulating less than optimal hormone signal transduction. To examine this point further, we studied the effect of FSH-β 33–53 on the basal secretion of estradiol in the cultured rat Sertoli cell system.

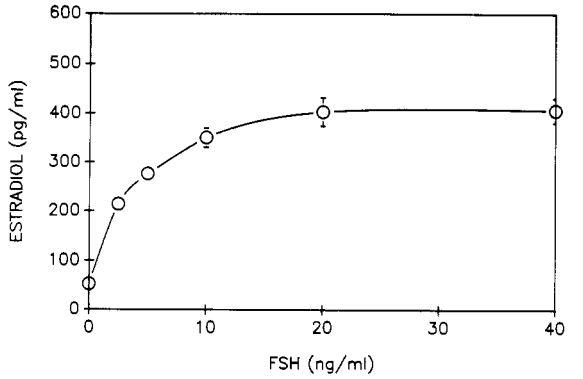

FIGURE 6: Stimulation of estradiol biosynthesis by Sertoli cells: Sertoli cells were isolated from testis of 16-day-old immature rats and maintained in culture 72 h prior to treatment. Androstenedione (0.04 mM) and Mix (0.1 mM) were added, and cells were incubated 24 h with different concentrations of FSH. Maximum response was obtained with 20 ng/mL FSH (mean ± SE, n = 3).

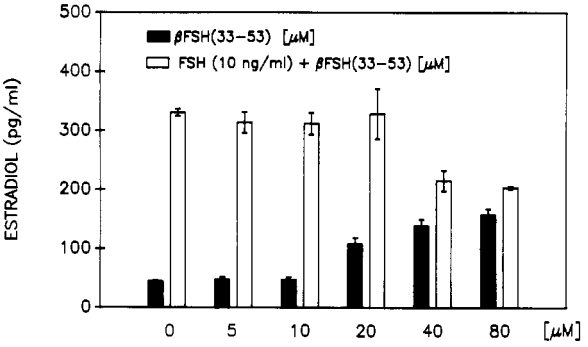

FIGURE 7: Effect of hFSH-β 33–53 on estradiol biosynthesis: the biosynthesis of estradiol by cultured rat Sertoli cells, in the absence (filled bars) or presence (open bars) of oFSH (10 mg/mL), at different peptide concentrations is shown (mean ± SE, n = 3). The peptide was able to stimulate estradiol biosynthesis up to 40% of the maximal response. At higher doses (40–80 μM), the peptide was a partial antagonist of FSH. Similar results were obtained with the alkylated hFSH-β 33–53 peptide.

As can be seen (Figure 7), hFSH-β 33–53 significantly stimulated basal levels of estradiol synthesis, with a maximal stimulation seen at about 80 μM. This agonist effect on basal levels of estradiol synthesis probably explains why the peptide cannot further diminish FSH-stimulated estradiol biosynthesis at concentrations in excess of 40 μM. It appears, therefore, that hFSH-β 33–53 functions as a partial agonist with respect to basal estradiol levels, and as a partial antagonist of FSH-stimulated estradiol biosynthesis in the cultured Sertoli cell system. The ability of the peptide to affect signal transduction and promote steroidogenesis in vitro is also noteworthy in view of the apparent absence of an absolute requirement for the carbohydrate components present in the β subunit to achieve this effect.

Role of Cys51 of hFSH-β 33–53 in Inhibition of FSH-Stimulated Estradiol Biosynthesis. The binding of FSH to its receptor is inhibited by treatment of membranes with reducing agents such as DTT (Reichert & Abou-Issa, 1976). Therefore, the presence of a free R–SH group at position 51 in the hFSH-β 33–53 peptide might lead to nonspecific binding inhibition through reduction of the FSH receptor on cultured Sertoli cells. In addition, the medium required for Sertoli cell culture (DMEM-F12) contains cysteine and cystine (0.1 mM each) so that formation of an hFSH-β Cys(51)–S–S–Cys derivative seemed likely. We prepared the latter derivative of hFSH-β 33–53 and determined conditions for its identification, using reverse-phase HPLC. This procedure was also utilized to monitor the forms of hFSH-β 33–53 generated

