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Topographic analysis of human follicle-stimulating hormone- β using anti-peptide antisera

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Summary

The purpose of this study was to identify peptide sequences of human follicle-stimulating hormone- β (hFSH β) which are accessible subsequent to association with hFSH α in heterodimeric hFSH. Antisera were raised against synthetic peptides (Ab^{pep}) corresponding to hFSH β sequences 1–20, 16–36, 33–53, 49–67, 66–85, 81–100 and 98–111. The topography of hFSH β was studied by testing the binding of these antisera to hFSH β and hFSH captured by monoclonal antibodies (MAb) in an enzyme-linked immunosorbent assay (ELISA). When hFSH and hFSH β were captured by the same MAb, binding of Ab^{16–36}, Ab^{33–53}, Ab^{81–100} and Ab^{98–111} to hFSH was significantly lower compared to hFSH β . However, compared to other Ab^{pep}, binding of Ab^{35–53} to hFSH was strong. Similar results were obtained when hFSH was captured by an α -specific MAb (10.3A6). Using 10.3A6, it was also possible to demonstrate significant binding of Ab^{49–67} to hFSH. The data suggests that residues in regions 33–53 and 49–67 of hFSH β appear to be accessible in the heterodimeric hFSH in addition to the glycosylated region of 1–15. Regions 16–36, 33–53, 81–100 and 98–111 of hFSH β appear to contain subunit contact-associated sequences which are either masked or structurally altered subsequent to association with hFSH α in the heterodimeric hFSH.

Introduction

Human follicle-stimulating hormone (hFSH) is comprised of two subunits, hFSH α and hFSH β

in noncovalent association. This pituitary glycoprotein plays an important role in the development of ovarian follicles and initiation of spermatogenesis in the testis. In order for FSH at physiological levels (nM) to bind its receptor and subsequently initiate signal transduction, hFSH must be presented to its receptor in heterodimeric form. The association of hFSH α and hFSH β in the heterodimeric FSH molecule likely results in masking or possibly conformational alteration of the surfaces of the two subunits, but this has not been demonstrated directly. Preliminary success in obtaining crystals of human chorionic gonadotropin has been reported (McPherson

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region 33–53, and to a side regions 49–67 and 990). We also reported that part of the peptide involved in subunit conformation in hFSH β 81–111 is also (Vakharia et al., 1991). We hypothesized that peptides which are masked on with hFSH α subunit would not bind to anti-hFSH β in antisera raised against hFSH β . The aim was to determine whether antisera raised from the hFSH β sequence between hFSH β and hFSH α approach was previously used in regions in hFSH α that are not to association with hFSH (Weiner et al.,

hFSH β (AFP-4911B) and hFSH α (AFP-2041889) (100 μ g/mg) were obtained from the National Hormone and Pituitary Program (National Hormone and Pituitary Program, Baltimore, MD, USA). hFSH α was immunized in frozen human pituitary (100 μ g).

Antisera and specificities of hFSH β and hFSH α -specific antisera were published elsewhere (Weiner et al., 1991). In this study, 10 times more hFSH than hFSH α of the antibody-bound hFSH in phase radioimmunoassay (Weiner et al., 1990). MAb 10.3A6 binds to hFSH β and also to hFSH α in solution phase and binds hFSH preferentially to hFSH α in solution phase.

Anti-hFSH β antiserum
hFSH β -2 (AFP-2041889) was obtained from the

National Hormone and Pituitary Program (Baltimore, MD, USA). The antiserum was reported to have been raised in a rabbit by immunizing with immunoaffinity purified hFSH β . In ELISA, the minimum moles of hFSH β and hFSH required by this antiserum at a dilution of 1:1500 to obtain an optical density value of 0.1 after 1 h of the addition of substrate was comparable (3.125 ng and 6.25 ng respectively). This is in contrast to liquid phase RIA, where 41-fold more of hFSH than hFSH β is required to displace 50% of the 125 I-hFSH β bound to the antiserum (Vakharia et al., 1991).

Peptide synthesis

Solid-phase synthesis of hFSH β peptides 1–20, 16–36, 33–53, 49–67, 66–85, 81–100 and 98–111 representing overlapping sequences of hFSH β was carried out. Details of the synthesis and purification of these peptides are given elsewhere (Vakharia et al., 1990, 1991).

