A detailed study of the L2 β long-loop region of human chorionic gonadotrophin suggests it to be spatially close to, but not part of, the receptor-binding site

D D Jagtap, S D Mahale, A K Mishra, T D Nandedkar
and K S Iyer

Peptide and Protein Research Laboratory, Institute for Research in Reproduction (ICMR), Jehangir Merwanji Street, Parel, Mumbai 400 012, India
(Requests for offprints should be addressed to K S Iyer; Email: pprrl203@rediffmail.com)

A K Mishra is now at Department of Biochemistry and Molecular Pharmacology, 375 Plantation Street, University of Massachusetts Medical School, Worcester, Massachusetts 01655, USA

Abstract

An in-depth study of the L2 β long-loop region of human chorionic gonadotrophin (hCG), earlier identified to be a conformational bionutralization epitope and receptor-binding site of the hormone, was carried out. The linear 38–57 hCG β peptide and the corresponding cyclic disulphide peptide were synthesized and antipeptide antibodies developed. Binding studies with antibodies to the linear peptide, and with hCG β , hCG and human LH suggest that part of the region is buried at the α/β interface and part exposed in hCG. Observation of the surface exposure of residues 47–53 from the crystal structure of hCG was

confirmed by epitope mapping studies of the region. The region is not unique to hCG as a majority of the antibodies to both the linear and cyclic peptides did not exhibit the required specificity. Competitive inhibition studies with the linear and cyclic peptides failed to show inhibition of radiolabelled hCG binding to its receptors. However, both the antipeptide antibodies were able to bionutralize the hormone in an *in vivo* assay. Taken together, these results seem to indicate that the L2 β long-loop region is not a receptor-binding site of hCG but spatially close to it.

Journal of Endocrinology (2002) **172**, 311–320

Introduction

Human chorionic gonadotrophin (hCG) is a member of the glycoprotein hormone family, of which luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone are the other members. The glycoprotein hormones are all heterodimers composed of, within a species, a common α -subunit non-covalently associated with a hormone-specific β -subunit. But there is a high degree of sequence similarity ($\sim 85\%$) in the first 114 amino acids between the β -subunits of hCG and human LH (hLH) but hCG β differs in that it has a carboxy terminus extension of 31 amino acids. The homology between hCG and hLH reflects a common biological function as both proteins bind the same receptor. The crystal structure of hCG (Lapthorn *et al.* 1994, Wu *et al.* 1994) reveals that each of its two different subunits has similar topology, with a long loop and two hairpin loops on either side of a cystine knot. During formation of the heterodimer, the cystine knots of the subunits come together with the long loop of one of the subunits aligning with the hairpin loops of the other subunit giving an asymmetric structure to the molecule.

The region 38–57 hCG β forming the long loop of the β -subunit (L2 β) has been the subject of intense activity for many years before the elucidation of the crystal structure of hCG. The linear peptide 38–54 hCG β was found to elicit very low levels of antibodies to hCG in rabbits, whereas the corresponding cyclic disulphide peptide, wherein the Cys residues at 38 and 57 were looped, was found to be highly immunogenic eliciting high levels of antibodies to hCG (Stevens *et al.* 1986). Subsequently, it was shown that antibodies to the cyclic disulphide peptide Cys(38–57) had the ability to bionutralize hCG in an *in vivo* assay (Stevens 1996). Keutmann *et al.* (1987) postulated the region to be a major receptor-binding region of hCG on the basis of their observation that the cyclic peptide was able to stimulate steroidogenesis in rat Leydig cells and also strongly inhibit binding of whole hCG to its receptor. On the other hand, Campbell *et al.* (1991) found the receptor-binding activity of the chimeric hormone unaffected when the entire loop 38–57 hCG β sequence was replaced with the corresponding human FSH β (hFSH) sequence. The observation was similar when 45–53 segment of this region was replaced by the hFSH sequence (Shao *et al.* 1996). These contradictory results prompted us to undertake a

