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Effect of modification of all loop regions in the α- and β-subunits of human choriogonadotropin on its signal transduction activity[1]

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Abstract

Human choriogonadotropin (hCG), according to its three dimensional structure as determined by X-ray diffraction, has three β-hairpin loops each in the α and β subunit designated as α_1, α_2 α_3 and β_1 β_2 and β_3, respectively. Since similar β-hairpin loops in NGF and TNFβ have been implicated in their direct interaction with the receptor, it prompted the present investigation to determine the role of such loops in receptor binding and post-receptor signaling events in hCG. Based on the three dimensional structure of hCG, radical mutations were introduced in the α loops by replacing hydrophobic α18Phe and α74Phe by hydrophilic Thr residues in the α_1 and α_3 loops, respectively, and positively charged α45Lys by negatively charged Asp in the helical segment in the α_2 loop. The β loops were mutated by replacement of the β_1, β_2 and β_3 sequences with the corresponding hFSH sequences. These replacements included β22Gly, β24Pro and β25Val with Glu, Arg and Phe in β_1, ^{45}Leu Gln Gly Val Leu Pro Ala Leu Pro^{53} with Tyr Lys Asn Pro Ala Arg Pro Leu Ile in β_2 and ^{73}Pro Arg Gly with Ala His His in the β_3 loop. Six mutants, hCG$\alpha_1\beta$, hCG$\alpha_2\beta$ and hCG$\alpha_3\beta$ and hCG$\alpha\beta_1$, hCG$\alpha\beta_2$ and hCG$\alpha\beta_3$, were obtained by co-infection of the insect High-Five cells with baculovirus containing mutant α or β cDNAs and that containing complimentary wild type β or α cDNAs. The mutants were almost completely secreted in the culture medium and were over expressed at levels ranging between 4.5 to 29 μg/ml indicating that mutations had no effect on the secretion or subunit assembly of hCG. In order to remove any contaminating β-subunit, the culture medium was passed through a column of an hCGβ-specific monoclonal antibody, B158. The receptor binding activity of the mutant hCG$\alpha_1\beta$, in which α18Phe was replaced with Thr, increased almost 200% relative to rehCG. Similarly, increase in the cAMP and progesterone stimulation by the mutant ranged between 150 to 200%. This increase is believed to be due to a short range conformational change in the mutant as a result of the mutation rather than direct involvement of α18Phe in the receptor binding. The evidence in support of this was derived from the fact that the affinity or interaction between the two subunits was impaired as indicated by the first order rate constant of hCG$\alpha_1\beta$ ($k_m = 4.1 \times 10^{-2}$ min^{-1}) at pH 3.0 at 23°C which is one order of magnitude greater relative to rehCG ($k_w = 4.6 \times 10^{-3}$ min^{-1}). All other mutations had no effect on the receptor binding or signal transduction of hCG indicating that, unlike NGF or TNFβ, β-hairpin loops in hCG were not directly involved in receptor binding or post-receptor signaling events. However, since the mutation in the α_1 loop affects the receptor binding site, its presence in the vicinity of the α_1 loop is highly likely.

Keywords: Choriogonadotropin; Glycoprotein hormone; β-Hairpin loops; Site directed mutagenesis

Abbreviations: BSA, bovine serum albumin; FSH, follitropin; hCG, human choriogonadotropin; rehCG, recombinant hCG; LH, lutropin; NGF, nerve growth factor; M.O.I., multiplicity of infection; P.I., post infection; PCR, polymerase chain reaction; RIA, radioimmunoassay; RRA, radioligand receptor assay; TNF, tumor necrosis factor.

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1. Introduction

Human choriogonadotropin belongs to a family of glycoprotein hormones including lutropin (LH), follitropin (FSH), and thyrotropin (TSH). They are all heterodimeric proteins made up of two subunits desig-

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nated as α and β. Within an animal species the α-subunits are identical in amino acid sequence and the β-subunits are different except that they all have six similarly spaced disulfide bonds in their amino acid sequences [1]. The heterodimers are biologically active but the individual subunits do not seem to have any biological activity. The subunits can be dissociated from the heterodimer with complete loss of biological activity and can be reconstituted with the restoration of almost full biological activity [2]. Thus, the intra- and inter-species hybrid hormones can be prepared readily; the biological activity of the hybrid hormone is dictated by the β-subunit [3]. The biological activity of all these hormones is mediated by cAMP. HCG and hLH have been reported to stimulate also the production of IP_3 from phosphatidyl inositol 4,5-bisphosphate [4–6]. Both hCG and hLH stimulate the production of progesterone. The molecular mechanism of signal transduction by the hormones involves interaction with the target cell surface receptors belonging to the seven transmembrane domain superfamily of receptors. Recently, the three dimensional structure of the chemically deglycosylated hCG by X-ray diffraction has been reported. The proposed structure is predominantly a β-structure with three helical segments, two in the α- and one in the β-subunit [7,8]. The antiparallel β-strands in each of the α- and β-subunits are joined by three hairpin loops designated as α_1, α_2 and α_3 in the α-subunit and β_1, β_2 and β_3 in the β-subunit. Both α and β subunit structures like NGF, TGFβ and PDGFβ contain the so-called cysteine knot motif [7,8]. Recently, the crystal structure of NGF has revealed the presence of three pairs of antiparallel β-strands connected by β-hairpin loops. Among the four members of the neutrotropin family, the loops contain the most variable amino acid residues and have been implicated in the interaction between NGF and its various receptor subtypes [9]. Similarly, the crystal structure of the soluble human 55 kDa TNF receptor and human TNF complex indicates the involvement of the β-hairpin loops in the interaction between the ligand and the receptor [10,11]. These observations prompted us to investigate the role of four β-hairpin loops and two other loops in the receptor binding and signal transducing properties of hCG. In order to avoid any disruption in the proper folding of the heterodimer, radical single residue mutations or analagous substitutions rather than deletions were introduced. All three loop sequences in the β-subunit were individually substituted by the corresponding sequences in hFSHβ. Since all the α-subunits in the various human hormones have identical amino acid sequences, radical single residue mutations were introduced by replacing invariant residues, Lys with Asp at position 45 in the α_2 loop and by replacing Phe residues at positions 18 and 74 with a hydrophilic residue, Thr in the α_1 and α_3 loops, respectively, in the α-subunit. The hCGα and hCGβ mutants were co-expressed with wild type hCGβ and hCGα respectively in baculovirus insect cell expression system [12]. The modification of the loops both in α and β-subunits of hCG had no effect on the subunit assembly or on the secretion of the heterodimer. The hCG mutant, $hCG\alpha_1\beta$ with mutation in the α_1 loop was found to be 200%-fold more active in receptor-binding and cAMP and progesterone stimulation than wild type rehCG. This increase in activity is believed to be due to a conformational change as demonstrated by weaker interaction between the mutated α and the wild type β-subunit. All other mutants of hCG including $hCG\alpha_2\beta$, $hCG\alpha_3\beta$, $hCG\alpha\beta_1$, $hCG\alpha\beta_2$ and $hCG\alpha\beta_3$ did not show any significant difference from the wild type hCG in receptor binding and signal transduction properties. The results of the present studies clearly demonstrate that, unlike NGF [9] and TNFβ [10], the loops in hCG are not directly involved in the receptor binding or in post-receptor signaling events. However, α18Phe in the α_1 loop plays a pivotal role in maintaining the proper folding of the subunits and is probably located in the vicinity of the receptor binding site.

