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Effect of modification of the β-hairpin and long loops simultaneously in both α- and β-subunits on the function of human choriogonadotropin: part II

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Abstract

According to the X-ray diffraction, human choriogonadotropin has four β-hairpin and two long loops, equally distributed in each of the α and β subunits. Radical mutations such as the replacement of α18Phe and α74Phe with Thr in the α_1 and α_3 loops respectively and the replacement of α45Lys with Asp in the α_2 loop in the α-subunit were introduced while the loop sequences in the β-subunit were replaced with the corresponding sequences in hFSHβ. Nine different double mutants with simultaneous mutations in both the α and β loops including hCG$\alpha_1\beta_1$, hCG$\alpha_1\beta_2$, hCG$\alpha_1\beta_3$, hCG$\alpha_2\beta_1$, hCG$\alpha_2\beta_2$, hCG$\alpha_2\beta_3$, hCG$\alpha_3\beta_1$, hCG$\alpha_3\beta_2$ and hCG$\alpha_3\beta_3$ were partially purified from insect High-Five cells. As previously reported (Shao et al., 1996, Mol. Cell. Endocrinol. 122, 173–182), the mutation in the α_1 loop in the mutant, hCG$\alpha_1\beta$, the mutants hCG$\alpha_1\beta_1$ and hCG$\alpha_1\beta_3$ caused 200% increase in the receptor binding, cAMP and progesterone stimulation. The mutant, hCG$\alpha_1\beta_2$ and all other mutants behaved like the recombinant hCG (rehCG) in the receptor binding and post-receptor signaling activities. The molecular cause for this increase is probably due to a conformational change in the heterodimers caused by the mutation in the α_1 loop. This conclusion is based on the results of the dissociation studies of the mutants heterodimers which indicated a decreased affinity between the subunits. The first order rate constants for the dissociation of the mutants hCG$\alpha_1\beta_1$, hCG$\alpha 1\beta_2$ and hCG$\alpha_1\beta_3$ were 3.7×10^{-2} min^{-1}, 1.4×10^{-2} min^{-1} and 4.6×10^{-2} min^{-1} respectively, as compared with 4.6×10^{-3} min^{-1} for the rehCG. It seems from the data that α18Phe is located in, or in proximity to the receptor binding site and probably plays a critical role in maintaining either directly or indirectly its conformational integrity. © 1997 Elsevier Science Ireland Ltd.

Keywords: Choriogonadotropin; Site directed mutagenesis; Receptor binding; Signal transduction

1. Introduction

Human chorionic gonadotropin (hCG) is one of the members of a family of glycoprotein hormones which also includes lutropin (LH), follitropin (FSH) and thyrotropin (TSH). All are structurally similar comprised of two noncovalently associated subunits designated as α and β [1]. Despite differences in the tissues of their synthesis and in their functional properties, they all share the same α-subunit [1]. The molecular mechanism of their action involves an initial interaction with the seven transmembrane domain type receptors followed by the activation of adenylate cyclase [2,3] and possibly phospholipase C [4–6]. A number of studies based on the chemical modification [7,8], site directed mutagenisis [9,10] and the use of synthetic peptides [11,12] have been carried out to identify the receptor binding domain of hCG. The importance of the inter-cysteine loop sequence in the β-subunit between residues 93 and 100 was first recognized by Ward and Moore [13], who proposed that variable charge in the loop could act as