Linear [Cys(Acm)^{38,57}] 35-57 hCGβ peptide

Cyclic Cys(38-57) 35-57 hCGβ peptide

Figure 1 Structure of the peptides synthesized for the study.

detailed study of this region. The linear peptide amide [Cys(Acm)^{38,57}] 35-57 hCGβ and the corresponding cyclic disulphide peptide amide Cys(38-57) 35-57 hCGβ (Fig. 1) were synthesized and studied for their ability to inhibit radiolabelled hCG binding to its receptor. Anti-peptide antibodies to both the linear and cyclic disulphide peptides were also developed to study their ability to bind hCGβ, hCG and hLH and to bionutralize hCG. Our findings are described and discussed in this paper.

Materials and Methods

Materials

Fluorenylmethyloxycarbonyl (Fmoc)-amino acids were purchased from Bachem (Bubendorf, Switzerland). Fmoc-amino acid pentafluorophenyl esters, Fmoc-amino acid 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine esters and Ultrosyn C resin were procured from Pharmacia-LKB (Nottingham, Notts, UK). 1-Hydroxybenzotriazole was purchased from Advanced Chemtech (Louisville, KY, USA). 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate was obtained from Sigma (St Louis, MO, USA). Piperidine was purchased from Merck (Darmstadt, Germany). 1,2-Ethanedithiol (EDT) and thioanisole were procured from Aldrich (Milwaukee, WI, USA). N,N-Di-isopropylethylamine was purchased from Fluka (Buchs, Switzerland). Na¹²⁵I was purchased from Amersham Pharmacia (Amersham, Bucks, UK). Iodination grade hCG and biological grade hCG and hLH

were gifts from the National Hormone and Pituitary Program (Bethesda, MD, USA). hCGβ was a gift from Professor G P Talwar, National Institute of Immunology, New Delhi, India. Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were procured from DIFCO Laboratories (Detroit, MI, USA). Iodogen and *o*-phenylenediamine were bought from Pierce (Rockford, IL, USA). Microtitre plates were obtained from Nunc (Roskilde, Denmark). Goat anti-rabbit immunoglobulin-peroxidase conjugate was procured from Genei (Bangalore, India).

Peptide synthesis

Synthesis of [Cys(Acm)^{38,57}] 35-57 hCGβ peptide amide The linear 35-57 hCGβ peptide was synthesized as the amide by a solid-phase method using Fmoc chemistry and a continuous flow mode on a Biolynx peptide synthesizer (LKB, Cambridge, Cambs, UK). The assembled peptide was cleaved from the resin with simultaneous cleavage of the side-chain protecting groups by treatment with the cleavage cocktail, trifluoroacetic acid:phenol:water:thioanisole:EDT (80:5:7:5:5:2:5) for 6 h at room temperature. The crude peptide was purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a preparative column (Hi-Pore, C18, 300Å, 25 × 2.15 cm; Bio-Rad, Hercules, CA, USA) using a gradient system consisting of 0.1% trifluoroacetic acid in water and acetonitrile. Homogeneity of the peptide was checked by analytical RP-HPLC on a Lichrosorb

Figure 2 Antibody titres of the 6th to 10th week bleeds from rabbits (A) 2135 and (B) 2138. Rabbits were immunized with the DT conjugate of the linear peptide 35–57 human chorionic gonadotrophin (hCG β) using complete Freund's adjuvant for primary immunization and incomplete Freund's adjuvant for the booster immunization after 3 weeks. Animals were bled weekly and titres measured by ELISA.

RP-18 column (25 $\times$ 0.4 cm). Amino acid composition of the peptide was determined by amino acid analysis of the acid hydrolysate of the peptide. Molecular weight of the peptide was determined by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer.

