

ANTIGENIC REGIONS ON THE β CHAIN OF HUMAN CHORIONIC
GONADOTROPIN AND DEVELOPMENT OF HORMONE SPECIFIC ANTIBODIES

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ABSTRACT

Five peptides corresponding to four regions of the β chain of human chorionic gonadotropin (hCG), were synthesized, purified and characterized. The four regions studied were selected on the basis of sequence differences between the β chain of hCG (β hCG) and the β chains of related hormones. The peptides were found to bind rabbit and mouse anti-hCG antibodies as well as rabbit anti- β chain antibodies, but did not bind antibodies against the α chain or against other hormones. All the peptides, even in their free form, were able to elicit high titer antisera in both rabbits and mice. In all cases, anti-peptide antisera bound to the immunizing peptide as well as to the native hCG and the isolated β chain. These anti-peptide antisera did not bind to unrelated peptides, the α chain of hCG or to other hormones with very similar β chains such as human luteotropic hormone (hLH), ovine luteotropic hormone (oLH) and equine chorionic gonadotropin (eCG). Since the areas represented by these peptides elicit antibodies that are specific for human β hCG, they can formulate the basis for the development of discriminatory reagents for the β chain of hCG.

INTRODUCTION

Human chorionic gonadotropin (hCG)⁺ is a hormone composed of two subunits with a total molecular weight of 37,000 (1). The complete amino acid sequence for both subunits is available (2,3). The subunits are linked by disulfide bonds and lose biological activity when separated. The α subunit (92 amino acids) is almost identical to the corresponding chain of other hormones such as human luteinizing hormone, follicle stimulating hormone, and thyroid stimulating hormone (4). However, the β subunit of hCG (145 amino acids) shows some sequence differences when compared to the β subunits of the other hormones (5).

The α and β subunits are translated from separate mRNAs and there is an abundance of the α subunit in contrast to the β subunit which is almost

absent from the serum of gestating women (6,7). The α subunit has been produced in vitro from a placental mRNA, resulting in a glycosylated molecule with the expected molecular weight (8,9).

Human chorionic gonadotropin is synthesized in the blastocyst and can be detected even before implantation or immediately thereafter, six to ten days after ovulation (10-14). Its concentration in serum reaches its peak at the end of the third month of pregnancy and falls to very low levels at the end of the second trimester (15). Due to the important role of hCG in various physiological and pathological processes, several techniques have been developed for its qualitative and quantitative determination in human tissues (16-19). Early detection of normal and ectopic pregnancy has been greatly improved with the availability of more sensitive screening methods (20). Furthermore, interest in the use of hCG antigenic peptides for the construction of an antipregnancy vaccine has stimulated efforts directed towards the identification of the antigenic regions of β hCG (21).

The determination of hCG in serum is not only of obvious importance in pregnancy, but is also useful for the diagnosis and monitoring of neoplasms which produce the hormone (22,23). Research in the past has been difficult because antisera and even monoclonal antibodies raised against hCG cross-react extensively with pituitary and structurally related hormones due to extensive homology (24). However, due to the fact that the last 30 amino acids at the C-terminus of β hCG are not present in hLH and homologous hormones, it has been possible, utilizing peptides from this region as immunogens, to develop antibodies with low or no cross-reactivity (25-27).

Studies have been undertaken in this laboratory that are aimed at understanding the antigenic properties of β hCG at the submolecular level. Synthetic peptides corresponding to areas which show amino acid differences when compared with homologous hormones, were synthesized. The possibility that these areas function as antigenic sites, when the native molecule is used as the immunizing antigen, was examined. Also, the peptides were used as immunogens and proved to be a useful tool in the development of hCG-specific antibodies.

