Identification of a Follicle-stimulating Hormone Receptor-binding Region in hFSH-β-(81–95) Using Synthetic Peptides*

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Pituitary and placental glycoprotein hormones are heterodimers with α-subunits of identical primary structure, but dissimilar β-subunits. Regions of structural similarity between the β-subunits might be involved in interaction with the homologous α-subunits, and regions of structural dissimilarity could, therefore, be candidates for receptor interactions. A restrained matrix dot-plot analysis identified hFSH-β-(8–32) and hFSH-β-(55–65) as candidates for interaction with α-subunit. Therefore, by subtraction, hFSH-β-(33–54) and hFSH-β-(66–111) seemed candidates for regions of interaction with receptor. In a previous report we demonstrated that hFSH-β-(33–53) represented a receptor-binding region of hFSH-β. Analysis of structural parameters (flexibility, surface probability, secondary structure prediction, etc.) indicates similarities between hFSH-β-(33–53) and hFSH-β-(85–95), suggesting the latter might be the component of hFSH-β-(61–111) interacting with the receptor. Testing of 11 synthetic peptides, corresponding to the primary structure of hFSH-β, demonstrated that hFSH-β-(81–95)-peptide amide, as well as hFSH-β-(31–45)-peptide amide, were unique in ability to inhibit [125I]hFSH to FSH receptor in calf testis membranes (Sluss et al., 1986; Schneyer et al., 1988) and that hFSH-β-(33–53)-peptide amide, which encompasses these tetrapeptides, inhibits binding with increased potency (Andersen et al., 1987). hFSH-β-(33–53)-peptide amide binds to FSH receptor, behaving as a partial agonist of FSH with regard to stimulation of basal estradiol production and as a partial antagonist of FSH-stimulated estradiol biosynthesis (Santa Coloma et al., 1990). In order to examine the hypothesis of a second receptor-binding region around hFSH-β-(85–95), to better delineate the already identified receptor-binding region (hFSH-β-(33–53)) and to search for possible additional binding regions, we examined 11 overlapping peptides corresponding to the primary structure of the FSH-β-subunit. Our results support the notion that an additional receptor-binding region is located in the hFSH-β-(81–95) sequence.

EXPERIMENTAL PROCEDURES

Materials—Highly purified human FSH (LER-1781-2, 4000 IU/mg) was used for calf testis membrane radioreceptor assay. Purified ovine FSH (LER-1996-S, 8 NIH-FSH-S1 units/mg) was used in Sertoli cell culture assay to stimulate aromatase activity. Iodoacetamide, cysteine, and dithiothreitol were obtained from Sigma. All solvents and buffers utilized in the chromatographic procedures were high performance liquid chromatography grade reagents and all other chemicals used were of the highest grade commercially available.

Peptide Synthesis, Purification, and Characterization—Synthetic peptides corresponding to regions of hFSH-β-subunit were prepared by Multiple Peptide Systems, San Diego, CA. Synthesis was by the solid-phase method (Merrifield, 1963) using the tert-butoxycarbonyl protection scheme. The resulting peptide amides were purified by us, individually, were very poor. Receptor-binding regions, therefore, appear to be characterized by a particular and complex arrangement of secondary structure motifs, surface probability, and flexibility.
Radioligand Receptor Assay (RRA)—Calf testis membranes containing FSH receptors were prepared as described elsewhere (Dattra-treymurty et al., 1987). The RRA was performed as previously described (Schneyer et al., 1986) with the following modifications: 3 mg wet weight of membrane preparation, 2.5 ng of radioligand, and samples or buffer (50 mM HEPES, 250 mM sucrose, 5 mM MgCl₂, adjusted to pH 6.5) to a total volume of 0.25 ml, were incubated for 18 h at 30 °C. After incubation, hormone bound to membrane receptor was separated from unbound hormone by centrifugation (30,000 × g, 15 min). Nonspecific binding was defined as radioligand bound in the presence of peptide plus a 300-fold excess of unlabeled hFSH.