2. Materials and methods

Monoclonal antibody B158 was prepared in the laboratory and was highly specific for hCGβ with no detectable cross-reactivity with hCGα or hCG [13]. The rabbit polyclonal antibodies against hCG and its subunits, α and β raised in the laboratory had titers varying between 1 to 20 000 and 1 to 1 000 000 for 30% binding with ^{125}I-labeled hormones [14]. hCG and its subunits were prepared in the laboratory [15]. hCGα and hCGβ cDNAs were obtained by screening cDNA library prepared from BeWo cells [16]. All restriction enzymes were purchased from Biolab or Promega. Ultima DNA polymerase was from Perkin Elmer. T_4 DNA ligase and DNA sequencing kit were obtained from USB. DEAE-Affi-Gel blue and Affi-Gel 10 were from BioRad. The insect fall army spodoptera cell line Sf9 was obtained from ATCC and cultured at 27°C, as previously described [3], in Grace's medium, pH 6.2 (Gibco) modified with lactalbumin hydrolysate 3.3 g/l and TC yeastolate, 3.3 g/l containing 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml fungizone (Gibco). Insect High Five cells were obtained from Invitrogen and cultured in serum-free medium Ex Cell 405 (JRH).

2.1. Construction of recombinant baculovirus vectors and expression of hCG mutants

The mutant cDNAs containing modified hairpin loops of hCGα and hCGβ were produced by PCR

method. Primers used for the various mutants of hCGα and hCGβ cDNAs were synthesized in the oligonucleotide facility of SUNY at Buffalo. The PCR products of hCGα were subcloned into the *Eco*RI and *Bam*HI restriction sites in pVL1392, and those of hCGβ were subcloned into the *Eco*RI and *Bgl*II sites in pVL1393. To confirm the mutations, the DNA sequencing [17] was performed with double-stranded plasmid DNA. The baculovirus linear DNA and pVL1392-hCGα or pVL1393-hCGβ were co-transfected into insect Sf9 cell line using cationic liposome (Invitrogen). The recombinant viruses with mutant hCGα or hCGβ cDNA incorporated into the viral genome through the in vivo homologous recombination were identified visually in the infected insect cell monolayer with agarose overlay [3] followed by RIA and immunoblotting with the anti-hCGα or anti-hCGβ antibody.

In order to express hCG mutants, the recombinant viruses containing the mutant α or β subunits were co-infected with wild type hCGβ or hCGα viruses, respectively, in 2×10^6 High Five insect cells/ml in 20 ml of serum-free medium (JRH) at an M.O.I. of 5. The cells were harvested in 60 h P.I.

2.2. Analytical techniques

The discontinuous gel system of Laemmli [18] with 5% stacking gel at pH 6.8 and 12% separation gel at pH 8.8 was used. The gel was blotted on the nitrocellulose membrane for immunoblotting. Antibodies for immunoblotting were diluted 50 times less than that of the titer for RIA. Blotting was performed in 50 mM PBS, pH 7.4, containing 2% BSA.

2.3. Purification of monoclonal anti-hCGβ antibody, B158, by FPLC and preparation of immunoaffinity adsorbent for immunoaffinity chromatography

Purification of the monoclonal antibody from mouse ascitic fluid was carried out by DEAE-Affi-Gel blue chromatography as described by Bruck et al. [19]. A 5-ml aliquot of anti-hCGβ (B158) was passed through an approximately 40 ml bed volume of Affi-Gel Column. The IgG fractions were eluted with a 300 ml of 0–100 mM NaCl gradient in 50 mM Tris–HCl buffer, pH 7.2. The column fractions were monitored at 280 nm as well as by SDS-PAGE and silver staining. The purified monoclonal antibodies were conjugated to Affi-Gel 10 (BioRad). A 10-mg sample of the antibody, B158, in 15 ml of the coupling buffer (0.1 M $NaHCO_3$, pH 8.0) was mixed with 10 ml of Affi-Gel 10 prewashed with the cold buffer. The coupling was performed by rocking the reaction mixture end over end at 4°C for 4 h. The residual active sites were blocked by treating with 1 M monoethanolamine HCl (pH 8.0) at 4°C for 1 h [20]. The coupling efficiency was about 80% as determined from the amount of the unused antibody recovered. A 2-ml sample of each of the hCG mutant-expressed in insect cells was passed through a 2-ml bed volume of the immunoaffinity adsorbent equilibrated with 20 ml of 50 mM Tris–HCl, pH 8.0 to ensure the removal of hCGβ contaminant from hCG. The column was eluted with the same buffer and the eluate was examined by Western immunoblotting.

2.4. Radioimmunoassay (RIA) and radioligand receptor assays (RRA)

The chloramine T method was used for radioiodination [20]. The RIA was performed using [^{125}I]hCG and anti-hCG antibodies as described previously [14]. All solutions for RIA were made in 50 mM PBS, pH 7.4 containing 0.1% BSA. The incubation mixture (300 μl) contained 30 000 cpm of [^{125}I]hCG, anti-hCG antibody (1:1 000 000 dilution) and varying concentrations of hCG or its mutants. The incubation was performed overnight at 4°C after which the amount of bound [^{125}I]hCG was determined by precipitation of the antigen-antibody complex with 10% PEG-8000. The RRA was carried out using [^{125}I]hCG and the crude rat ovarian LH/CG receptor essentially as described previously [21]. The pseudo pregnant ovaries and ovarian receptor were prepared as described earlier [22]. The incubation mixture (300 μl) contained 30 000 cpm of [^{125}I]hCG and 100 μl of the crude hCG/LH receptor suspension. The incubation was done overnight at 4°C in the absence or presence of varying concentrations of either wild type or mutant hCG. The ligand-receptor complex was precipitated by 10% PEG-8000 using γ-globulin as a carrier protein.