Polyclonal rabbit anti-hFSH β peptide antiserum (Ab^{pep})

Antisera to hemocyanin-coupled peptides (Ab^{pep}) were developed in rabbits. Synthetic peptides 1–20, 16–36, 33–53, 49–67, 66–85, 81–100 and 98–111 were coupled to hemocyanin (H-1757, Sigma, St. Louis, MO, USA) using the water-soluble coupling reagent 1-ethyl-3-(3-methylaminopropyl)carbodiimide HCl (EDAC; E7750, Sigma, St. Louis, MO, USA) according to the method described elsewhere (Weiner et al., 1990). The first three immunizations of 100 μ g of the coupled peptide were at intervals of 15 days. Subsequently, 1 mg of the coupled peptide was immunized at intervals of 15 days. Blood samples were collected before every immunization. The blood sample collected prior to the fourth immunization of 1 mg of peptide was used in the present study.

Titer and peptide specificity of Ab^{pep}

The titer of Ab^{pep} was determined in an ELISA by testing the binding of different dilutions of Ab^{pep} to 1 μ g peptide adsorbed on wells of Immulon I plates. The details of the ELISA method are described elsewhere (Vakharia et al., 1990, 1991). In brief, peptides dissolved in 0.05 M

Tris buffer, pH 9.5 were coated on the wells of Immulon I plates at 37°C for 2 h. After the incubation period 2.5% nonfat milk solution (Carnation milk powder, Carnation Co., Los Angeles, CA, USA) in bicarbonate buffer was added for 1 h at room temperature (RT) to block the free sites in the wells. Plates were then washed 3 times and Ab^{pep} in binding buffer were added to wells and incubated at 37°C for 1 h. After incubation the plates were washed and alkaline phosphatase-labeled goat anti-rabbit immunoglobulin, diluted 1:2000 in binding buffer, was added and incubated for 1 h at RT. The wells were washed again and substrate (*p*-nitrophenylphosphate) was added. The yellow color that developed after the addition of substrate was quantitated using a Dynatech plate reader, with a 410 nm filter. Ab^{pep} bind to peptides with high affinity, therefore the color development was rapid, allowing the optical density in the wells to be determined as early as 30 min after the addition of the substrate.

The highest dilution of the antiserum giving an optical density greater than 0.1 after correcting for the nonspecific control, 30 min after the addition of the substrate, was considered the titer of the antiserum. The peptide specificity of each of the Ab^{pep} was determined by testing the binding of a 1:50 dilution of each of the antisera to 1 μ g/well of different peptides. If an Ab^{pep} cross-reactivity at 1:50 dilution was observed with hFSH β peptides against which the animal was not immunized, then a working dilution of the Ab^{pep} was determined which showed no nonspecific cross-reactivity with other peptides.

Binding of Ab^{pep} to hFSH β and hFSH captured by hFSH β -specific and hFSH α -specific monoclonal antibodies respectively

Immulon I plates were precoated with 1–3 μ g/well of protein-A immunopurified hFSH β -specific MAb 3G3, and hFSH α -specific MAb 10.3A6, by incubating overnight at 4°C in bicarbonate buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, 3 mM sodium azide), pH 9.6. After the incubation period, the contents of the wells were discarded and 2.5% nonfat milk solution (Carnation milk powder 200 μ l/well) in bicarbonate buffer was added for 1 h at RT to block the free sites in the wells. After washing, 50

ng of hFSH β in bicarbonate buffer was added to wells containing hFSH β -specific MAb, and 100 ng of hFSH in bicarbonate buffer was added to wells containing hFSH α -specific MAb. After an overnight incubation at 4°C, the plates were washed and Ab^{pep} and preimmune sera were added to both hFSH β and hFSH containing wells and the subsequent part of the assay was carried out as described above. The dilutions of Ab^{pep} used were determined above. Wells containing preimmune sera served as nonspecific controls, the optical density values of which were subtracted from the corresponding wells containing Ab^{pep}. Absorbency values that were greater than 2 at the time of record were assigned the numeric value of 2.0.