In Vitro Bioassay for FSH Activity—Assessment of FSH agonist or antagonist activity was based on ability of peptides to affect either basal or luteinizing hormone-stimulated conversion of androstenedione to estradiol by cultures of Sertoli cells taken from immature rat testis (Grasso and Reichert, 1980). Medium was changed 48 h after isolation of Sertoli cells and initial incubation. After an additional 24 h, ovine FSH or test peptides were added to the cells, and incubation was allowed to proceed for another 24 h. The final incubation medium contained 0.7 mM methylisobutylxanthine, and 0.4 mM androstenedione as substrate for aromatase. Measurement of estradiol was done by radioimmunoassay as previously described (Grasso and Reichert, 1989), using antiserum generously made available by Dr. Gordon Niswender, Department of Physiology and Biophysics, Colorado State University, Fort Collins, CO.

Other Procedures—Alignment of sequences, sequence comparisons (dot-plot), secondary structure predictions, and hydrophilicity, flexibility, surface location, and antigenicity analysis was done using the Genetic Computer Group programs (Devereux et al., 1984). Statistical analyses were performed using the BMDP program (BMDP Software, Los Angeles, CA) on a VAX 9500 computer.

RESULTS

Assessment of Receptor-binding Domains by Computer Analysis—As outlined under the Introduction and considered further under “Discussion,” regions of homology among β-subunits of glycoprotein hormones may be involved in subunit interaction and regions of low sequence homology might contain receptor-binding regions. Analysis of aligned hFSH and hCG β-subunit sequences indicated several regions of sequence homology (Fig. 1). Using a constrained dot-plot approach a simpler profile was obtained, suggesting two possible α-subunit binding regions (Fig. 2) located between amino acids 8–32 and 55–65. There also appeared to be two likely receptor-binding regions on the hFSH-β-subunit. The first theoretical binding region, suggested by the dot-plot analysis (Fig. 2), was located between amino acids 33 and 53. In a separate study, based on a different approach (see “Discussion”), we have already reported that the sequence 33–53 contains a receptor-binding region for FSH (Andersen et al., 1987; Santa Coloma et al., 1990). The location of the second theoretical receptor-binding region on hFSH-β was not well defined by the alignment of sequences or the dot-plot analysis. A broad region with receptor-binding potential was suggested by dot-plot analysis to be between amino acids 66 and 111. To obtain evidence for more precise location of the second binding region, several structural and physicochemical character-
FIG. 3. Prediction of secondary structure and properties of hFSH-β-subunit. Programs PEPTIDESTRUCTURE and PLOTSTRUCTURE from the CGC package (Devereux et al., 1984) were used. Shaded areas were added to indicate zones with highest values (hydrophilicity > 1.3, antigenic index > 1.2, flexibility > 1, surface probability > 5). Frames indicate positions for hFSH-β-(31-45) and hFSH-β-(81-95).

FIG. 4. Overlapping synthetic peptides used to map the hFSH-β-subunit for receptor-binding regions.

FIG. 5. Inhibition profile for synthetic peptides. Eleven overlapping peptides corresponding to hFSH-β (Fig. 4) were incubated at 0.8 mM concentration with calf testis membranes in the presence of radiolabeled FSH. Bars represent average values from three separate experiments, each by triplicate (standard errors are indicated). hFSH-β-(31-45), a fragment of the already identified 33-53 region (Santa Coloma et al., 1990) and hFSH-β-(81-95) were the more potent inhibitors. The results suggest the presence of a second binding region in hFSH-β-(81-95).

By using the regression coefficients it was possible to define a linear function, referred to as “binding index,” BI = 526.8 + 2.178 (S) - 381.6 (F) + 40.8 (H) - 48.6 (E) - 40.9 (T). The predicted values of this function, together with experimental values, are represented in Fig. 6. These parameters seem to have questionable significance when considered individually (Table I), but taken together, appear to contain sufficient information to describe or predict receptor-binding regions with some reliability. Therefore, we calculated the predicted values for peptides corresponding to the N-terminal region,
coefficients. The predicted values (Fig. 6A) suggest that the sites of carbohydrate attachment (Asn-7 and Asn-24) and carbohydrate residues play an important role in FSH signal transduction (Ryan et al., 1988; Matzuk et al., 1989). Therefore, we do not exclude the possibility that additional receptor-binding regions may exist around the sites of carbohydrate attachment in the fully glycosylated molecule, which could not be expressed in the inhibition assay due to the low solubility of the synthetic peptides corresponding to this region or to the absence of carbohydrates. The latter possibility seems less probable because binding activity is only slightly affected by carbohydrate removal (Matzuk et al., 1989).