2.5. Secretion of hCG mutants

Ten ml of High Five cells were co-infected with the α and β recombinant viruses. Then, 1-ml aliquots were taken out at different time intervals and centrifuged at 5000 revs./min for 5 min. The supernatant and the pellet were used to determine the amounts of the extra- (secreted) and intra-cellular hormone respectively. The pellet was suspended in 0.5 ml of 50 mM Tris–HCl buffer, pH 8.0, and the reaction mixture was homogenized. The homogenate was centrifuged at 10 000 revs./min for 5 min. Both supernatants were analyzed for the hormone by radioreceptor assay.

2.6. Determination of cAMP and progesterone

MA-10 cells were used for these assays [12]. The cells were grown in 50 mm dishes and washed twice with Waymouth's medium (Gibco) containing 0.1% BSA. The cells were incubated in 1 ml of the medium containing 0.5 mM 3-isobutyl methyl xanthine (IBMX)

with varying concentrations of hCG or its mutants for 1 h at 37°C. After washing three times with ice-cold PBS containing 0.5 mM IBMX, the cells were scraped in 0.5 ml of 5 mM EDTA in 0.1 M HCl containing 0.5 mM IBMX and the plates were rinsed with 0.2 ml of the solution. The cell lysates were then boiled for 5 min and centrifuged at 15000 revs./min for 15 min. After neutralization with 70 μl of 1 M Tris–HCl (pH 9.5) and centrifugation, 50-μl aliquots of the supernatants were used directly for cAMP estimation by cAMP binding assay using [^{3}H]cAMP and cAMP binding protein provided with the assay kit (Amersham). For progesterone assay, the cells were pre-washed and incubated with varying amounts of hCG or its mutants for 4 h at 37°C with the same medium without IBMX. Subsequently, the medium was collected and small aliquots were used directly for progesterone estimation by radioimmunoassay using [^{125}I]progesterone and anti-progesterone antibody supplied in the assay kit (ICN).

2.7. Dissociation studies of rehCG and the mutant, $hCG\alpha_1\beta$

To a 200 μl sample of rehCG (15 ng/μl) or its mutant in 50 mM Tris–HCl, pH 8.0, was added 400 μl of 0.2 M glycine–HCl, pH 2.4, containing 0.15 M KCl to adjust the final pH of the solution to 3.0. The reaction mixture was kept at 23°C. Aliquots of 80 μl were removed at various time intervals ranging from 0 min to 120 min and immediately neutralized to pH 7.5 by the addition of 1 M Tris–HCl, pH 9.5. The rate of dissociation of the rehCG or its mutant, $hCG\alpha_1\beta$ was monitored by following the appearance of hCGβ and/or the disappearance of the rehCG or the mutant by immunoblotting using rabbit anti-hCGβ antibody and by measuring hCGβ by an hCGβ-specific RIA using [^{125}I]hCGβ and a highly specific monoclonal antibody against hCGβ, B158 [13].

3. Results

3.1. Construction of baculovirus transfer expression vectors containing mutated hCGα and hCGβ cDNAs, pVL1392-hCGα cDNAs and pVL1393-hCGβ cDNAs

In order to evaluate the role of β-hairpin and two other loops on the receptor binding and signal transduction of hCG, a series of hCGα and hCGβ mutants were prepared by PCR methodology. hCGα cDNA or hCGβ cDNA was used for mutagenesis by two sequential PCRs using Ultima DNA Polymerase (Perkin Elmer). Various residues in the β-hairpin loops which were modified specifically by site directed mutagenesis are shown in Fig. 1. In the first step as shown in Fig. 2A, two PCRs were carried out and the products of both PCR were used as templates to perform the secondary PCR. The secondary PCR product containing hCGα cDNA was subcloned into pVL1392 at *Eco*RI and *Bam*HI sites (Fig. 2B) and that containing hCGβ cDNA was subcloned into pVL1393 at *Eco*RI and *Bgl*II sites (Fig. 2C). α18Phe and α74Phe in β-hairpin loops α_1 and α_3 were replaced by Thr and α45 Lys in loop α_2 was substituted by Asp (Fig. 1). In the β-subunit, the loops β_1, β_2 and β_3 were replaced by corresponding hFSH sequences (Fig. 1). The presence of these mutations in hCGα and hCGβ sequences and the absence of any undesirable mutations were verified by nucleotide sequencing using dideoxy chain termination method [17]. The recombinant viruses containing the mutated hCGα or hCGβ cDNAs were generated by transfection of Sf9 insect cells with transfer vectors containing mutated hCGα or hCGβ cDNAs and AcMNPV linear DNA using cationic liposomes method. Five to ten plaques for each construct, visually identified on agarose overlay were screened for the expression of hCGα or hCGβ mutants by RIA using [^{125}I]hCGα and anti-hCGα or [^{125}I]hCGβ and anti-hCGβ, respectively. The recombinant viruses giving the highest level of expression were subsequently used for the expression of the heterodimers.

3.2. Expression and secretion of hCG mutants

In order to prepare hCG mutants modified in the loops in the α and β subunits, the High Five cells were co-infected with recombinant mutant viruses containing mutants of α or β loops, α_1, α_2 and α_3 or β_1, β_2 and β_3

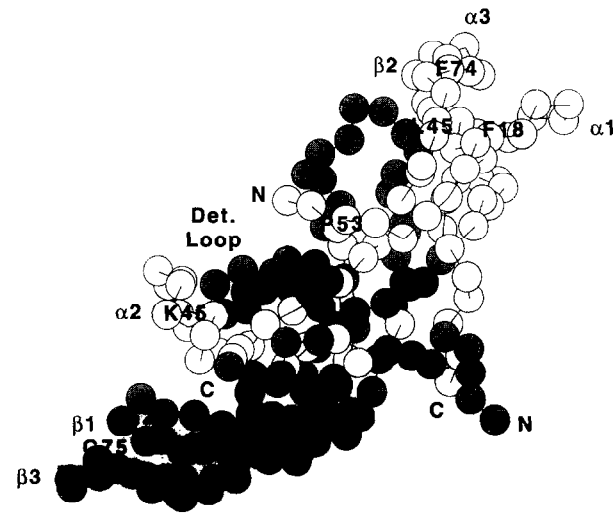


Fig. 1. Structure of hCG using Imdad program, hCGα (red) and hCGβ (green). Residues mutated in the six loops are shown in the figure. F18, K45, F74 in the α_1, α_2 and α_3 loops were mutated to TTD, respectively. G22, P24, V25 in β_1 loop, 45LOGVLPALP53 (●●●●) in β_2 loop and P73, R74, G75 in β_3 loop were substituted by ERF, YKNPARPLI and AHH, respectively.