In order to objectively affirm a positive result, statistical analysis of these data was performed. Briefly, an analysis of variance was performed for all anti-peptide antibody pairs (preimmune, immune sera) within each treatment. For example, in Fig. 1, hFSH β would be one treatment with eight pairs. Then an independent sample *t*-test was used to determine if the difference between each pair was different from zero. The level of significance was set at *P* = 0.05 before the analy-

sis was done. Thus, a positive result is the difference between preimmune and immune sera that is significantly different from zero at the *P* = 0.05 level.

In order to determine if the amount of hFSH β and hFSH bound to capture MABs was comparable the following assessment was made. MABs 3G3 and 10.3A6 at 3 μ g/well concentrations were coated on Immulon I plates as described above. To 3G3 coated wells unlabeled hFSH β (3.0–6000 ng) and 0.5 ng of ¹²⁵I-hFSH β (80,000 cpm) were added in a final volume of 60 μ l. To wells coated with 10.3A6 unlabeled hFSH (3.0–6000 ng) and 1.4 ng of ¹²⁵I-hFSH (80,000 cpm) were added also in a final volume of 60 μ l. After overnight incubation at 4°C, the wells were washed with cold phosphate-buffered saline (PBS) pH 7.5 and each well was counted in a gamma counter. The amount of hormone bound to MAB at each concentration was determined by analysis of the results of the ligand binding isotherm (Munson and Rodbard, 1978). The amount of hFSH β and hFSH captured by 3G3 was also determined.

Results

General characteristics of Ab^{pep}

Table 1 lists the titer of each anti-peptide antiserum determined using an ELISA format where wells were coated with unconjugated peptides. The cross-reactivity of each Ab^{pep} with other hFSH β peptides is illustrated in Table 2. All Ab^{pep} except Ab^{16–36}, Ab^{49–67} and Ab^{81–100} bound only to immunized peptide or, as expected, to neighboring peptides with overlapping peptide sequence. Ab^{16–36}, Ab^{49–67} and Ab^{81–100} showed slight cross-reactivity with peptides 49–67, 16–36 and 49–67 respectively; however, with further dilution of these Ab^{pep} no cross-reactivity was detectable (Table 3). The optical density values of wells containing preimmune sera and peptides which ranged from 0.012 to 0.062 for dilutions (1:500 to 1:75,000), were automatically subtracted from the respective wells containing Ab^{pep} and peptides. The absorbance values of wells with Ab^{pep} and peptide after the correction for preimmune sera were always greater than 0.10.

TABLE 1

TITER OF ANTI-PEPTIDE ANTISERA FOR THE RESPECTIVE IMMUNIZED PEPTIDES DETERMINED IN AN ELISA

Immunon I plates were coated with 1 μ g peptide. Absorbance (410 nm) was determined after a 30 min incubation with substrate.

Antisera	Titer ^a
1– 20	10,000
16– 36	5,000
33– 53	75,000
49– 67	75,000
66– 85	75,000
81–100	75,000
98–111	500

^a Values are expressed as the reciprocal of the end-point dilution of duplicate determinations.

TABLE 2

CROSS-REACTIVITY OF ANTISERA (1:50 DILUTION) WITH hFSH β PEPTIDES

Antisera	hFSH β peptide 1–20 Optical density
1– 20	0.79 \pm 0.08
16– 36	
33– 53	
49– 67	
66– 85	
81–100	
98–111	

Binding of Ab^{pep} to hFSH β and hFSH α -specific and hFSH β -specific MABs

To test the binding of Ab^{pep} to hFSH α (hFSH α -specific MAB) and hFSH β (hFSH β -specific MAB) either by hFSH β -specific MAB or hFSH α -specific MAB, hFSH is bound to the MAB surface, leaving some surfaces to react with Ab^{pep}.