MATERIALS AND METHODS

Materials

Human chorionic gonadotropin was obtained from Ayerst Laboratories, New York, NY. Some HCG, β hCG and α hCG were kindly provided by Dr. Robert

Synthetic peptides corresponding to segments of the β subunit of hCG

<u>Peptide</u>	<u>Residues</u>	<u>Structure</u>
1A	6-12	Arg-Pro- <u>Arg</u> -Cys- <u>Arg</u> -Pro-Ile-Gly
1B	6-12	Arg-Pro- <u>Arg</u> -Gly- <u>Arg</u> -Pro-Ile-Gly
2	76-84	Val- <u>Asn</u> -Pro-Val-Val-Ser-Tyr- <u>Ala</u> -Val-Gly
3	87-93	Ser-Gly- <u>Gln</u> -Gly- <u>Ala</u> -Leu-Gly
4	111-19	Asp- <u>Asp</u> -Pro-Arg- <u>Phe</u> - <u>Gln</u> - <u>Asp</u> - <u>Ser</u> - <u>Ser</u> -Gly

FIGURE 1

Amino acid sequences of the β hCG peptides synthesized for this study. Underlined residues represent sequence differences relative to the corresponding regions of the β chain of hLH. Note that in peptide 1B Cys in position 9 is replaced by Gly.

Ryan, Mayo Clinic, Rochester, MN. Samples of hLH, eCG and oLH were generous gifts from Dr. Darrell N. Ward, M.D. Anderson Hospital, Houston, Texas. The β hCG peptides (Figure 1) were synthesized by the solid phase method and purified by ion-exchange chromatography as described (28). Peptide purity was monitored by high voltage paper electrophoresis and confirmed by HPLC. Peptide composition was confirmed by amino acid analysis (Beckman, System 6300). The peptides were homogeneous by high voltage electrophoresis and HPLC and their amino acid compositions (Table I) agreed well with those expected from their sequences.

Coupling of the peptides to protein carriers

Peptides were conjugated to protein carriers by a method previously described (29). Briefly, bovine serum albumin or lysozyme (Sigma, St. Louis, MO) were succinylated at their amino groups followed by activation of the carboxyl groups by conversion to their p-nitrophenyl esters. A solution containing the peptide is then added to the activated carrier and the reaction is carried out for 12 hours. This procedure avoids polymerization of the carrier protein or the peptide (29). The extent of peptide coupling is determined by amino acid analysis of the acid hydrolyzate of the peptide-carrier conjugates.

TABLE I

Amino acid analysis of the synthetic peptides.

Amino acid composition (residues/molecule)

Amino acid	Peptide 1A	Peptide 1B	Peptide 2	Peptide 3	Peptide 4
Asp			1.17(1)		2.96(3)
Ser			1.10(1)	.91(1)	1.82(2)
Glu				1.02(1)	1.03(1)
Pro	1.89(2)	1.96(2)	1.30(1)		.98(1)
Gly	.94(1)	1.97(2)	.86(1)	3.04(3)	1.00(1)
Ala			.84(1)	1.12(1)	
Cys	.82(1)				
Val			4.30(4)		
Ile	1.15(1)	.99(1)			
Leu				.96(1)	
Tyr			.87(1)		
Phe					.99(1)
Arg	2.98(3)	3.15(3)			1.13(1)

a Numbers in parenthesis represent values expected from the primary structure of the peptides (Figure 1).

Results represent the average of three analyses. Values of serine have been extrapolated to zero hydrolysis time.

Immunizations

Male BALB/C, SW or ICR mice (Jackson Laboratory, Bar Harbor, ME), were immunized intramuscularly with 50 μ g of free peptide or peptide conjugate in PBS, emulsified with an equal volume of Freund's complete adjuvant. The animals were boosted thereafter monthly, in a similar manner, except that incomplete Freund's adjuvant was used.

Radioadsorbent titrations

Synthetic peptides were coupled to CNBr-activated Sepharose CL-4B by a method previously described (30). Immunoglobulin fractions of the antisera were precipitated in 33% ammonium sulfate, dialyzed and purified by anion exchange chromatography. The IgG fractions were labeled with 125 I by the chloramine T method (31), and quantitated to allow standardization with all antisera. Antibody binding to peptide-adsorbents was determined by titrating a fixed amount of 125 I-labeled immune IgG with varying amounts of adsorbents as described (30).