Synthesis of cyclic Cys(38–57) 35–57 hCG β peptide amide The linear [Cys(Acm)^{38,57}] 35–57 hCG β peptide was first deacetamidomethylated with mercuric acetate (Veber *et al.* 1972) and crude dithiol peptide was purified by gel filtration on Sephadex G-25. This was followed by oxidation of the thiol peptide in a dilute solution (6.08 μ M in phosphate buffer, pH 7.4) with three equivalents of potassium ferricyanide (Stevens *et al.* 1986). The cyclic peptide was purified by Sephadex G-25 gel filtration chromatography and its homogeneity checked by analyti-

cal RP-HPLC. Amino acid composition and molecular weight of the cyclic peptide were also determined.

Preparation of peptide–diphtheria toxoid (DT) conjugates

Both the linear (4 mg, 1.6 μ m) and cyclic disulphide loop (1.3 mg, 0.54 μ m) peptides were conjugated separately to DT by the glutaraldehyde method (Walter *et al.* 1980). DT and the peptide in the molar ratio 1:30 were dissolved in 0.5 ml phosphate-buffered saline (PBS; pH 7.4) and treated with 20 mM glutaraldehyde solution (1 ml for 30 mg DT) in water added dropwise with stirring. After 30 min, 0.1 vol. 1 M glycine was added and the stirring was continued for another 30 min. The reaction mixture was loaded onto a Sephadex G-25 column and eluted with 0.1 M PBS. Fractions containing the conjugate were pooled, concentrated by ultrafiltration and stored at 4 °C.

Figure 3 Competitive inhibition studies of the binding of anti-35–57 hCG β antibodies to the immobilized peptide by the free peptide and an unrelated peptide. Various concentrations (3–100 ng) of the free peptide or the unrelated peptide were preincubated with antiserum 1 (10th week bleed) at 1:16 000 dilution before addition to the peptide immobilized in microtitre wells for ELISA.

A second batch of the conjugate of the linear peptide to DT was prepared using a molar ratio 1:20 of DT to peptide. Amino acid analysis of the conjugate revealed a molar ratio of 1:10.

Immunization protocol

Two adult male rabbits each for the linear and cyclic peptides were immunized with the peptide–DT conjugates. Primary immunization was carried out intradermally at multiple sites with the peptide–DT conjugate (50 μ g peptide/rabbit assuming 25% conjugation of the peptide to DT) emulsified with CFA. After 3 weeks, a booster immunization was given intradermally at multiple sites with the same amount of the peptide–DT conjugate emulsified with IFA. Six weeks after the primary immunization, animals were bled once in a week until the antibody titres started to decline. Subsequently, four more rabbits were immunized with the second batch of the conjugate of the linear peptide to DT (using 174 μ g conjugate containing 50 μ g of the peptide/rabbit/immunization) following the same protocol.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to measure the anti-peptide antibody titres and also to measure the extent of binding of the anti-peptide antibodies to hCG β , hCG and hLH. Microtitre wells were

incubated overnight at 4 °C with 100 μ l of a solution of either the linear peptide, cyclic peptide, hCG β , hCG or hLH in carbonate buffer (pH 9.4) at a concentration of 10 μ g/ml. The remaining sites were blocked with 1% gelatin in PBS containing 0.05% Tween-20 at 37 °C for 2 h. Serial dilutions of the antisera were added to the wells followed by incubation at 37 °C for 1.5 h. This was followed by the addition of goat anti-rabbit immunoglobulin–peroxidase conjugate and further incubation at 37 °C for 1 h. The enzyme activity was measured using *o*-phenylenediamine and hydrogen peroxide as the substrate. For competition experiments, serial dilutions of the free peptide or an unrelated peptide were preincubated with the antisera before addition to the microtitre wells.

Purification of antisera

Crude immunoglobulins were obtained from the antisera by repeated precipitation with saturated ammonium sulphate solution. This was followed by affinity chromatography on a protein A–Sepharose gel column to yield the pure IgG fractions which were diluted to serum equivalents with PBS.