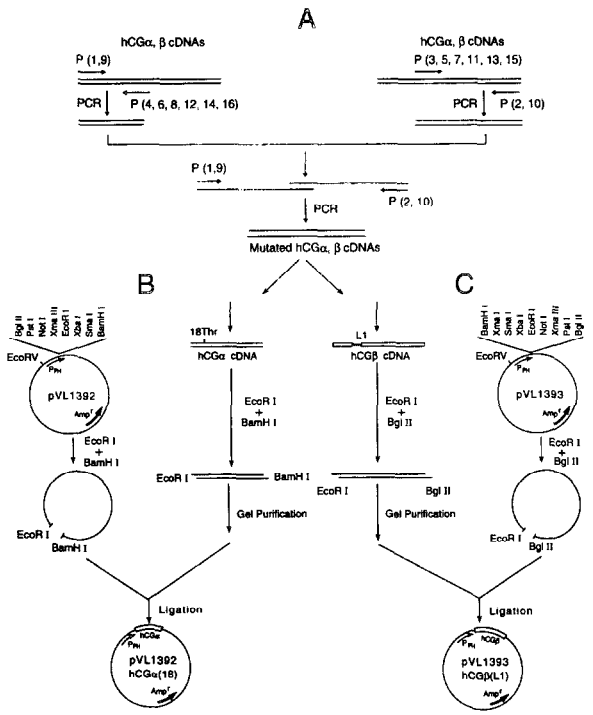


Fig. 2. (A) Strategy employed in PCR. The numbers represent the primers (Table 1) used in the preparation of the mutated hCGα or β cDNAs. (1) 5'CGC GGA ATT CAA TGG ATT ACT ACA 3', (2) 5'CGC GGA TCC GCA GTG GAA CAA GCT 3', (3) 5'AA AAC CCA TTC ACA TCC CAG CCG GGT G 3', (4) 5'CGG CTG GGA TGT GAA TGG GTT TTC C 3', (5) 5'AGG TCC AAG GAC ACG ATG TTG GTC C 3', (6) 5'CAA CAT CGT GTC CTT GGA CCT TAG 3', (7) 5'ATG GGG GGT ACT AAA GTG GAG AAC 3', (8) 5'CTC CAC TTT AGT ACC CCC CAT TAC 3', (9) 5'CGG AAT TCC TTT GCT CGG GTC ACG 3', (10) 5'GAA GAT CTG CGG ATT GAG AAG CCT 3', (11) 5'TGT GGA GAA GGA GGA ATG CCG ATT CTG CAT CAC CGT CAA CAC C 3', (12) 5'GAC GGT GAT GCA GAA TCG GCA TTC CTC CTT CTC CAC AGC CA 3', (13) 5' AAG AAC CCA GCA AGA CCT CTT ATC CAG GTG GTG TGC AAC TAC 3', (14) 5'ATA AGA GGT CTT GCTGGG TTC TTG TAC ACG CGG GTC ATG GTG G 3', (15) 5'CTC CCT GGC TGC GCA CAT CAT GTG AAC CCC GTG GTC TCC 3', (16) 5'ACC ACG GGG TTC ACA TGA TGT GCG CAG CCA GGG AGC CGG ATG 3'. (B) Construction of transfer vector, pVL1392-hCGα cDNA containing hCGα cDNA mutated at nucleotides corresponding to amino acid residue 18. Other transfer vectors of hCGα cDNA mutated at amino acids 45 and 74 were made similarly. See the text for details. (C) Construction of transfer vector, pVL1393-hCGβ cDNA containing hCGβ cDNA mutated at nucleotides corresponding to amino acids in hairpin loop 1 by those of hFSH. Other transfer vectors of hCGβ cDNA mutated at hairpin loops 2 and 3 were made in the same manner. See the text for details.

and the wild type hCGβ or hCGα virus, respectively. The co-infection of the cells with wild type hCGα and β viruses was also carried out as a control. The various mutants with mutations in the α and β loops are designated as $hCG\alpha_1\beta$, $hCG\alpha_2\beta$ and $hCG\alpha_3\beta$ and $hCG\alpha\beta_1$, $hCG\alpha\beta_2$ and $hCG\alpha\beta_3$, respectively. The expression of the heterodimers was monitored by Western immunoblotting of the culture media (Fig. 3) using a mixture of rabbit anti-hCG and anti-hCGβ and by an RIA using [^{125}I]hCG and anti-hCG. The various mutant preparations thus obtained were found to be contaminated with free β-subunit as shown by their immunoblots (Fig. 3A, lanes 3–5; Fig. 3B, lanes 5–7). The level of the free β-subunit in the mutant preparations was considerably reduced when 10 to 20% excess of α virus was used for co-infection with the β virus.