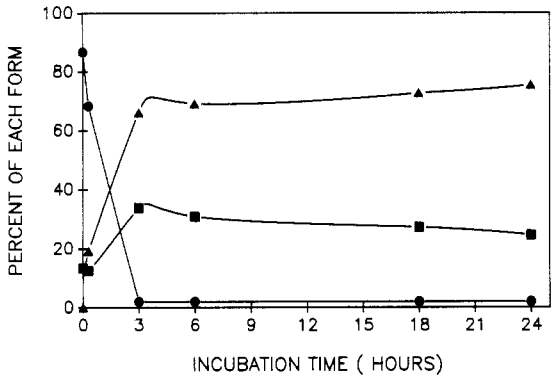

FIGURE 8: Time course of hFSH-β 33–53 monomer conversion to dimer and cysteinyl derivative in culture medium DMEM-F12: the hFSH-β 33–53 peptide (0.1 mM) was incubated for different times under exactly the same conditions used during Sertoli cell culture (DMEM-F12, 37 °C, 5% CO_2). At the indicated times, the medium was analyzed by HPLC and the precentage of each form determined (mean ± SE, n = 2). (●) Monomer; (■) dimer; (▲) cysteinylated peptide. After 3 h, almost all the monomer was converted to dimer (30%) and cysteinylated peptide (70%).

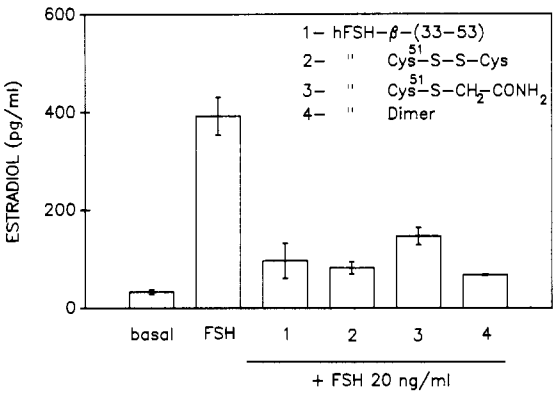

FIGURE 9: Inhibition of FSH-stimulated estradiol biosynthesis in cultured rat Serotoli cells by hFSH-β 33–52 monomer, dimer, alkylated, and cysteinyl derivatives: the different derivatives formed upon incubation in DMEM-F12 were synthesized, purified by HPLC, and tested at 0.1 mM concentration. All the peptides had inhibitory activity (mean ± SE, n = 3), indicating that the reductive R–SH group of Cys51 was not responsible for the inhibitory effect.

under the conditions of Sertoli cell culture. As can be seen in Figure 8, the culture medium causes a rapid conversion of monomer to dimer as well as a rapid formation of the hFSH-β Cys(51)–S–S–Cys derivative, so that at the end of incubation, the latter is the predominant species present (about 80%), along with a small amount (about 20%) of peptide dimer.

Although incubation of hFSH-β 33–53 with the culture medium of Sertoli cells (DMEM-F12) resulted in a mixture of forms, the peptide, at an initial concentration of 0.1 mM, inhibited FSH (20 ng/mL) stimulation of estradiol synthesis (Figure 9). When, hFSH-β 33–53 was allowed to dimerize at pH 7.5 prior to addition to the assay system, the preformed dimer also inhibited FSH stimulation of steroidogenesis (Figure 9). Similar results were obtained when hFSH-β Cys(51)–S–S–Cys (formed by incubation with cysteine) was tested (Figure 9). Under these circumstances, it was not possible to assign the steroidogenic nor the inhibitiory activity to any particular species (monomer, dimer, or cysteinylated monomer). However, due to the irreversibility of the alkylation, the carboxymethylated peptide cannot form peptide dimer or cysteinyl derivative in the culture medium. hFSH-β Cys(51)–S–CH_2–$CONH_2$ (formed by alkylation with iodoacetamide) inhibited the estradiol biosynthesis (Figure 9). In addition, the effects on basal or FSH-stimulated estradiol biosynthesis, obtained when utilizing the alkylated peptide (data not shown), were similar to those obtained with the underivatized peptide (Figure 7). These results suggest that the free sulfhydryl group is not involved in the activity of hFSH-β 33–53.