Plate binding assays

Flexible PVC microtiter plates (Micro Test III, BD-Falcon, Lincoln Park, NJ) were coated with protein or peptide-carrier conjugate overnight at 4°C using solutions (5 µg in 50 µl) in 0.01 M sodium phosphate buffer, pH 7.0 (PBS). After blocking additional binding sites with 1% bovine serum albumin (BSA) in PBS for 1 hour at 37°C, the plates were incubated for 3 hours at 37°C with the appropriate dilution of antisera in PBS containing 0.1% BSA. After washing with PBS, the wells were incubated for 3 hours at 37°C with 50 µl (1:500 dilution in PBS-BSA) of amplifier antibody, affinity purified rabbit-anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN), and then washed. ¹²⁵I-labeled Protein A (Amersham, Arlington Heights, IL), appropriately diluted in PBS-BSA buffer, was added (200,000 CPM/well) and the plates were incubated for 2 hours at 37°C. The unbound protein was washed off, the wells were cut out and bound radioactivity measured in a gamma counter.

RESULTS

Binding of anti-hCG, anti β and anti α chains to the peptides

Rabbit and mouse ¹²⁵I-labeled anti-hCG antibodies bound, to various extents, to each of the peptide adsorbents. For both species, anti-hCG antisera showed preferential binding to peptide 1A (Table II). It should be noted that for peptide 1B the binding is reduced, probably due to the substitution of Cys by Gly. In this particular case this single amino acid substitution is able to disturb anti-protein antibody binding to a peptide representing an immunodominant region. Antibodies raised against the β chain of hCG also show higher binding to peptide 1A. However, antibodies against the α chain of hCG did not exhibit significant binding to any of the peptides as shown in Table II. For peptide 2 (residues 76-84) significant binding was observed with mouse and rabbit anti-hCG antibodies, as well as rabbit anti-β chain antibodies (Table II). As expected, no binding to peptide 2 was detected with rabbit anti-α chain antibodies. Finally, peptides 3 (residues 87-93) and 4 (residues 111-119) also bound anti-hCG antisera. Higher binding, to both peptides, was observed with mouse anti-hCG antiserum and lower but significant binding with rabbit anti-hCG antiserum. However, it should be noted that higher binding to peptides 2,3 and 4 was obtained

TABLE II

Reactivity of anti-hCG antibodies with synthetic peptides

Binding of Anti-hCG Antibodies (Δ cpm)

Antigen	Mouse Anti-hCG	Rabbit Anti-hCG	Rabbit Anti- β hCG	Rabbit Anti- α hCG
hCG	17828	25213	11995	15933
β hCG	7783	13571	16592	2330
α hCG	11456	12235	312	20528
Pept. 1A	6037	3397	3586	142
Pept. 1B	1341	1628	2998	469
Pept. 2	3715	1375	2347	254
Pept. 3	2362	817	1268	462
Pept. 4	3378	758	1160	345
NPa	75	37	0	31
BSA	0	0	0	0
SuBSAb	0	0	0	0

a Nonsense Peptide [30]

b Succinylated BSA

Radioimmunoassay titrations were carried out using a fixed amount of 125 I-labeled antibodies and increased amounts of adsorbents (from 12.5 to 200 μ l of 1:1 suspension in PBS). This summarizes the values obtained with 200 μ l of an adsorbent suspension (1:1, (v/v)). Results were obtained from two separate experiments, each done in triplicate and varied \pm 3% or less.

with antibodies against the isolated β chain, probably indicating either some slight difference in the immune recognition of the free β chain or, more likely, a difference between the two individual rabbits. In all cases 125 I-labeled antibodies against unrelated proteins were used as controls. These antibodies did not bind to hCG or any of its peptides. In addition a nonsense peptide, of the same size but unrelated sequence, was used as a negative control in all the radioadsorbent assays. No binding to this peptide was detected with the antibody preparations used in this study. Hen egg lysozyme, BSA and succinylated BSA were coupled to sepharose and employed as controls for antisera binding to hCG and its subunits. Again, no binding of the anti-hCG antisera to the control proteins was observed.

Binding studies with anti-peptide antibodies

After demonstration that anti-hCG antibodies bind to the synthetic peptides, the possibility of anti-peptide antibody binding