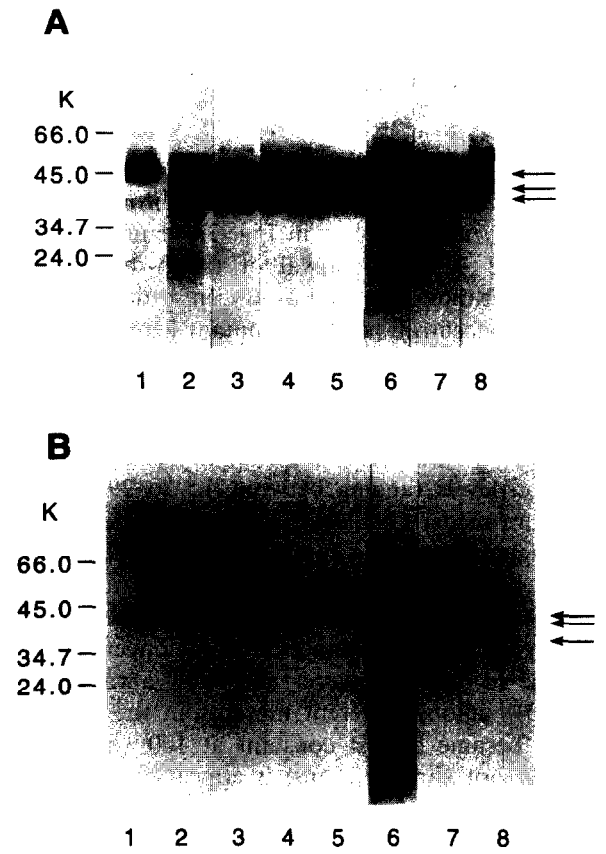


Fig. 3. (A) Immunoblots of hCG mutants, $hCG\alpha_1\beta$, $hCG\alpha_2\beta$ and $hCG\alpha_3\beta$. Thirty μl of the culture media before and after passing through an immunoaffinity column of hCGβ-specific monoclonal antibody, B158, were run on SDS-PAGE in 12% polyacrylamide gel. After transferring to nitrocellulose membrane, the protein bands were visualized using rabbit anti-hCG antibody and goat anti-rabbit IgG-conjugated alkaline phosphatase. Lane 1, rehCG; lane 2, rehCGβ; lanes 3, 4 and 5, $hCG\alpha_1\beta$, $hCG\alpha_2\beta$ and $hCG\alpha_3\beta$ before and lanes 6, 7 and 8, $hCG\alpha_1\beta$, $hCG\alpha_2\beta$ and $hCG\alpha_3\beta$ after passing through the immunoaffinity column, respectively. Three arrows from top to bottom represent the heterodimer, native hCGβ, and recombinant hCGβ respectively. (B) Immunoblots of hCG mutants, $hCG\alpha\beta_1$, $hCG\alpha\beta_2$ and $hCG\alpha\beta_3$. Thirty μl of the culture media before and after passing through an immunoaffinity column of hCGβ specific monoclonal antibody, B158, were run on SDS-PAGE in 12% polyacrylamide gel. After transferring to nitrocellulose membrane, the protein bands were visualized by using rabbit anti-hCG and goat anti-rabbit IgG-conjugated alkaline phosphatase. Lanes 1 and 8, rehCGβ and rehCG, respectively; lanes 2, 3 and 4, $hCG\alpha\beta_1$, $hCG\alpha\beta_2$ and $hCG\alpha\beta_3$ after and lanes 5, 6 and 7, before passing through the immunoaffinity column, respectively. Three arrows from top to bottom represent the heterodimer, native hCGβ, and recombinant hCGβ, respectively.

Table 1
Receptor binding and signal transduction properties of the mutants of the β-hairpin loops in the α- or β-subunits of human choriogonadotropin

Mutants hCG	Expression levels (μg/ml)[a,b]	% Binding activity[b]	% cAMP activity[b]	% Progesterone activity[b]
αβ	33.2	100	100	100
$\alpha_1\beta$	12.2	213[c]	153[c]	156[c]
$\alpha_2\beta$	5.3	76	79	80
$\alpha_3\beta$	29.3	88	93	88
$\alpha\beta_1$	17.6	87	94	84
$\alpha\beta_2$	20.4	76	85	80
$\alpha\beta_3$	4.5	84	86	99

[a]Determined by RIA.
[b]Average of two experiments.
[c]Average of three experiments. Variation between two experiments was less than 10%.

The free β-subunit could be removed from the mutant preparations by immunoaffinity chromatography as shown by immunoblots in Fig. 3A, lanes 6–8; Fig. 3B, lanes 2–4. The removal of the β-subunit from these preparations was necessary in order to determine the concentration of the mutant heterodimers by RIAs, using [^{125}I]hCG and anti-hCG. The dose response curves of the mutants were parallel to that of the rehCG indicating that they all shared common epitopes. The mutants were over-expressed with the expression levels ranging between 4.5 to 29 μg/ml of the culture media (Table 1). The high expression levels strongly suggest that the subunit assembly was not affected by the mutations. The modification of the loops also did not have any significant effect on the secretion of the heterodimers over a longer time period. The secretion, however, did show a variation of 30 to 60% among different mutants in the first 12 h P.I. but became almost constant at 100% after 24 h P.I. (data not shown). Thus, it appears that after 24 h P.I., the mutant heterodimers were secreted as soon as they were synthesized and only very little if any were retained intracellularly.

3.3. Effect of modification of the loops in the α- and β-subunits of hCG on its receptor binding

The receptor binding activity of the mutants hCG$\alpha_1\beta$, hCG$\alpha_2\beta$, hCG$\alpha_3\beta$ and hCG$\alpha\beta_1$, hCG$\alpha\beta_2$ and hCG$\alpha\beta_3$ was determined by radioreceptor assays using [^{125}I]hCG and rat ovarian membrane suspension. The mutant hCG$\alpha_1\beta$ in which Phe18 was replaced in the α_1 loop by Thr had about 200% increase in its receptor binding activity as compared with rehCG (Fig. 4A). This result was highly reproducible. All other mutants did not show any significant change in their receptor binding activity (Fig. 4A and B). It appears from the data that the β-hairpin loops were not directly involved in the receptor binding. However, the effect of mutation of α18Phe on receptor binding would suggest that α_1 loop might be in the vicinity of the binding site. The data on the receptor binding activity of the mutants are summarized in Table 1. The increase in the activity of the mutant involving the α_1 loop was probably due to a conformational change as discussed below.