Since the various Cys51 derivatives lacking a free sulfhydryl group inhibited the estradiol response, it seems clear that the free sulfhydryl group at Cys51 in the hFSH-β 33–53 peptide is not required for receptor binding inhibition and that the peptide does not inhibit the FSH response through nonspecific reduction of receptor disulfide bonds. These findings are consistent with the absence of free sulfhydryl groups in the intact glycoprotein hormones (Ryan et al., 1987).

Discussion

The β subunits of the gonadotropic hormones share regions of primary structure that are highly conserved. Since FSH, LH, TSH, and hCG also have a common α subunit, it seems likely that the regions on the β subunit sharing highest sequence homology are those involved in binding to the α subunit. The regions with lowest homology or highest sequence variability would include receptor binding regions (Pierce & Parsons, 1981). By analysis of aligned sequences, it is possible to identify several regions of the β subunit that could be involved in interaction with the α subunit. These regions include hFSH-β 12–32, 52–67, 76–79, or 76–87. Neither the N-terminal nor the C-terminal regions are thought to play an important role in the interaction between subunits, or between hormone and receptor (Ryan et al., 1988). As a result, using the subtractive approach, there appear three likely receptor binding regions in the hFSH-β subunit. These are sequences 33–51, 68–75, and a region near the C-terminus, possibly 87–94.

Ward and Moore (1979) have postulated that the HCG β subunit region 93–100 may be the determinant of hormone specificity among the glycoprotein hormones and designated this region the "determinant loop". This determinant loop concept has proved useful in attempts to understand details of the interaction of the glycoprotein hormones and receptors. Recently, Keutmann et al. (1989) reported that a synthetic peptide corresponding to this region inhibited hCG binding to receptor but did not stimulate testosterone production in cultured rat Leydig cells. Keutman et al. (1987) found that synthetic peptides corresponding to 38–57 of the hCG-β subunit (comparable to hFSH-β 32–51) inhibited hCG binding to receptor, and also stimulated testosterone production in Leydig cell cultures. These results suggest that multiple receptor binding sites exist on hCG and that all receptor binding sites on hCG are not functionally associated with postbinding events.

We have recently identified a putative receptor binding site on the hFSH-β subunit, hFSH-β 34–37, and suggested that multiple receptor binding sites may also exist on hFSH (Schneyer et al., 1988). Because of the complex heterodimeric glycoprotein structure of FSH, such a prediction would not seem unreasonable. One would not expect, therefore, that a small peptide region as represented by hFSH-β 33–53 could possess all of the structural and binding characteristics required for a quantitative expression of the hormone effect as elicited by the hFSH α/β hologlycohormone.

A two-dimensional graphic representation of secondary structure predictions (Chou & Fasman, 1978) for hFSH-β 33–53 is shown in Figure 10A–D. In addition to structural features, the graphics indicate zones of maximum hydrophilicity and hydrophobicity (Figure 10A) and the probability for surface exposure of amino acids (Figure 10B) as well as