Epitope mapping of the region 35–57 hCG β

Overlapping peptides 35–42, 39–46, 43–50 and 47–57 spanning the region 35–57 hCG β were synthesized and

Figure 4 Binding studies of anti-35–57 hCG β antibodies to hCG. hCG (100 ng/well) was immobilized in the microtitre wells and incubated with serial dilutions of the antisera 1–6 (6th week bleed) at 37 °C for 1.5 h before addition of the goat anti-rabbit immunoglobulin–peroxidase conjugate for further incubation followed by colour development by the addition of *o*-phenylenediamine.

then assessed for their ability to competitively inhibit the binding of anti-35–57 hCG β antibodies to immobilized 35–57 hCG β peptide by ELISA.

Mouse uterine weight gain bioassay

The mouse uterine weight gain bioassay (Levin & Tyndale 1937) was used in a modified form (Das *et al.* 1976) to test the *in vivo* hCG-neutralizing capacity of the antipeptide antibodies. Prepubertal female mice of the Swiss strain, 20–22 days old and weighing 7–8 g, were placed in groups of five to six mice each. The test solutions (0.2 ml) were injected intraperitoneally once daily for 3 days. Autopsy was done on day 4. The uteri were dissected out, blotted free of fluid and weighed. For standardization, hCG at a total dose of 0.75, 1.5, 3.0, 6.0 and 12.0 IU was used to draw the dose–response curve. A total dose of 3 IU was found to produce four times the increase in uterine weight over that of saline-treated controls. This concentration of hCG was used for neutralization experiments. For this purpose, 1:10 diluted affinity-purified antisera were incubated with hCG at 37 °C for 2 h before injecting into the animals.

Radioreceptor assay (RRA)

RRA was used to assess the ability of the peptides to inhibit 125 I-hCG from binding to LH/CG receptor.

Corpora luteal membrane LH/CG receptor homogenate was prepared from sheep ovaries (Selvaraj & Moudgal 1993). Aliquots (100 μ l) of the receptor homogenate were incubated with the peptides in various concentrations (0.003×10^{-3} M to 2.343×10^{-3} M) or unlabelled hCG (0.625×10^{-10} M to 151.87×10^{-10} M) in Tris–HCl buffer (0.05 M, pH 7, 200 μ l) containing MgCl₂ (10 mM) and bovine serum albumin (0.1%) at 37 °C for 15 min in a shaking waterbath. This was followed by the addition of 100 μ l 125 I-hCG (hCG was radioiodinated by the iodogen method (Fraker & Speck 1978)) and further incubation for a further 2 h. Reaction was terminated by the addition of 1.25 ml chilled Tris–HCl buffer. Assay tubes were spun at 3000 r.p.m. for 30 min, the pellets were washed with chilled buffer once and the pellet-bound radioactivity counted.

Statistical analysis

All results are expressed as means $\pm$ S.E.M. Statistical significance was evaluated using one-way analysis of variance with Duncan's multiple range test. A value of $P < 0.05$ was considered statistically significant.

Results

Solid-phase synthesis of the linear peptide [Cys(Acm)^{38,57}] 35–57 hCG β followed by cleavage and purification

Figure 5 Binding studies of anti-35–57 hCG β antibodies to hCG β . hCG β (100 ng/well) was immobilized in the microtitre wells and incubated with serial dilutions of the antisera 1–6 (6th week bleed) at 37 °C for 1.5 h before addition of the goat anti-rabbit immunoglobulin–peroxidase conjugate for further incubation followed by colour development by the addition of o-phenylenediamine.

yielded a homogeneous peptide. Amino acid analysis of the acid hydrolysate of the peptide showed the presence of the expected amino acids in the proper ratios. Molecular weight of the peptide as determined by MALDI-TOF mass spectrometry was found to be 2556.4 against an expected value of 2558.1. Potassium ferricyanide oxidation of the thiol peptide, obtained after deacetamidomethylation of the linear peptide followed by purification, resulted in a homogeneous product which tested positive for the presence of disulphide bond in the 2-nitro-5-thiosulphobenzoate test (Thannhauser *et al.* 1987), had a different retention time in analytical RP-HPLC as compared with the linear peptide and was found to have a molecular mass of 2413.9 (calculated molecular weight of the cyclic disulphide peptide amide is 2413.93).