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

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a determinant of specificity, positive for hCG/LH and negative for FSH and TSH. The importance of the determinant loop was demonstrated by constructing chimeras of hCG and FSH [14,15]. It was found that the hCG/LH receptor binding activity was reduced when the determinant loop sequence in hCG was replaced by the corresponding sequence in FSH. Synthetic peptide approach also supported this finding [11,12]. Another inter-cysteine loop sequence between residues β38 and β57 was found to have receptor binding and progesterone stimulating activity [16]. However, when this sequence in hCG was replaced by the corresponding FSH sequence, no change in the receptor binding activity of the hormone was observed [14] indicating that this sequence may not contain the receptor binding site. By a similar approach as used to study the receptor binding site in the β-subunit, two sequences, α88–92 at the carboxy-terminus [17] and α40–50 [18,19], were found to be involved in receptor binding. The removal of the carboxy-terminal α88–92 essentially eliminated the receptor binding activity of the hormone [17]. According to the structure of hCG as determined by X-ray diffraction, the residues α40–50 are adjacent to the 'determinant' loop, β93-100 and this might explain its role in the receptor binding. The three dimensional structure of hCG, proposed by X-ray diffraction studies, indicates a predominant β-structure with three helical segments, two in the α- and one in the β-subunit [19,20]. The antiparallel β-strands in each of the α and β-subunits are joined by the β-hairpin and long 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 subunits contain the so-called cysteine knot motif. Similarly, the crystal structures of nerve growth factor (NGF) [21] and tumor necrosis factor (TNF) [22] have revealed the presence of cysteine knot motif and three pairs of antiparallel β-strands connected by β-hairpin loops. The crystal structure of the soluble human 55 kd TNF receptor–human TNFβ complex indicates the involvement of the β-hairpin loops in the interaction between the ligand and the receptor [23]. These findings prompted us to investigate the role of β-hairpin loops in the receptor binding and signal transduction of hCG. All three loop sequences in the β-subunit were individually substituted by the corresponding sequences in hFSHβ. Since all the α-subunits in various human hormones have identical amino acid sequences, radical single residue mutations were introduced by replacing α45Lys with Asp in the α_2 loop and αPhe18 and αPhe74 with a hydrophilic residue, Thr, in the α_1 and α_3 loops respectively. It may be noted that all these mutations in the α and β subunits with the exception of the one in β_2 loop and their effect on the hormone action have not been previously investigated except by cross-linking studies in which α45Lys was implicated in the receptor and β-subunit binding [24]. Although the effect of modification of each loop individually on the heterodimer formation, receptor binding and signal transduction has been previously reported [25], the purpose of the present investigation was to determine if the simultaneous modification of the loops in both α and β subunits will alter the receptor binding and post-receptor signaling properties of the heterodimers. In this communication, we report the preparation of the heterodimers with simultaneous mutations in the α and β loops by the coexpression of both hCGα and hCGβ mutant cDNAs in baculovirus expression system and their effect on the folding, receptor binding and signal transduction. The results of these studies indicate that the mutations did not have any effect on the subunit folding and assembly of the heterodimer although there was a reduction in their level of expression relative to the rehCG. All mutants containing the mutation in the α_1 loop except hCG$\alpha_1\beta_2$ had 2–3-fold enhancement in their receptor binding and signal transduction activities as compared with the rehCG. Thus, the simultaneous mutation in the β_1 or β_3 loop did not alter the results obtained by an individual mutation in the α_1 loop. However the mutation in the β_2 loop did have an adverse effect on the mutation in the α_1 loop in the heterodimer, hCG$\alpha_1\beta_2$. The mutant hCG$\alpha_1\beta_2$ and all other double mutants had no detectable difference in their receptor binding and signal transduction properties from those of the wild type rehCG indicating that these loop regions were not involved directly or indirectly in the receptor binding and signal transduction. It appears from the data that α18Phe is located either in or in the vicinity of the receptor binding site and plays an important role in maintaining the conformational integrity of hCG.

2. Materials and methods

The insect Sf9 and High-Five cells and mouse Leydig tumor MA-10 cells were cultured as previously described [25].

2.1. Construction of vectors, transfection, expression and partial purification of hCG mutants

The mutant cDNAs containing modified hairpin and the long loops of hCGα and hCGβ were produced by polymerase chain reaction (PCR) method using the appropriate primers as previously described [25]. The PCR products of hCGα were subcloned into the EcoRI and BamHI restriction sites in pVL 1392, and those of hCGβ were subcloned into the EcoRI and BglII sites in pVL 1393. The baculovirus linear DNA and pVL 1392-hCGα or pVL 1393-hCGβ were co-transfected [25,26,30] into insect Sf9 cells using cationic liposomes (Invitrogen). The mutant viruses were amplified in Sf9

cells but the expression of the mutants was carried out in High-Five cells. Each recombinant virus containing the mutant α or β cDNA was co-infected with the recombinant virus (2 ml) containing the mutant β or α cDNA respectively in 2×10^6 High-Five cells/ml in 20 ml of serum free medium, Ex Cell 405, at a multiplicity of infection (M.O.I.) of 5. The cells were harvested after 60 h post-infection (P.I.) 2-ml sample of each mutant expressed in insect cells was passed through a 2-ml bed volume immunoaffinity adsorbent containing monoclonal antibody, B-158 [27]. The column was eluted with two bed volumes of 50 mM Tris–HCl, pH 8.0. The flow through and the eluate were examined by Western immunoblotting [25].

2.2. Analytical techniques, immunological and biological assays and dissociation kinetics

SDS-PAGE was performed in the discontinuous gel system of Laemmli with 5% stacking gel at pH 6.8 and 12% separation gel at pH 8.8 [25]. Western immunoblotting, radioimmuno, radioreceptor assays, [28,29] and the determination of cAMP and progesterone were carried out as previously described [25]. The kinetics of dissociation of rehCG and the mutants, hCG$\alpha_1\beta_1$, hCG$\alpha_1\beta_2$ and hCG$\alpha_1\beta_3$ at pH 3 and their dissociation rate constants were determined as previously described [25].