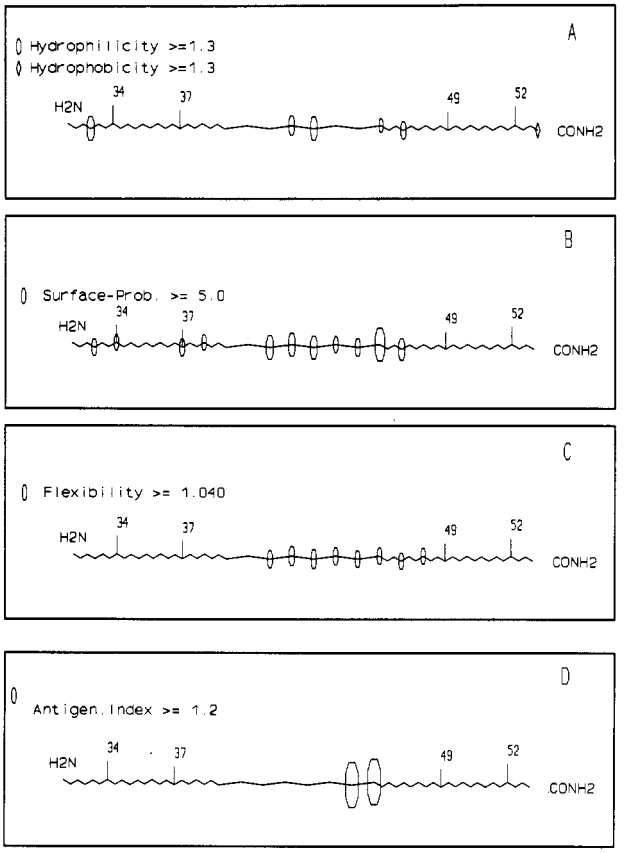

FIGURE 10: Secondary structure analysis of hFSH-β 33–53: the analysis has been done by using the GCG program (Devereux et al., 1984). The figure includes Chou and Fasman analysis of secondary structure (wavy lines β sheets and straight lines random coil) together with hydrophilicity and hydrophobicity (A), surface probability (B), flexibility (C), and antigenic index (D). No α helix was predicted. The peptide seems to be random coil oriented and highly flexible in the center, with β-sheet structure at the extremes. The high surface probability and antigenic index predict that the peptide should be surface-oriented and highly antigenic.

maximum flexibility (Figure 10C) and likely antigenicity (Figure 10D). From such an analysis, it is possible to anticipate that hFSH-β 33–53 is a highly unstructured and flexible peptide that is exposed on the surface of FSH and may represent, therefore, an antigenic determinant of the hormone. We have previously reported a component of the exposed region, hFSH-β 34–37 (TRDL), to represent an antigenic determinant of FSH (blocks interaction between FSH and a hormone-specific polyclonal antibody) and proposed it to represent an FSH receptor binding region of the hFSH β subunit (Schneyer et al., 1988).

The high flexibility and absence of structure predicted for hFSH-β 33–53 by the Chou and Fasman analysis suggest that the peptide is capable of adopting numerous conformations in solution (Wright et al., 1988). The ability of this peptide to inhibit binding of FSH to membrane receptor with homogeneous (parallel) binding inhibition isotherms indicates that hFSH-β 33–53, as well as its dimer form and various derivatives, is capable of assuming a conformation compatible with a productive receptor interaction. The low affinity constant for binding of radioiodinated peptide to membrane receptor as determined by Scatchard analysis (K_d = 5.5 × 10^{-5} M) compared to that of the hFSH α/β holohormone (K_d = 10^{-10} M) is not surprising if multiple binding sites and the possibility of several peptide conformers in solution are considered. In addition, because the carboxymethylated peptide also inhibits the estradiol response, the inhibition can be attributed to the peptide itself and not to the reductive properties of the sulfhydryl group at Cys51. The binding of the radioiodinated peptide provides the first direct evidence for the interaction of a segment of the hFSH-β subunit with the FSH receptor.

The hFSH-β 33–53 peptide, in addition to functioning as a binding antagonist, stimulated estradiol synthesis in cultured rat Sertoli cells. Maximum stimulation obtained with the peptide was about 50% of maximal FSH stimulation, and the peptide also inhibited the steroid response generated by FSH. Thus, in addition to functioning as a binding antagonist, hFSH-β also functions as a partial agonist of FSH action. The steroidogenic effect of the synthetic peptide also indicates that although the α subunit and the carbohydrate structure of FSH-β are required for complete expression of hormone action and quantitatively maximal steroidogenic effects, they are not absolutely required for effective signal transduction. The effectiveness of small peptides to interact with the FSH receptor with expression of antagonist/partial agonist effects suggests that analogues of hFSH-β 33–53, as well as of other receptor binding regions as they are identified, may lead to generation of derivatives with properties of potential usefulness in understanding and controlling reproductive processes.