Immunization of two rabbits with the first batch of linear peptide–DT conjugate resulted in good titres (titre of the 6th week bleed antiserum: rabbit 2135, 1:6000; rabbit 2138, 1:16 000; titre is defined as the dilution of the antiserum showing a net absorbance of 1 at 492 nm) of anti-peptide antibodies in both the animals. Figure 2 depicts antibody profiles (6th week bleed) of antiserum 1 (rabbit no. 2135) and antiserum 2 (rabbit no. 2138). Subsequent immunization of four additional rabbits with the second batch of linear peptide–DT conjugate yielded comparatively lower titres (titres of 6th week bleed antisera varied from 1:500 to 1:2000) of anti-peptide antibodies

(antisera 3–6 from rabbit nos 2744, 2749, 2753 and 2754 respectively). In competitive inhibition studies, homologous peptides competitively inhibited the binding of the anti-peptide antibodies to the immobilized peptides while unrelated peptides did not (Fig. 3). Out of the six antisera to the linear peptide only antiserum 1 exhibited significant binding to hCG while the remaining five antisera had low to almost negligible levels of hCG-binding antibodies (Fig. 4). As for binding to hCG β , four out of the six antisera showed higher levels of binding as compared with binding to hCG (Fig. 5). Again, antiserum 1 exhibited exceptionally high binding to hCG β . Except for antiserum 1 which showed negligible binding to hLH, the remaining antisera exhibited similar binding to hLH as to hCG. Epitope mapping studies revealed that peptide 47–57 could competitively inhibit the binding of anti-35–57 hCG β antibodies to the 35–57 hCG β peptide, whereas the remaining three peptides 35–42, 39–46 and 43–50 were inactive (Fig. 6). Immunization of two rabbits with the cyclic peptide–DT conjugate resulted in high titres (titres of 6th week bleed antisera: antiserum 7 (rabbit 2338) and antiserum 8 (rabbit 2339), 1:35 000) of anti-peptide antibodies to the cyclic peptide. Both the antisera exhibited binding to hCG β , hCG and hLH. Significantly higher binding was observed with hCG β and lower binding with hLH as compared with hCG. In the *in vivo* mouse uterine weight gain bioassay for hCG, anti-peptide antibodies to

Figure 6 Epitope mapping of the region 35–57 hCG β . Competitive inhibition studies of the binding of anti-35–57 hCG β antibodies to the immobilized 35–57 hCG β peptide by the peptides 35–42, 39–46, 43–50, 47–57, 35–57 and an unrelated peptide. Various concentrations (10 ng to 10 μ g) of the peptides were preincubated with the antiserum 6 (6th week bleed) at 1:1000 dilution before adding to the immobilized peptide in the microtitre wells for ELISA.

both the linear (antiserum 1) and cyclic (antiserum 7) peptides effected a decrease in hCG-induced uterine weight gain (Fig. 7). In RRA, neither the linear nor the cyclic peptides inhibited the binding of radiolabelled hCG to its ovarian receptors (Fig. 8).

Discussion

Our laboratory recently attempted to map the receptor-binding regions of hCG by screening the disulphide peptides (six disulphide peptides corresponding to the six disulphide bonds in the β -subunit) of its β -subunit for their ability to inhibit radiolabelled hCG binding to its ovarian receptors (Mishra *et al.* 2001). The results suggest the involvement of the regions around the disulphide bonds Cys(9–57) and Cys(23–72) in receptor binding of hCG. This study did not include the 38–57 hCG β region as the disulphide assignments from the crystal structure of hCG did not propose a disulphide linkage between Cys residues at 38 and 57. Earlier work (Stevens *et al.* 1986, Keutmann *et al.* 1987) on this region had focused mainly on the cyclic disulphide peptide, probably because the disulphide assignments at that point in time proposed a Cys(38–57) disulphide bond (Mise & Bahl *et al.* 1981). Also, no definite conclusions could be drawn regarding the

involvement of this region in the receptor binding of hCG. Our laboratory therefore felt the need to carry out a systematic study of this region, using both the linear and cyclic peptides and the corresponding antipeptide antibodies.