Effect of hFSH-β-(81-95)-peptide Amide on Estradiol Synthesis in Cultured Rat Sertoli Cells—We also examined the effect of hFSH-β-(81-95)-peptide amide on the conversion of androstenedione to estradiol in cultures of Sertoli cells taken from testes of immature rats. We studied the effect of this peptide on basal secretion of estradiol and on FSH-stimulated estradiol biosynthesis. hFSH-β-(81-95)-peptide amide significantly increased basal levels of estradiol synthesis, with maximal effect (60% of maximal response induced by ovine FSH) seen at about 50 μM (partial agonist) (Fig. 7). A similar partial agonist activity was observed with the 21-mer synthetic peptide hFSH-β-(33-53)-peptide amide (Santa Coloma et al., 1990). Another 15-mer peptide, corresponding to hFSH-β-(31-45), had no effect on basal estradiol levels when tested at similar concentrations (not shown), indicating that the stimulation on basal levels induced by hFSH-β-(81-95)-peptide amide is sequence-specific. A synergistic effect on FSH-

which were not included in the calculation of the regression coefficients. The predicted values (Fig. 6A) suggest that hFSH-β-(31-45) may be also involved in the interaction with the FSH receptor. The observed value (Fig. 6A) may not reflect a strong inhibition because of the low peptide solubility. All the peptides corresponding to the N-terminal region of hFSH-β (sequences 1-15, 11-25, and 21-35) were only partially soluble. hFSH-β-(1-15) and hFSH-β-(11-25) contain the sites of carbohydrate attachment (Asn-7 and Asn-24) and carbohydrate residues play an important role in FSH and luteinizing hormone (hCG) signal transduction (Ryan et al., 1988; Matzuk et al., 1989). Therefore, we do not exclude the possibility that additional receptor-binding regions may contain the sites of carbohydrate attachment (Asn-7 and Asn-24) and carbohydrate residues play an important role in FSH and luteinizing hormone (hCG) signal transduction (Ryan et al., 1988; Matzuk et al., 1989). Therefore, we do not exclude the possibility that additional receptor-binding regions may

| Variable | Regression coefficient | T-statistic | 2-tail p | Contribution to $R^2$ |
|----------|------------------------|------------|----------|-----------------------|
| Intercept | 526.0 ± 140.4 | 3.55 | 0.002 | 0.004 |
| Surface probability | 2.178 ± 1.203 | 1.81 | 0.077 | 0.02 |
| Flexibility | $-381.6 ± 150.7$ | -2.33 | 0.021 | 0.05 |
| Helix | $40.81 ± 8.900$ | 4.59 | $<0.001$ | 0.15 |
| Extended | $-48.57 ± 8.495$ | -5.72 | $<0.001$ | 0.23 |
| Turn | $-40.91 ± 5.856$ | -6.98 | $<0.001$ | 0.35 |

a Variable AI was removed because it prunednant information (the function to estimate AI includes hydrophilicity, surface probability, helix, Garnier's, and Chou-Fasman parameters and is highly correlated with variables hydrophilicity and turn). Variable hydrophilicity was excluded from regression because of its low significance ($p > 0.5$).

b Due to solubility problems, values corresponding to peptides hFSH-β-(1-15), (11-25), and (21-35) were not included for calculation of regression coefficients. Values for peptide parameters hydrophilicity, AI, surface probability, and flexibility were obtained as average values for each peptide, and values for Garnier's parameters were taken as follows: the parameter was equal to 0 if 0 < % < 25, 1 if 25 < % < 50, 2 if 50 < % < 75, and 3 if 75 < % = 100, where % is the percentage of α, extended, turn, or coil structure. Values are means ± S.E.