Initial experiments showed that 1 μ g/well of capture MAB gave appreciable binding of Ab^{pep} and hFSH such that the binding of Ab^{pep} to hFSH was estimated. Using NIDDI, it was determined that a 2-fold

TABLE 3

DILUTIONS OF ANTI-PEPTIDE ANTISERA (1:50 DILUTION) WITH hFSH β PEPTIDES

Antisera	hFSH β peptide 1–20 Optical density
1– 20 (1:100)	0.62 \pm 0.08
16– 36 (1:100)	
33– 53 (1:100)	
49– 67 (1:100)	
66– 85 (1:100)	
81–100 (1:1000)	
98–111 (1:50)	

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volume of 60 μl . After
4°C, the wells were
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TABLE 2

CROSS-REACTIVITY OF ANTI-PEPTIDE ANTISERA TESTED AT THE SAME DILUTION FOR hFSH β PEPTIDESAntisera (1:50 dilution) were tested against peptides (1 μg /well) coated on Immulon I plates.

Antisera	hFSH β peptides						
	1-20	16-36	33-53	49-67	66-85	81-100	98-111
	Optical density (410 nm)						
1-20	0.79 \pm 0.08	0.16 \pm 0.02					
16-36		1.00 \pm 0.04		0.11 \pm 0.00			
33-53			1.14 \pm 0.018	0.32 \pm 0.06			
49-67		0.29 \pm 0.03		1.43 \pm 0.01	0.18 \pm 0.02		
66-85					1.16 \pm 0.07		
81-100				0.17 \pm 0.05	0.45 \pm 0.02	0.55 \pm 0.04	
98-111							0.85 \pm 0.04

Binding of Ab^{pep} to hFSH and hFSH β captured by hFSH α -specific and hFSH β -specific MAb

To test the binding of Ab^{pep} to hFSH β associated with hFSH α (hFSH), hFSH was captured either by hFSH β -specific MAb (3G3) or by an hFSH α -specific MAb 10.3A6. In the latter format, hFSH is bound to MAb via its α -subunit, leaving some surfaces of the β -subunit available to react with Ab^{pep}.

Initial experiments determined that coating 3 μg /well of capture MAb was sufficient to gain appreciable binding of Ab^{pep} to captured hFSH β and hFSH such that differences if any in the binding of Ab^{pep} to hFSH β and hFSH could be estimated. Using NIDDK-anti-hFSH β it was determined that a 2-fold molar excess of hFSH to

hFSH β yielded sufficient amounts of hFSH and hFSH β captured for Ab^{pep} binding and this was incorporated into the design. Finally, when the amount of hFSH β and hFSH captured by MAb was assessed, Scatchard analysis revealed that the amount of hFSH β captured by 3G3 and hFSH captured by either 10.3A6 or 3G3 was comparable (data not shown).

The binding of Ab^{pep} to captured hFSH β and hFSH is illustrated in Fig. 1 and Fig. 2. The results where both hFSH β and hFSH were captured by MAb 3G3 are illustrated in Fig. 1. Ab¹⁶⁻³⁶, Ab³³⁻⁵³, Ab⁸¹⁻¹⁰⁰ and Ab⁹⁸⁻¹¹¹ bound to hFSH β ($P = 0.05$). Ab³³⁻⁵³ also gave a strong signal for hFSH although at an earlier reading it was significantly lower than hFSH β (see also

TABLE 3

DILUTIONS OF ANTI-PEPTIDE ANTISERA SHOWING SPECIFICITY FOR IMMUNIZED hFSH β PEPTIDESAll antisera at respective dilutions were tested against peptides (1 μg /well) coated on Immulon I plates.

Antisera	hFSH β peptides						
	1-20	16-36	33-53	49-67	66-85	81-100	98-111
	Optical density (410 nm)						
1-20 (1:100)	0.62 \pm 0.03						
16-36 (1:100)		0.93 \pm 0.02					
33-53 (1:100)			1.05 \pm 0.02				
49-67 (1:100)				1.25 \pm 0.01			
66-85 (1:100)					1.01 \pm 0.06		
81-100 (1:1000)						0.35 \pm 0.01	
98-111 (1:50)							0.73 \pm 0.06