The study consisted of assessing the ability of the linear [Cys(Acm)^{38,57}] 35–57 hCG β peptide and the corresponding disulphide loop peptide Cys(38–57) 35–57 hCG β to inhibit the binding of radiolabelled hCG to its ovarian receptors. The rationale for including the disulphide loop peptide in the study is to have a peptide with the loop structure stabilized by the disulphide bridge, in the event that the linear peptide by itself cannot maintain the overall shape and conformation of the loop region. The study also included screening of antipeptide antibodies to both the linear and cyclic peptides for their ability to bind hCG β , hCG and hLH. The results may throw light on the involvement of this region in heterodimer formation with the α -subunit, on the surface exposure of this region in hCG and hLH and on the subtle differences in the conformation of this loop region in hCG and hLH, as there are differences in the amino acid residues between hCG β and hLH β at positions 42 (Thr $\rightarrow$ Met), 47 (Gly $\rightarrow$ Ala) and at 51 (Ala $\rightarrow$ Pro) of this region. As antibodies to receptor-binding regions may be expected to neutralize the bioactivity of the hormone by blocking its binding

Figure 7 Effect of antibodies to linear 35–57 hCG β and cyclic Cys(38–57) 35–57 hCG β peptides in hCG-induced mouse uterine weight gain assay. Affinity-purified antibodies from antiserum 1 (6th bleed) and antiserum 8 (6th bleed) were used for the assay. The numbers in the bars show the number of animals in each group. Mice were injected intraperitoneally with saline (C), hCG (3 IU) or hCG+antipeptide antibodies over a period of 3 days. Autopsy was done on day 4 and the uteri were dissected out, blotted and weighed on a torsion balance.

to the receptor, the *in vivo* bionutralizing ability of the antipeptide antibodies was also checked.

The region 38–57 hCG β is probably surface exposed in hCG as most of the antisera to the linear peptide and the two antisera to the cyclic peptide exhibited binding to the heterodimeric hormone, although to varying extents. However, the consistent lower levels of binding to hCG as compared with hCG β by a majority of the antibodies indicate that part of the 38–57 region is perhaps buried at the interface or that there is an alteration in the conformation of this region when the β -subunit combines with the α -subunit to form the native hormone. Crystal structure of hCG (Lapthorn *et al.* 1994) shows the residues

Figure 8 Peptides or hCG in various concentrations in 0.05 M Tris–HCl buffer (pH 7) were incubated with corpora luteal membrane LH/CG receptor homogenate at 37 °C for 15 min followed by the addition of ¹²⁵I-hCG and further incubation for 2 h. Pellet-bound radioactivity was counted after terminating the reaction, centrifugation and washing the pellets. hCG (▲), linear [Cys (Ac)^{38,57}] 35–57 hCG β peptide (■) and cyclic Cys(38–57) 35–57 hCG β peptide (●).

40–46 buried at the α/β interface and the residues 47–53 exposed at the surface forming a wedge-shaped extrusion.

Epitope mapping of the region 35–57 hCG β using overlapping peptides 35–42, 39–46, 43–50 and 47–57 seems to indicate that the majority of the antibody populations are directed to the whole peptide, perhaps to a conformational epitope. This may be so because (i) the homologous peptide was able to completely inhibit the binding of the antisera to the immobilized peptide at very low concentrations of the free peptide (the IC₅₀ values observed with the various antisera ranged from 0.012×10^{-6} M to 0.28×10^{-6} M) and (ii) out of the four overlapping peptides tested, three did not show any inhibition while the fourth peptide inhibited partially with IC₅₀ values ranging from 1.03×10^{-6} M to 85.8×10^{-6} M. But it is very interesting to note that the peptide exhibiting positive inhibition turns out to be 47–57, the very same region that is observed to be exposed in hCG as per its crystal structure. It is therefore logical to conclude that binding of the anti-35–57 hCG β antibodies to hCG is through the 47–57 region.