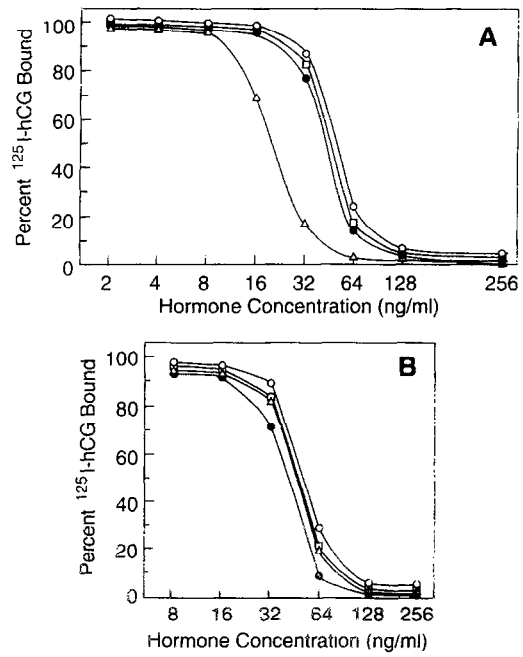


Fig. 4. (**A**) Radioligand receptor assay of hCG mutants, hCG$\alpha_1\beta$, hCG$\alpha_2\beta$ and hCG$\alpha_3\beta$. About 100 ng of the crude LH/CG receptor from rat ovary was incubated with 30 000 cpm of [^{125}I]hCG with varying concentrations of the recombinant hCG (●), hCG$\alpha_1\beta$ (△), hCG$\alpha_2\beta$ (○) and hCG$\alpha_3\beta$ (□) overnight at 4°C. The bound [^{125}I]hCG was precipitated with PEG-8000 and the radioactivity was counted by a Beckman gamma counter. (**B**) Radioligand receptor assay of hCG mutants, hCG$\alpha\beta_1$, hCG$\alpha\beta_2$ and hCG$\alpha\beta_3$. About 100 ng of the crude LH/CG receptor from rat ovary was incubated with 30 000 cpm of [^{125}I]hCG with varying concentrations of recombinant hCG (●), hCG$\alpha\beta_1$ (△), hCG$\alpha\beta_2$ (○) and hCG$\alpha\beta_3$ (□) overnight at 4°C. The bound [^{125}I]hCG was precipitated with PEG-8000 and measured by a Beckman gamma counter.

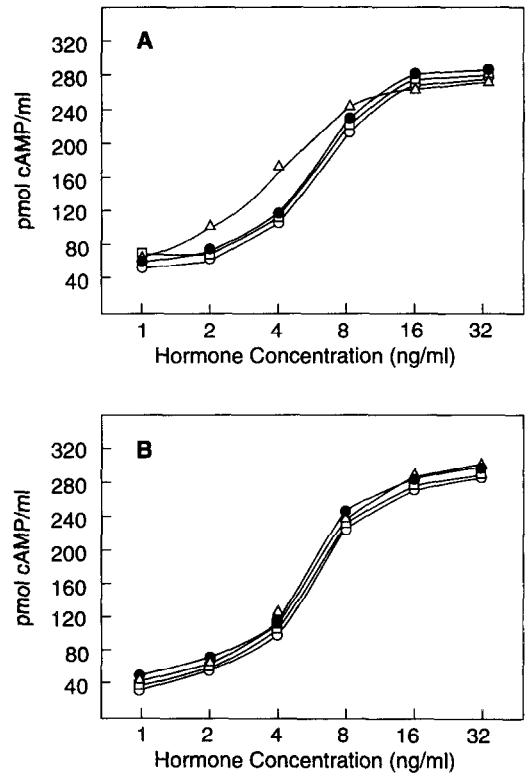


Fig. 5. (A) Stimulation of cAMP by hCG mutants, $hCG\alpha_1\beta$, $hCG\alpha_2\beta$ and $hCG\alpha_3\beta$ in Leydig MA-10 cells. The concentration-dependent stimulation of cAMP in MA-10 cells by hCG (●), $hCG\alpha_1\beta$ (△), $hCG\alpha_2\beta$ (○) and $hCG\alpha_3\beta$ (□) was determined by incubating the cells with various concentrations of hCG, $hCG\alpha_1\beta$, $hCG\alpha_2\beta$ and $hCG\alpha_3\beta$ at 37°C for 1 h. The cAMP produced was measured by protein binding assay. (B) Stimulation of cAMP by hCG mutants, $hCG\alpha\beta_1$, $hCG\alpha\beta_2$ and $hCG\alpha\beta_3$ in Leydig MA-10 cells. The concentration dependent stimulation of cAMP in MA-10 cells by hCG (○), $hCG\alpha\beta_1$ (△), $hCG\alpha\beta_2$ (○) and $hCG\alpha\beta_3$ (□) was determined by incubating the cells with various concentrations of hCG, $hCG\alpha_1\beta$, $hCG\alpha_2\beta$ and $hCG\alpha_3\beta$ at 37°C for 1 h. The cAMP produced was measured by protein binding assay.

3.4. Effect of modification of loops in the α and β-subunits of hCG on its signal transduction

The results of signal transduction studies which included the determination of cAMP and progesterone stimulation by the mutants $hCG\alpha_1\beta$, $hCG\alpha_2\beta$, and $hCG\alpha_3\beta$ and $hCG\alpha\beta_1$, $hCG\alpha\beta_2$, and $hCG\alpha\beta_3$ almost paralleled their receptor binding properties. The mutant hCGα1β in which Phe18 was substituted by Thr had almost 150 to 200% higher activity in the stimulation of cAMP and progesterone as compared with rehCG (Table 1, Fig. 5A and Fig. 6A). All other mutants did not show any alteration in their cAMP- or progesterone-stimulating activities (Table 1, Fig. 5A and B and Fig. 6A and B) due to the mutations. Thus, the data clearly demonstrate that the loop structures were not directly involved in signal transduction. This is particularly true in the case of the β-subunit in which almost the entire loop sequences were replaced by those of the corresponding hFSH sequences.

3.5. Effect of pH on the dissociation of the mutant, $hCG\alpha_1\beta$

Since the only mutation involving the replacement of α18 Phe with Thr in α_1 loop had an effect on the receptor binding and post-receptor signaling events, studies were carried out to determine the probable cause for this increase at a molecular level. It has been previously reported that a conformational change could result in reducing the affinity between the subunits [23]. The subunit interaction or affinity between the subunits was measured by studying the rate of dissociation of the mutant compared with the rehCG. The dissociation of $hCG\alpha_1\beta$ was carried out at 23°C at pH 3.0. The rate of dissociation was followed by monitoring the release of the β-subunit (Fig. 7) and/or the disappearance of