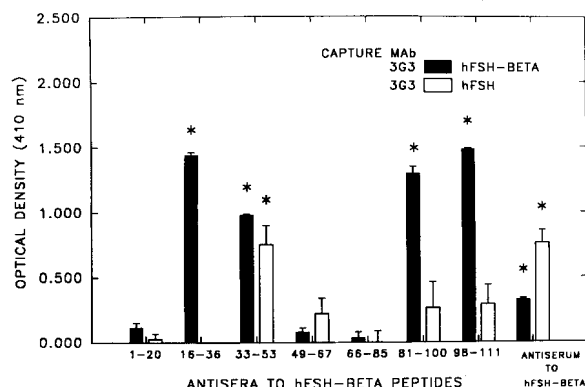


Fig. 1. Binding of Ab^{Pep} to hFSHβ (50 ng) and hFSH (100 ng) both captured by MAb 3G3 in an ELISA. See Materials and methods for the details. The dilutions of Ab^{Pep} used are listed in Table 3. NIDDK-anti-hFSHβ antiserum (1:1500) was used as a control to determine whether comparable amounts of hFSHβ and hFSH were available for Ab^{Pep} binding. An asterisk indicates when specific optical density values are significantly different from zero ($P < 0.05$).

discussion of Fig. 2). In this experiment it was not possible to demonstrate significant difference in the binding of Ab¹⁻²⁰ and Ab⁴⁹⁻⁶⁷ to either hFSHβ or hFSH. Ab⁶⁶⁻⁸⁵ bound neither hFSHβ nor hFSH.

Fig. 2 illustrates the results of experiments where hFSHβ was captured by MAb 3G3 and

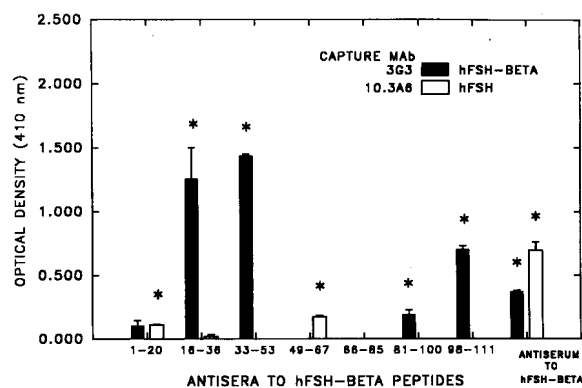


Fig. 2. Binding of Ab^{Pep} to hFSHβ (50 ng) captured by MAb 3G3 and hFSH (100 ng) captured by MAb 10.3A6 in an ELISA. See Materials and methods for the details. The dilutions of Ab^{Pep} used are listed in Table 3. NIDDK-anti-hFSHβ antiserum (1:1500) was used as a control to determine whether comparable amounts of hFSHβ and hFSH were available for Ab^{Pep} binding. An asterisk indicates when optical density values are significantly different from zero ($P < 0.05$).

hFSH was captured by MAb 10.3A6. The results collected 2 h after substrate addition were qualitatively identical to the results in Fig. 1. The binding of Ab¹⁶⁻³⁶, Ab³³⁻⁵³, Ab⁸¹⁻¹⁰⁰ and Ab⁹⁸⁻¹¹¹ to hFSHβ was higher than hFSH. Significant binding of Ab¹⁻²⁰ to both hFSHβ and hFSH was only observed when color development was allowed for a longer time. In that case, the binding of Ab¹⁻²⁰ to hFSHβ was no different from hFSH. Additionally, Ab⁴⁹⁻⁶⁷ showed significant binding to hFSH in wells coated with hFSH, obscured in Fig. 1 because that sequence is in the epitope of 3G3, as is hFSHβ 66-85. Other results shown here and conclusions are comparable to Fig. 1.

Discussion

In the present study anti-peptide antisera were raised against synthetic peptides corresponding to hFSHβ sequences and were tested for their binding to either FSHβ or hFSHα-associated hFSHβ (hFSH). In contrast to earlier work with hFSHα (Weiner et al., 1990), only limited binding of Ab^{Pep} to hFSHβ was observed if hFSHβ was coated directly onto the ELISA plates (not shown). However, when hFSHβ was captured by an hFSHβ-specific MAb, five of the seven Ab^{Pep} showed binding to hFSHβ detectable in 1-2 h. The inability of the remaining two (Ab⁴⁹⁻⁶⁷, Ab⁶⁶⁻⁸⁵) to bind hFSHβ captured by MAb 3G3 can be explained by the fact that sequences 49-67 and 66-85 of hFSHβ are also in the epitope recognized by 3G3 (Vakharia et al., 1990).