One of the objectives of this study was to look for any subtle differences in the conformation of this loop region in hCG and hLH as there are differences in the amino acid residues between hCG β and hLH β at positions 42, 47 and 51. Except for antiserum 1, all the other antisera including antisera to the cyclic peptide exhibited almost similar binding to both hCG and hLH. Antiserum 1 was exceptionally specific to hCG with very little cross-reactivity with hLH. Therefore, it appears that the region 35–57 hCG β is not unique to hCG.

In the RRA, neither the linear nor the cyclic peptides exhibited any inhibition of the binding of radiolabelled hCG to its ovarian receptor even at the highest concentration, i.e. 2.343×10^{-3} M of the peptides. This is in striking contrast to the report by Keutmann *et al.* (1987) who observed inhibition of ^{125}I -labelled hCG half-maximally at 1.51×10^{-4} M with the cyclic disulphide 38–57 hCG β peptide. The corresponding hLH β peptide was found to be eightfold more potent and both these peptides even stimulated testosterone production by rat Leydig cells. Subsequently, there were two observations which contradicted the above claim that 38–57 hCG β loop region forms a receptor-binding site in hCG. In chimeric hCG, where the entire loop 38–57 hCG β sequence (Campbell *et al.* 1991) or 45–53 hCG β sequence (Shao *et al.* 1996) was replaced with the corresponding hFSH β sequences, the receptor binding activity was found to be unaffected. Our data support the contention that the L2 β long-loop region of hCG is not a receptor-binding site.

Antibodies to both the linear and cyclic peptides, when tested in the *in vivo* mouse uterine weight gain bioassay for hCG, effected a decrease in hCG-induced uterine weight gain. The apparent difference in potency observed between the antibodies to linear and cyclic peptides was not statistically significant. Obviously, binding of the antibodies to hCG prevents it from interacting with the receptor to exert its biological action. As the corresponding peptides failed to show any binding affinity for the hCG receptors, hCG bionutralization activity cannot be attributed to direct blocking of the receptor-binding site in hCG by the antipeptide antibodies. On the other hand, if the region 38–57 hCG β is spatially close to the receptor-binding site, it is conceivable that binding of bulky antibody molecules to the region may sterically hinder hCG from interacting with its receptor, resulting in bionutralization of the hormone. In fact, the crystal structure of hCG (Lapthorn *et al.* 1994) positions the residues 47–53 proximal to the determinant loop region 93–100 hCG β which is proposed to be the receptor-binding site determining hormone specificity (Ward & Moore 1979) and our epitope mapping of the region 35–57 clearly showed that residues 47–57 are the hCG-binding epitope. The Cys(9–57) disulphide bond, identified by our laboratory to be involved in receptor binding of hCG, seems to be also in the close vicinity of the 38–57 loop region.

In conclusion, our studies on the 38–57 region which forms the L2 β long loop of hCG suggest the following: (i) part of the region is buried at the α/β interface and part exposed in hCG, our results confirm the observation from the crystal structure that the residues 47–53 are exposed in hCG; (ii) the region is not unique to hCG and (iii) the region is probably not involved in the receptor binding of hCG but it is spatially close to the receptor-binding site.

Acknowledgements

We would like to thank Dr H S Juneja, Ex-Director of our Institute for his sustained interest in the work and for constant encouragement. We would like to acknowledge the National Hormone and Pituitary Program for the generous supply of iodination grade hCG, biological grade hCG and hLH. We would like to thank Professor G P Talwar, Ex-Director, National Institute of Immunology, New Delhi, India for the gift of the hCG β subunit. We would also like to thank Ms Leena Tendulkar for technical assistance. Our thanks are also due to Ms S L Barde for secretarial assistance.