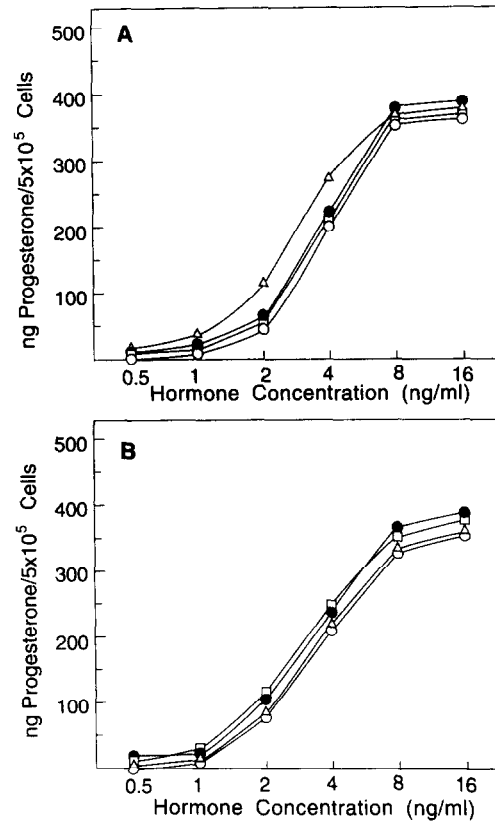


Fig. 6. (A) Effect of hCG mutants, $hCG\alpha_1\beta$, $hCG\alpha_2\beta$ and $hCG\alpha_3\beta$ on the steroidogenesis in MA-10 cells. MA-10 cells were exposed to varying concentrations of hCG (●), $hCG\alpha_1\beta$ (△), $hCG\alpha_2\beta$ (○) and $hCG\alpha_3\beta$ (□) at 37°C for 4 h. The progesterone produced in the medium was estimated by radioimmunoassay using [^{125}I]progesterone and anti-progesterone antibody. (B) Effect of hCG mutants, $hCG\alpha\beta_1$, $hCG\alpha\beta_2$ and $hCG\alpha\beta_3$ on the steroidogenesis in MA-10 cells. MA-10 cells were exposed to varying concentrations of hCG (●), $hCG\alpha\beta_1$ (△), $hCG\alpha\beta_2$ (○) and $hCG\alpha\beta_3$ (□) at 37°C for 4 h. The progesterone produced in the medium was estimated by radioimmunoassay using [^{125}I]progesterone and anti-progesterone antibody.

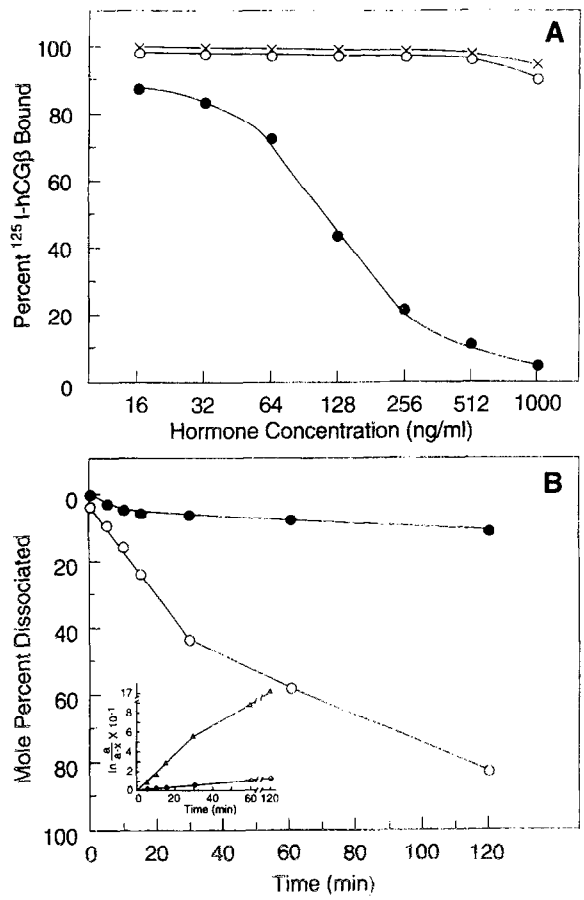


Fig. 7. (**A**) Radioimmunoassay of hCGβ subunit, rehCG and hCG mutant hCG$\alpha_1\beta$. Varying concentration of hCGβ (●), rehCG (○) and hCG$\alpha_1\beta$ (x) were incubated with 30 000 cpm of [^{125}I]hCGβ and the monoclonal anti-hCGβ antibody, B158, in 300 μl of 50 mM PBS, pH 7.4 containing 0.1% BSA. The bound and free tracer was separated by precipitation with PEG 8000. (**B**) Dissociation of rehCG (●) and its mutant hCG$\alpha_1\beta$ (○) into its subunits at pH 3 at 23°C. Dissociation at different time intervals was monitored by measuring the appearance of hCGβ by an hCGβ specific radioimmunoassay. See the text for details. Inset: A plot between ln $a/a-x$ and time (min) for the mutant (–△–) and rehCG (–●–) where a is the initial concentration and x is the amount dissociated at a certain time.

the heterodimer by Western immunoblotting (data not shown) using anti-hCGβ and anti-hCG antibodies. Similar studies were carried out with rehCG as a control. While dissociation of the rehCG did not begin until almost an hour, the mutant, hCG$\alpha_1\beta$ dissociated more than 50% during the same period (Fig. 7B). The rate of dissociation was quantitated by measuring the appearance of the β-subunit by RIA using a β-subunit specific monoclonal antibody, B158 and [^{125}I]hCGβ. Fig. 7A shows the dose-response curve for hCGβ. The rehCG as well as the mutant heterodimer did not show any cross-reactivity with the antibody. Fig. 7B is the percent dissociation of the mutant and rehCG as measured by the release of the β-subunit. In 2 h, about 80% of the mutant was dissociated while in the same period only about 10% of the rehCG was dissociated. The rate constant, k_m, for the mutant, 4.1×10^{-2} min^{-1}, was one order of magnitude greater than that of the rehCG, $k_w = 4.6 \times 10^{-3}$ min^{-1} (Fig. 7B inset). This indicates weaker affinity of binding of the subunits in the mutant probably due to a conformational change caused by the mutation. The change in conformation, in turn, might lead to the greater accessibility of the receptor binding site of the hormone and thereby enhanced receptor binding and signal transduction activity.