The present study had as its hypothesis that low binding of Ab^{Pep} to hFSH compared to hFSHβ is due to masking or induced mobility of amino acids in the corresponding hFSHβ sequences subsequent to association with hFSHα in the heterodimeric hFSH. We reasoned that a comparable or higher binding of Ab^{Pep} to hFSH than hFSHβ would suggest that amino acids in the corresponding hFSHβ sequences are accessible and not masked by hFSHα in heterodimeric hFSH.

From the Ab^{Pep}-binding data (Figs. 1 and 2) it was realized that the optical density signal following binding of Ab^{Pep} to hFSHβ was consistently greater than binding to hFSH. Scatchard analysis revealed that, after incubating 50 ng of hFSHβ

or 100 ng hFSH with comparable amounts of hFSHβ and hFSH, the binding were comparable to free hFSHβ represented in the control wells. The results of experiments with specific 10.3A6-captured hFSHβ concerns that low or no binding of hFSH in experiments with hFSHβ could be due to obstruction of hFSHβ by 3G3. By using information about region hFSHβ and the differences in binding to hFSH and hFSHβ, it was as when hFSH was captured by MAb (3G3).

The epitope on hFSHβ consists in part of peptide sequences 1-20 and 66-85 (Vakharia et al., 1990). It was expected no binding of Ab^{Pep} to hFSHβ corresponding to the data in Figs. 1 and 2. Ab⁴⁹⁻⁶⁷ and Ab⁶⁶⁻⁸⁵ did not bind to hFSHβ. Ab³³⁻⁵³ did bind to hFSHβ, suggesting that part of hFSHβ is accessible even when hFSH is bound. Ab³³⁻⁵³ contains antibodies with a sufficiently high titer to bind hFSHβ. To identify this binding of Ab³³⁻⁵³ and 3G3, representing overlapping sequences 49, 48-54, 55-62 and 66-85 (not shown). Ab³³⁻⁵³ bound to hFSHβ 48-54 and to a lesser extent to hFSHβ 35-42. 3G3 also bound to hFSHβ 48-54 and did not bind to hFSHβ. It seems reasonable to suggest that hFSHβ was masked by 3G3 through its retained specificities that were not 49 of MAb-captured hFSHβ.

One interpretation of the data is that in hFSHβ the sequences 48-54 and 35-42 acquire more than one binding site. In comparison in the binding of hFSH, captured by MAb 10.3A6, bound more strongly to hFSHβ. In our hypothesis this is because the 33-53 of hFSHβ is partially altered subsequent to capture by MAb 10.3A6.

Ab 10.3A6. The results of the addition were qualitatively similar to those shown in Fig. 1. The results of the binding of Ab⁸¹⁻¹⁰⁰ and Ab⁹⁸⁻¹¹¹ to hFSH were also similar to those of Ab 10.3A6. Significant binding of hFSH β and hFSH was observed in that case, the binding was not different from hFSH. The observed significant binding with hFSH, obscured in part by the presence of hFSH α is in the epitope of hFSH β . Other results shown are comparable to Fig. 1.

Anti-peptide antisera were prepared against peptides corresponding to the hFSH β sequence. The antisera were tested for their binding to hFSH α -associated hFSH β and hFSH. Earlier work with hFSH α showed that the limited binding of hFSH β was observed if hFSH β was captured by MAb 3G3 on ELISA plates (not shown). hFSH β was captured by MAb 3G3 and five of the seven Ab^{pep} were detectable in 1–2 h. The remaining two (Ab⁴⁹⁻⁶⁷ and Ab⁶⁶⁻⁸⁵) were not captured by MAb 3G3. It was also found that sequences 49–67 were also in the epitope of hFSH β (Vakharia et al., 1990).

As its hypothesis that hFSH compared to hFSH β or induced mobility of hFSH β corresponding hFSH β sequence association with hFSH α was not observed. We reasoned that a binding of Ab^{pep} to hFSH β would suggest that amino acids in hFSH β sequences are accessible to hFSH α in heterodimeric hFSH.