References

- Campbell RK, Dean-Emig OM & Moyle WR 1991 Conversion of human chorionic gonadotropin into follitropin by protein engineering. *PNAS* **88** 760–764.
- Das C, Salahuddin M & Talwar GP 1976 Investigations on the ability of antisera produced by Pr- β -hCG-TT to neutralize the biological activity of hCG. *Contraception* **13** 171–181.
- Fraker PJ & Speck JC Jr 1978 Protein and cell membrane iodinations with a sparingly soluble chloramide 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochemical and Biophysical Research Communications* **80** 849–857.
- Keutmann HT, Charlesworth MC, Mason KA, Ostrea T, Johnson L & Ryan RJ 1987 A receptor-binding region in human choriogonadotropin/lutropin β subunit. *PNAS* **84** 2038–2042.
- Lapthorn AJ, Harris DC, Littlejohn A, Lustbader JW, Canfield RE, Machin KJ, Morgan FJ & Isaacs NW 1994 Crystal structure of human chorionic gonadotropin. *Nature* **369** 455–461.
- Levin L & Tyndale HH 1937 The quantitative assay of follicle stimulating substances. *Endocrinology* **21** 619–628.
- Mise T & Bahl OP 1981 Assignment of disulphide bonds in the β -subunit of human chorionic gonadotropin. *Journal of Biological Chemistry* **256** 6587–6592.
- Mishra AK, Mahale SD & Iyer KS 2001 Mapping the receptor binding of regions of human chorionic gonadotropin (hCG) using disulfide peptides of its β -subunit: possible involvement of the disulfide bonds Cys⁹-Cys⁵⁷ and Cys²³-Cys⁷² in receptor binding of the hormone. *Journal of Peptide Research* **58** 17–26.
- Selvaraj N & Moudgal NR 1993 Development of an LH receptor assay capable of measuring serum LH/CG in a wide variety of species. *Journal of Reproduction and Fertility* **98** 611–616.
- Shao K, Purohit S & Bahl OP 1996 Effect of modification of all loop regions in the α - and β - subunits of human choriogonadotropin on its signal transduction activity. *Molecular and Cellular Endocrinology* **122** 173–182.
- Stevens VC 1996 Progress in the development of human chorionic gonadotropin antifertility vaccines. *American Journal of Reproductive Immunology* **35** 148–155.
- Stevens VC, Chou W, Powel JE, Lee AC & Smoot J 1986 The identification of peptide sequences of human chorionic gonadotropin containing a conformational epitope. *Immunology Letters* **12** 11–18.
- Thannhauser TW, Konishi Y & Scheraga HA 1987 Analysis for disulfide bonds in peptides and proteins. In *Methods in Enzymology*, Vol 143, pp 115–119. Eds WB Jakob & OW Griffith. New York: Academic Press, Inc.
- Veber DF, Milkowski JD, Varga S, Denkwalter RG & Hirschmann R 1972 Acetamidomethyl-A novel thiol protecting group of cysteine. *Journal of the American Chemical Society* **94** 5456–5461.

Walter G, Scheidtmann KH, Carbone A, Laudano AP & Doolittle RF 1980 Antibodies specific for the carboxy- and amino-terminal regions of simian virus 40 large tumor antigen. *PNAS* **77** 5197–5200.

Ward DN & Moore WT 1979 Comparative studies of mammalian glycoprotein hormones. In *Animal Models for Research on Contraception and Fertility*, pp 151–161. Ed. NJ Alexander. New York: Harper and Row.

Wu H, Lustbader JW, Liu Y, Canfield RE & Hendrickson WA 1994 Structure of human chorionic gonadotropin at 2.6 Å resolution from MAD analysis of the selenomethionyl protein. *Structure* **2** 545–558.

Received 29 August 2001

Accepted 4 October 2001