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Registry No. FSH, 9002-68-0; hFSH-β 33–53, 124341-78-2; hFSH-β 33–53, Cys(51)–S–S–Cys, 124354-90-1; hFSH-β 33–53, Cys(51)–S–CH_2–CO_2H, 124341-79-3; hFSH-β 33–53, Cys(51)–S–CH_2–$CONH_2$, 124341-80-6; androstenedione, 63-05-8; estradiol, 50-28-2; hFSH-β 33–53, Cys(51) dimer, 124581-05-1.

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Structure–Function Analysis of Mononucleotides and Short Oligonucleotides in the Priming of Enzymatic DNA Synthesis

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ABSTRACT: The reversed-phase chromatography technique was employed in the measurement of DNA synthesis at the primers $d(pT)_n$, $r(pU)_n$, $d(pA)_n$, and $r(pA)_n$ (n = 1–16) in the presence of template poly(dA) or poly(dT). DNA synthesis was catalyzed by *Escherichia coli* DNA polymerase I Klenow fragment, *Physarum polycephalum* DNA polymerase β-like, *P. polycephalum* DNA polymerase α, and human placenta DNA polymerase α. Values of K_m and V_{max} were measured as functions of the primer chain lengths. It was found that all mononucleotides and small oligonucleotides served as primers of DNA synthesis. Values of the logarithm of both K_m and V_{max} increased linearly until primers had attained a chain length of 9–12 nucleotides, where a break was observed. The incremental as well as the absolute values of K_m were interpreted in terms of free binding energies. These together with other data indicate that the 3′-ultimate nucleotide of the primer contributes a decisive amount of free energy of binding to DNA polymerase both from the nucleoside and from the phosphate moiety. The incremental increase is due to a complementary interaction between bases of primer and template buried in the binding cleft of the polymerase. It is also the ultimate nucleotide that determines whether the ribonucleotide or the deoxyribonucleotide is an efficient primer. It is of interest that the major results seem preserved for all four DNA polymerases. An energetic model for the binding of the template-primer was proposed and compared with available crystallographic data.

DNA polymerases catalyze the elongation of oligo- or polynucleotides at their 3′-terminus by covalent addition of nucleoside 5′-monophosphates from the triphosphates complementary to the base-pairing polynucleotide template strand (Kornberg, 1980). The length of these nucleotides serving as primers of DNA polymerization and their ribose or deoxyribose nature determine the success at which elongation occurs. The types of DNA polymerases that function in chromosome replication easily use RNA as primers while DNA repairing polymerases strongly prefer DNA. The length can be imagined to be associated with the number of noncovalent complementary interactions with a given template and probably with the polymerase binding cleft and thus with the efficiency holding the 3′-terminus in the position that is optimal for the catalytic addition of mononucleotides. This efficiency has been measured previously in experiments employing synthetic hook polymers (Fisher & Korn, 1979) and small primers with natural templates (Grosse & Krauss, 1984). The success of priming has been examined after routine acid precipitation of product DNA, usually after extensive primer elongation. The minimal number of nucleotides in these primers has been suggested to be three to five and four, respectively.

The crystal structure has been elucidated for *Escherichia coli* DNA polymerase I Klenow fragment (Ollis et al., 1985; Joyce & Steitz, 1987). From the structural coordinates as well as from model fitting, the binding site for DNA has been localized and also a site that binds nucleoside 5′-monophosphates. This site is very likely to be involved in the 3′–5′ exonuclease (proofreading activity; Kornberg, 1980) located on a small structural domain while the activity to synthesize DNA resides on the large domain of Klenow fragment (Freemont et al., 1986). The exact location of the primer 3′-terminus and of nucleoside 5′-triphosphates has remained mainly unsolved.

We have investigated the structure–function relation of synthetic ribo and deoxyribo primers for four representative DNA polymerases employing Michaelis–Menten kinetic analysis and measurement of products by reversed-phase chromatography. We find that all these polymerases catalyze DNA synthesis in the presence of primers as short as nucleoside 5′-monophosphates. From the results of a systematic structure–energy investigation, we have developed an energetic model of the template-primer binding site of DNA polymerases.

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