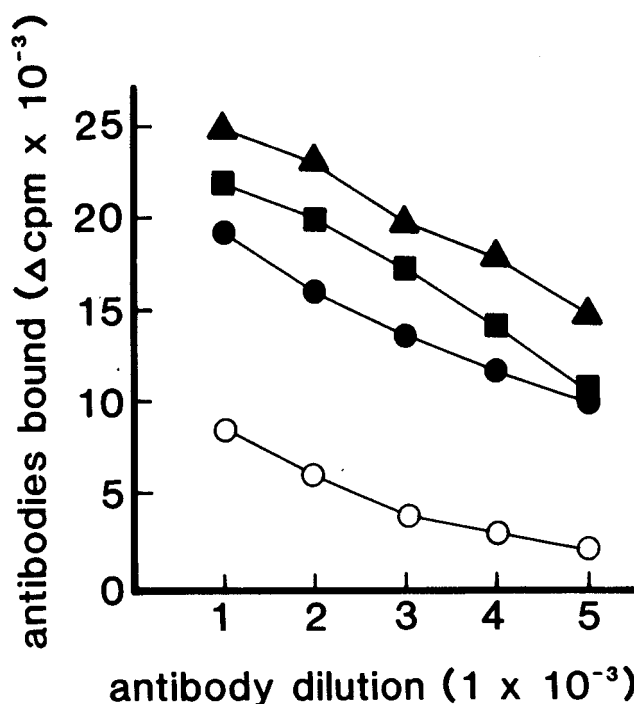


FIGURE 2

Titration curves showing binding of anti-peptide antibodies to native hCG. Binding was determined by RIA in hCG coated plates (see Materials and Methods). (○) anti-peptide 1A; (▲) anti-peptide 2; (●) anti-peptide 3; (■) anti-peptide 4.

to the native molecule was investigated. The potential that these region-specific antibodies could discriminate between hCG and hormones with very similar primary structures was also studied. For this purpose, inbred and outbred mice were injected with free peptides or with peptide-carrier conjugates and the titers monitored by solid phase plate RIA with conjugated peptides. All peptides stimulated very good antibody titers when injected either as free peptides or BSA conjugates. Figure 2 represents titration curves for anti-peptide antisera binding to hCG. As shown in Table III, high titers were obtained in all cases and binding to both hCG and its isolated β chain was very strong. On the other hand, no significant binding of anti-peptide antibodies to the α chains was observed. Anti-peptide antibodies against peptides 1A and 1B showed extensive cross-reactivity, further demonstrating that the substitution made was sufficient to disturb the antigenic recognition of the area, but not to a very significant

TABLE III

Reactivity of anti-peptide antibodies with hCG and related hormones.

Antigen	Binding of anti-peptide antibodies ^a (mean Δ cpm $\times 10^{-3} \pm$ SEM)				
	Anti-Pept. 1A	Anti-Pept. 1B	Anti-Pept. 2	Anti-Pept. 3	Anti-Pept. 4
hCG	4.49 \pm 0.52	6.87 \pm 0.44	18.58 \pm 0.56	12.02 \pm 0.87	10.72 \pm 0.98
ahCG	0.17 \pm 0.06	0.34 \pm 0.01	0.42 \pm 0.02	0.25 \pm 0.06	0.33 \pm 0.03
phCG	4.56 \pm 0.28	8.55 \pm 0.67	15.14 \pm 0.51	8.09 \pm 0.11	6.88 \pm 0.25
Pept 1A	54.74 \pm 1.35	28.30 \pm 0.34	0.02 \pm 0.01	0 ⁻	0.39 \pm 0.02
Pept 1B	35.98 \pm 0.72	43.22 \pm 2.24	0 ⁻	0	0.18 \pm 0.09
Pept 2	0.31 \pm 0.02	0.36 \pm 0.06	41.97 \pm 1.18	0.11 \pm 0.09	0.11 \pm 0.03
Pept 3	0.04 \pm 0.03	0.05 \pm 0.03	0 ⁻	49.64 \pm 1.37	0.18 \pm 0.03
Pept 4	0 ⁻	0.37 \pm 0.01	0	0.31 \pm 0.4	34.26 \pm 1.37
hLH	0.88 \pm 0.03	0.55 \pm 0.03	0.82 \pm 0.04	0.63 \pm 0.18	0.56 \pm 0.12
eCG	0.99 \pm 0.29	0.40 \pm 0.03	0 ⁻	0 ⁻	0.31 \pm 0.06
oLH	0.66 \pm 0.10	0.64 \pm 0.15	0.73 \pm 0.16	0.23 \pm 0.09	0.64 \pm 0.23
NP	0.03 \pm 0.01	0.09 \pm 0.07	0.03 \pm 0.01	0.01 \pm 0.00	0 ⁻
BSA	0 ⁻	0 ⁻	0 ⁻	0 ⁻	0
SuBSA	0	0	0	0	0

^a Antisera were obtained at 120 days after 4 injections with 50 μ g of synthetic peptide and tested by RIA as described in the text. No binding was detected when pre-immune sera was used (not shown). Each value represents the mean of 6 mice.

extent. More importantly, these anti-peptide antibodies exhibited little or no cross-reactivity with hLH, eCG, and oLH. These results are very encouraging and point to the strong probability that these areas, in addition to being antigenic sites, could be used as potential tools for the development of phCG-specific antibodies.