The data (Figs. 1 and 2) it was found that the density signal following the binding of hFSH β was consistently higher than hFSH. Scatchard analysis of the binding of 50 ng of hFSH β

or 100 ng hFSH with capture MAb, the resultant amounts of hFSH β and hFSH available for Ab^{pep} binding were comparable, i.e., the amounts of hFSH β represented in heterodimer (hFSH) was comparable to free hFSH β .

The results of experiments using hFSH α -specific 10.3A6-captured hFSH alleviated some concerns that low or no binding of Ab^{pep} to hFSH in experiments using 3G3-captured hFSH could be due to obstruction of some regions on hFSH β by 3G3. By using 10.3A6 additional information about region hFSH β 49–67 was obtained and the differences in the binding of other Ab^{pep} to hFSH and hFSH β were qualitatively the same as when hFSH was captured by hFSH β -specific MAb (3G3).

The epitope on hFSH β to which 3G3 binds consists in part of peptide sequences 33–53, 49–67 and 66–85 (Vakharia et al., 1990). We therefore expected no binding or reduced binding of the corresponding Ab^{pep} to captured hFSH β . From the data in Figs. 1 and 2 we observed no binding of Ab⁴⁹⁻⁶⁷ and Ab⁶⁶⁻⁸⁵ to captured hFSH β . But Ab³³⁻⁵³ did bind to captured hFSH β . This suggested that part of hFSH β 33–53 is still exposed even when hFSH is captured by 3G3 and that Ab³³⁻⁵³ contains antibody specificities that are of sufficiently high titer to bind that part of the protein. To identify this sequence we tested binding of Ab³³⁻⁵³ and 3G3 to short peptides representing overlapping sequences 28–35, 35–42, 43–49, 48–54, 55–62 and 63–69 of hFSH β (data not shown). Ab³³⁻⁵³ bound predominantly to peptide 48–54 and to a lesser extent to peptides 43–49 and 35–42. 3G3 also bound strongly to peptide 48–54 and did not bind to 43–49 or 35–42. Thus it seems reasonable to suggest that if region 48–54 was masked by 3G3 then perhaps Ab³³⁻⁵³ contained specificities that recognized sequences 43–49 of MAb-captured hFSH β .

One interpretation of this observation is that in hFSH β the sequences in region 33–53 can acquire more than one configuration. The comparison in the binding of Ab³³⁻⁵³ to hFSH β and hFSH, captured by MAb, suggested that Ab³³⁻⁵³ bound more strongly to hFSH β than hFSH. As per our hypothesis this would suggest that region 33–53 of hFSH β is partially masked or structurally altered subsequent to association with

hFSH α in the heterodimeric hFSH. As discussed before in detail (Vakharia et al., 1991), data obtained following enzyme digestion of ovine LH (Bousfield and Ward, 1988) have suggested that a similar region in other gonadotropins is at the subunit-contact site.

Ab⁶⁶⁻⁸⁵ did not bind to captured hFSH β or hFSH. If Ab⁶⁶⁻⁸⁵ does contain all the appropriate antibody specificities, but does not bind captured hFSH β then the data suggest that this region is indeed the epitope recognized by capture MAb 3G3 and is blocked by it (Vakharia et al., 1990). The inability to bind hFSH captured by MAb also suggests that this region of hFSH β is either obscured or structurally altered subsequent to association with hFSH α in the heterodimeric hFSH. In this regard, subunit-association studies also revealed that the preincubation of hFSH α with hFSH β -peptide 66–85 prevented further association of hFSH β with hFSH α (Vakharia et al., 1991).

Ab⁴⁹⁻⁶⁷ bound captured hFSH, suggesting that part of hFSH β 49–67 is still exposed even when in association with hFSH α in heterodimeric hFSH. Recent studies of Grasso et al. (1991a) have reported that synthetic peptides hFSH β 1–15 and hFSH β 59–65 induce uptake of Ca²⁺ into the liposomes. Implicit in those results is the notion that these sequences are exposed in hFSH.