The contribution to $R^2$ for each variable is the amount by which $R^2$ would be reduced if that variable were removed from the regression equation. The contents of α-helix (low) and β-extended (high) or turn (high) structure seem to be more important parameters to describe or predict binding sites in hFSH-β than surface probability or flexibility.
**DISCUSSION**

Ward and Moore (1979) postulated that the hCG β-subunit region 89-100 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 with their receptors. Recently, Keutmann et al. (1987) reported that a synthetic peptide corresponding to hCG-P-(38-57), comparable to hFSH-P-(32-51), inhibited hCG binding to receptor, but did not stimulate testosterone production in cultured rat Leydig cells. Keutmann et al. (1987) also found that synthetic peptides corresponding to hCG-β-(38-57), comparable to hFSH-β-(31-45), inhibited hCG binding to receptor, and stimulated testosterone production in Leydig cell cultures. In an earlier study (Sluss et al., 1986), we reported inhibition of 125I-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 sites, which included hFSH-β-(34-37) (TRDL). In a preliminary report (Andersen et al., 1987), we noted that an extended synthetic peptide encompassing TRDL and KTCT, hFSH-β-(33-53)-peptide amide, also inhibited 125I-hFSH binding to receptor, at a potency significantly greater than seen with either individual tetrapeptide. In a further study we obtained evidence that hFSH-β-(33-53)-peptide amide binds to the FSH receptor in a functional manner, affecting both basal and FSH-stimulated steroidogenesis (Santa Coloma et al., 1990).

Using sequence alignment and a "dot-plot" approach to localize nonhomologous regions between hFSH β and hCG β, and considering some parameters related to the hFSH-β-(33-53)-binding region (surface probability, hydrophilicity, etc.), we developed the hypothesis that a second binding region should be located around FSH-β-(85-85). This was the approximate region that Ward and Moore (1979) designated as the determinant loop (hFSH-β-(87-94)). Using 11 overlapping synthetic peptides corresponding to hFSH-β-subunit, we obtained experimental evidence suggesting that hFSH-β-(61-95)-peptide amide, which includes the predicted 85-95 region and the determinant loop, represents a second receptor-binding region. Due to solubility problems encountered with peptides corresponding to the N-terminal segment of hFSH-β-subunit in the RRA, we do not exclude the possibility that an additional binding site could exist in the N-terminal region, as postulated by Stewart and Stewart (1977) for luteinizing hormone. No evidence for interaction between the C-terminal region (amino acids 101-111) of hFSH-β-subunit and the FSH receptor were found.

Multiple linear regression analysis of the FSH-binding inhibition data identified parameters appropriate to describe these receptor-binding regions. Hydrophilicity was a poor estimator even when it was considered together with a number of other structural parameters. The suggestion that hydrophilicity is not adequate to describe (or predict) receptor-binding sites in the FSH molecule is interesting because it is widely held that receptor-binding sites are exposed at the surface, and therefore should be hydrophilic. Taken individually, surface probability (S), flexibility (F), or Garnier's parameters H (helix), E (extended), T (turn), and C (coil) were not sufficient to describe receptor-binding regions in FSH-β-subunit. A linear combination of these parameters, however, provides an improved prediction of receptor binding regions. Regression analysis also indicates that hydrophilicity and surface probability, flexibility, or antigenic index are highly correlated and therefore, all together provide redundant and probably irrelevant information to the regression model. Among the secondary structure parameters of hFSH-β, the percentage of predicted β-turns or extended structures and α-helices (with a contribution to R² of 0.35, 0.23, and 0.15, respectively) seem to be more meaningful than flexibility or surface probability. Perhaps the more interesting observation derived from the regression analysis is that, at least for hFSH-β-subunit, binding regions are characterized by a unique combination of different structural and physicochemical properties.

The identification of a second binding region was further confirmed by the ability of FSH-β-(81-95) to modify the response to FSH in Sertoli cell cultures. Since FSH is a complex molecule with two subunits, each probably contributing to the interaction with receptor, it would be surprising if a small peptide totally mimicked the effect of the intact hormone. However, the discovery of partial agonists was crucial to the development of several syntropic antagonists (Black, 1989). FSH-β-(33-53) and FSH-β-(81-95) peptides, are each partial agonists of FSH-stimulated estradiol biosynthesis. Selective and appropriate chemical modifications could lead eventually to the development of a full antagonist or agonist of FSH.

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