DISCUSSION

The work reported in this paper stemmed from the need for methods of discrimination between hormones with very similar primary structure. Since very little is known about the three-dimensional structure of these groups of hormones, our approach relied on the differences shown at the level of the covalent structure. Due to the

fact that the α chain of hCG and hLH are almost identical the differences in the β chain were exploited. When the β chains of various hormones were compared, a group of differences in amino acid sequence were noticed. Four different regions of the β hCG chain were selected because they possess two or more different residues relative to β hLH. Peptides were designed in such a way that these residues were placed in the center because substitutions occurring at the center of antigenic sites of proteins have been shown (32,33) to have more pronounced effect on antibody response and binding. The peptides synthesized in the present study are shown in Figure 1. An alternate to peptide 1A was also made in which Cys-9 was substituted by glycine (peptide 1B). This was done in order to prevent dimerization of the peptide and would help to test if such a substitution did not interfere with antibody binding as previously demonstrated for lysozyme (34), and serum albumin (35).

Previous studies have demonstrated that antisera raised against hCG are highly cross-reactive with hLH and related hormones (36). This fact is not surprising because the α chain of all these hormones (hLH, tSH, oLH, eCG, etc.) is almost identical and the β chain is very similar. However, since some differences do exist in the β chain, the occurrence of antigenic sites around these regions and their exploitation for the eventual development of specific polyclonal and monoclonal antibodies were examined. As shown in Table II all the peptides were recognized by anti-hCG and anti- β hCG antibodies raised in both mice and rabbits. No recognition was observed when the α chain was the immunogen. These results indicate that these regions contain either complete or major parts of antigenic sites, when the whole hCG molecule is the injected antigen, because a substantial amount of the immune response is directed toward the areas represented by these four peptides. Other unrelated peptides are not recognized. Peptide 1A (residues 6-12) is recognized by large amounts of antibodies, relative to those recognizing the other peptides, suggesting that this area must be a strong antigenic site in the intact molecule. Since these regions are able to bind antibodies against the whole hormone or against its β chain, they must reside on the surface of the molecule (37,38). Therefore, antibodies against the peptides representing these regions would be expected to bind to the whole protein (32).

It is clear that when the synthetic peptides are used as the immunogen, the antibodies raised can recognize the corresponding areas in the intact molecule and also the β chain (Table III). The structurally similar hormones hLH, eCG and oLH as well as the α chain of hCG are not recognized by any of these anti-peptide antisera.

The area including the residues represented by peptide 4 (residues 111-119), has previously been shown to be useful for the development of hCG-specific antibodies (39,40). However, peptide 4 is much shorter than the peptides used in those studies. This peptide is an obvious candidate due to its absence in hLH. Those studies, together with the present data emphasize the importance of the C-terminal region of β hCG in the development of hCG specific reagents.

Monoclonal antibodies directed against these synthetic peptides have been developed and are now being characterized and the results will be reported in a subsequent publication. These antibodies could be useful in the improvement of tests for the specific detection of hCG and its β chain. This fine discrimination is very important in the very early detection of pregnancy and ectopic neoplasms which produce hCG. In addition, the T cell responses (cytotoxic, helper, and delayed-type hypersensitivity) toward these peptides and its relevance in the immune response against the whole molecule in vivo are currently under investigation in this laboratory.

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+ Abbreviations used are: hCG, human chorionic gonadotropin, hLH, human luteotropic hormone; tSH, thyroid-stimulating hormone; oLH, ovine luteotropic hormone; eCG, equine chorionic gonadotropin and when they are preceded by α and β it denotes the subunit of the respective hormone; PBS, 0.01 M sodium phosphate, pH 7.2, in 0.15 M NaCl; BSA, bovine serum albumin.