Ab¹⁶⁻³⁶ bound captured hFSH β but not hFSH. This suggests that amino acids in peptide region 16–36 of hFSH β are also at the subunit contact site. Earlier biochemical studies have shown that Tyr³⁷ of hCG β cannot be nitrated, iodinated or perturbed by solvent in the native hCG molecule but can be affected in the free hCG β molecule (Ryan et al., 1987). Those data suggest that the highly conserved region 31–37 which includes amino acids CAGY³⁷ of hCG β is positioned at the α - β subunit contact site. Tryptophan fluorescence of the β subunit of LH or FSH is perturbed upon combination with the α subunit (Ryan et al., 1987; Sanyal et al., 1987). The decrease in tryptophan fluorescence is inhibited by peptides containing CAGY amino acid residues. A recent study using peptides corresponding to the β subunit of human thyroid-stimulating hormone reported that association of α and β human chorionic gonadotropins was inhibited by

peptides including the CAGY sequence (Bergert et al., 1991). Since it is likely that hFSH and hCG fold similarly and have a similar overall conformation, it seems reasonable to suggest that CAGY-containing sequences of hFSH β are masked or altered by hFSH α in hFSH. For hFSH β the comparable conserved region which contains tryptophan as well as CAGY amino acid residues is 25–31. This sequence is contained in peptide 16–36.

Ab^{81–100} and Ab^{98–111} bound captured hFSH β and, to a lesser extent, hFSH. This suggested that residues within regions 81–100 and 98–111 of hFSH β are either masked or structurally altered by hFSH α in the heterodimeric hFSH. The present study confirms and extends the results of those subunit-association experiments and also of epitope mapping studies. In the latter study MAb with higher affinity for hFSH β than hFSH were mapped to regions within 81–100 and 98–111 suggesting that these are subunit-contact associated epitopes and that these regions are either masked or altered subsequent to association with hFSH α in heterodimeric hFSH (Vakharia et al., 1991). We have not been able to demonstrate that the synthetic peptides hFSH β 81–100 or hFSH β 98–111 inhibit binding of hFSH to receptor in ¹²⁵I-hFSH-receptor-displacement assay (Vakharia et al., 1990). However, studies of Santa-Coloma and Reichert (1990) have shown such inhibition by hFSH β 81–95. The disparity in the results may lie in the use of different peptide preparations. The corresponding loop region peptide (93–100) derived from the hCG β sequence has been shown to inhibit receptor binding (Keutmann et al., 1989). However, unlike hFSH β 81–95, hCG β 93–100 did not stimulate steroid production in the target organ. Keutmann et al. (1989) have suggested that hCG β 93–100 has no significant ordered structure, is less rigid and poorly antigenic compared to hCG β 38–57 and that it associates with hCG α to form a topographical receptor-binding domain in the whole hormone. Using β subunit chimeras and mutants Campbell et al. (1990) have predicted that hCG β 47–51, hCG β 89–92 and hCG β 106–108 are receptor-binding sites. Substitution of hFSH β 88–108 amino acids in place of hCG β 94–115 resulted in a hormone analog identical to hFSH in

its ability to bind and stimulate FSH receptor (Campbell et al., 1991). However, as discussed by them (Campbell et al., 1991) hCG β 94–115 (hFSH β 88–108) is also near α subunit and that induced change in the conformation of α -subunit and hFSH β 88–108 or hCG β 94–115 in the heterodimer may facilitate receptor binding. More recent site-directed mutagenesis experiments of the hCG β 93–100 loop region where Arg⁹⁴, Arg⁹⁵ and Asp⁹⁹ were replaced by different amino acids having a different charge property or by amino acids represented by the corresponding position in the hFSH β sequence suggested that these amino acids were important for receptor binding (Chen et al., 1991; Fang and Puett, 1991; Xia et al., 1991). They also suggested that these amino acids either represent contact site or conformational determinants on the β subunit. The conclusions of the present study and those described in our earlier study (Vakharia et al., 1991) confirm and extend those studies discussed above.

The present data and the information in the literature clearly lead us to suggest that although some regions of hFSH β are exposed in the heterodimeric hFSH, a significant proportion of the remaining hFSH β molecule in heterodimeric hFSH is altered or masked when compared with hFSH β which is not associated with hFSH α . It seems imperative that the crystal structures of gonadotropin subunits as well as heterodimer be elucidated in order to fully appreciate their fine structures.

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