4. Discussion

According to the three dimensional structure of hCG determined by X-ray diffraction, the α and β subunit each has three loops, α_1, α_2, and α_3, and β_1, β_2 and β_3, respectively [7,8]. The objective of the present studies was to investigate the role of these loops in the subunit assembly, folding, receptor binding and signal transduction function of the hormone. The mutations were designed to bring about radical changes in the loops without risking the proper folding and assembly of the subunits. Consequently, single residue radical mutations in the α loops and replacement of the β loops with the analogous sequences in hFSH were carried out (Fig. 1). It is clear from the receptor binding data that both folding and subunit assembly were not affected. It may be pointed out that these mutations with the exception of β_2 loop have not been previously investigated. The present data clearly demonstrate that unlike NGF [9] and TNF [10,11] which have similar hairpin loop structures, these loops are not directly involved in receptor binding or signal transduction. With the exception of the mutant hCG$\alpha_1\beta$ in which α18Phe was replaced with Thr, all other mutants with the mutations in α_2 (α45Lys to Asp) and α_3 (α74Phe to Thr) and in β_1, β_2 and β_3, with corresponding hFSH amino acid residues, did not have any effect on the physicochemical and biological properties of hCG. The replacement of α18Phe caused a two-fold increase in the receptor binding (Fig. 4A) and cAMP (Fig. 5A) and progesterone stimulation (Fig. 6A). This increase in the receptor binding and signaling activity appears to be due to a conformational change in the heterodimer caused by the mutation. The evidence in support of this conclusion is derived from dissociation studies of the mutant at low pH [23]. The dissociation rate of the mutant, hCG$\alpha_1\beta$ at pH, 3.0 was one order of magnitude greater than that of the wild type hCG (Fig. 7B inset). The enhanced dissociability of the mutant relative to rehCG implies weaker interaction between the two subunits. This result is consistent with the structure of hCG determined by X-ray diffraction [7,8]. The α and β subunits each have a hydrophobic core which is reported to stabilize the loop structures in each subunit of hCG [7,8]. The α-subunit

core consists of α17Phe, α18Phe, α25Ile, α68Val, α70Val, α71Met, α74Phe and α76Val. All three phenylalanines in the α-core are clustered together and are partially exposed to the surface. There are hydrophobic contacts between the triplet phenylalanine cluster and the β-subunit residues, β44Val and β45Leu. The replacement of α18Phe by a hydrophilic residue, Thr, probably results in weakening its interaction with β44Val and β45Leu and thereby weakening the subunit association. This would explain the higher dissociability of the mutant containing α18Thr in place of Phe. We believe that its enhanced receptor binding activity is due to a short range conformational change affecting the receptor binding site probably located in close proximity to α18Phe, although the possibility of a long range conformational effect cannot be ruled out. Moyle et al. [24] have proposed, by epitope mapping of the hormone when it is complexed with the receptor, that the receptor binding site is located on loops one and three of the β-subunit in the groove created between the two subunits of the hormone. This proposal can only be reconciled by our data if one assumes the structure of the heterodimer like a pair of 'scissors', in which the two blades and two handles representing loops α_1 and α_3 and β_1 and β_3, respectively, on either side of the hinge, i.e. two cystine knots superimposed on each other, represent two α loops, α_1 and α_3 with β_2 loop on one side and two β loops, β_1 and β_3 with α_2 loop on the other side (Fig. 1). The weakening of affinity between the two subunits caused by α18Phe mutation to Thr on one side of the cystine knot would weaken the interaction between β_1 and β_3 loops and α_2 loop on the opposite side by a long range conformational change and thus render the receptor more accessible to the hormone. It is interesting to note that the mutant hCG$\alpha_1\beta_2$ in which the β_2 loop sequence was replaced with that of the corresponding hFSH sequence, had a greater conformational change as indicated by alteration in its cross-reactivity with anti-hCG antibody (data not shown). In this mutant, β45Leu in hCGβ was replaced with Tyr. Tyrosine being less hydrophobic than Leu due to the presence of phenolic hydroxyl caused a weakening of the affinity between the two subunits. It is surprising that the replacement of α74Phe in α_3 loop, which is also a part of the phenylalanine triplet, to Thr did not affect the receptor binding and signaling activity of the hormone. Although the three dimensional structure of hCG by X-ray diffraction predicted the cluster of triplet phenylalines to be partly exposed, it appears from our data that α74Phe must be exposed considerably more than α18Phe since its replacement with Thr did not significantly affect the receptor binding or signal transduction. The precise orientation of α17Phe is not known. The mutation of α45Lys to Asp, one of the residues in the helical segment in loop 2 in the α-subunit had no effect on the receptor binding and signal transduction. Although it was a radical mutation, it apparently did not disrupt the helical structure. In contrast, the replacement of the adjoining α44Lys to Ala drastically reduced the receptor binding and signal transduction activity [25].

The mutants in which the β_1, β_2 and β_3 loops of hCGβ were replaced with the corresponding hFSHβ sequences had no significant effect on the receptor binding or signal transduction functions of the hormone. In the β_1 loop, β22Gly, β24Pro and β25Val were replaced by Glu, Arg, and Phe, respectively, the residue 23 being cystine in hCG. Although two of these mutations were radical, they did not affect the biological properties of the hormone. Thus, it appears that these residues neither are near or in the receptor and subunit binding sites nor do they affect the conformation of these sites. The lack of involvement of these residues in the subunit assembly is in agreement with the X-ray diffraction structure of hCG [7,8]. The replacement of β73Pro, β74Arg and β75Gly in loop 3 by Ala, His and His, respectively, also did not affect the receptor binding and signal transduction. Although, according to the crystal structure of hCG, these residues form hydrogen bonds with α75, α76 and α77, the formation of the heterodimer was not affected by these mutations. It may be noted β73Pro and β75Gly are conserved in various mammalian LH β-sequences [1] but their replacement did not result in any change in the in vitro biological properties of the hormone. The sequence of the long β_2 loop, ^{45}LeuGlnGlyValLeuProAlaLeuPro^{53} was replaced by the hFSH sequence ^{45}TyrLysAsnProAlaArgProLeuIle^{53}. The leucine residue at position 52 was not substituted by the corresponding Lys in hFSH. The receptor binding and signaling activities of the hormone were not affected by this mutation although this sequence is a part of the β38 to β57 sequence which was previously reported by Keutman et al. [26] to be active in receptor binding and steroid stimulation in their synthetic peptide studies. Our data are in agreement with that of Moyle et al. [24,27,28] who replaced the entire loop (β38 to β57) sequence with the corresponding hFSH sequence and did not find any loss of receptor binding activity of the hormone. The replacement of β45Leu to Tyr in β_2 loop however, did cause a conformational change as described above in the mutant hCG$\alpha_1\beta_2$ in which α18Phe was also replaced with Thr (data not shown).

In summary, unlike NGF and TNF, the three β-hairpin loops in each of the α and β subunits were not directly involved in receptor binding and signal transduction. The replacement of α45Lys to Asp in the helical segment in the α-subunit had no effect on the function of the hormone. Subunit assembly and secretion of the heterodimers by these mutations in β-hairpin loops were also not affected. The mutation of α18Phe to Thr in loop α_1 increased the receptor binding

and post-receptor signaling activity two-fold. This increase was attributed to a short-range conformational effect on the receptor binding site which is believed to be located in close proximity to α18Phe.

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