3. Results

3.1. Expression of hCG mutants

To express hCG mutants, the High-Five insect cells were co-infected with recombinant mutant-viruses, constructed as previously described [25], containing α_1, α_2 or α_3 and β_1,β_2 or β_3 mutant cDNAs respectively. The α_1, α_2 and α_3, and β_1, β_2 and β_3 are designations of the mutated loops in the α and β subunits of hCG respectively. In the α loops, single residue radical mutations of the invariant amino acid residues in the α-subunits of the various mammalian species were introduced. In the α_1 and α_3 loops, α18Phe and α74Phe respectively were replaced with Thr, while α45Lys in α_2 was replaced with Asp. In the β loops, the sequences in all three loops were replaced with the corresponding hFSHβ sequences. The various mutations introduced in the α and β loops are given in Table 1 and Fig. 1. The following nine mutants, hCG$\alpha_1\beta_1$, hCG$\alpha_1\beta_2$, hCG$\alpha_1\beta_3$, hCG$\alpha_2\beta_1$, hCG$\alpha_2\beta_2$, hCG$\alpha_2\beta_3$, hCG$\alpha_3\beta_1$, hCG$\alpha_3\beta_2$ and hCG$\alpha_3\beta_3$ were prepared. The expression of the heterodimers was monitored by Western immunoblotting of the culture media (Fig. 2) using rabbit anti-hCG and anti-hCGβ antibodies as well as by RIA using [^{125}I]hCG and anti-hCG. As shown by their immunoblots (Fig. 2A,B,C, lanes 2–4) hCG mutants thus prepared were contaminated with the β-subunit. In order to determine the concentration of the mutants by

Table 1
Mutations* in the α and β loops of hCG

Loop	Mutation
α_1	18 Phe–Thr
α_2	45 Lys–Asp
α_3	74 Phe–Thr
β_1	22 Gly Cys Pro Val 25–Glu Cys Arg Phe
β_2	45 Leu Gln Gly Val Leu Pro Ala Leu Pro 53–Tyr Lys Asn Pro Ala Arg Pro Leu Ile
β_3	73 Pro Arg Gly 75–Ala His His

* See Ref. [25] for details.

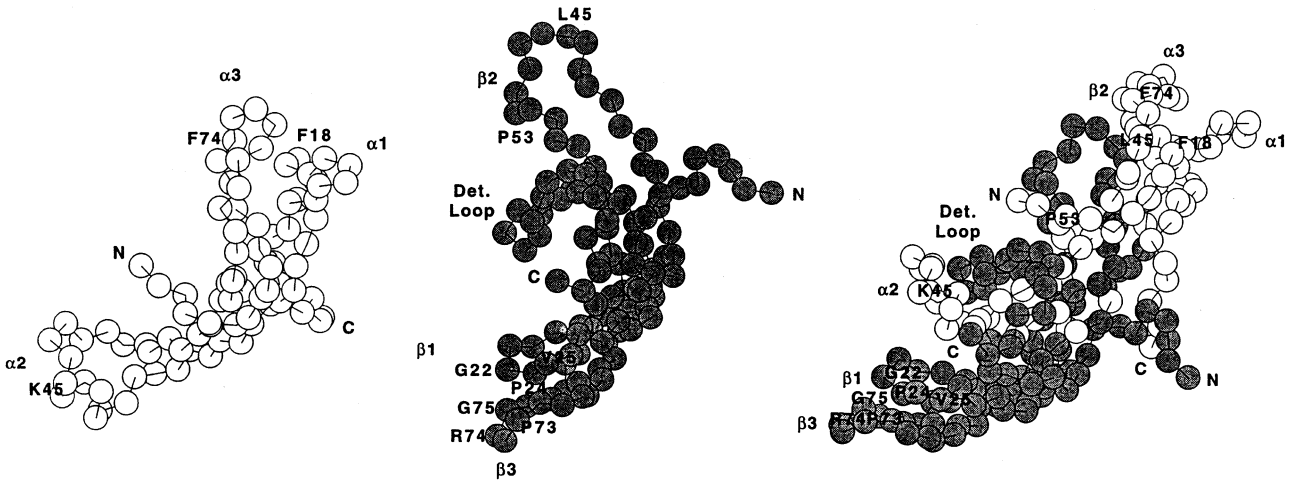


Fig. 1. Amino acid residues mutated in the β-hairpin and long loops of hCG. In the α-subunit (○), F18 and F74 in α_1 and α_3 loops respectively were replaced with T and K45 with D. Residues replaced in the β-subunit (●), 45–53, LQGVLPALP with YKNPARPLI in the long β_2 loop, G22P24V25 with ERF in β_1 loop and P73R74G75 with AHH. Relative positions of all residues mutated in hCG (○ and ●), Det. loop, determinant loop of inter-cysteine residues 93–100 in the β-subunit. Structures generated using Imdad.

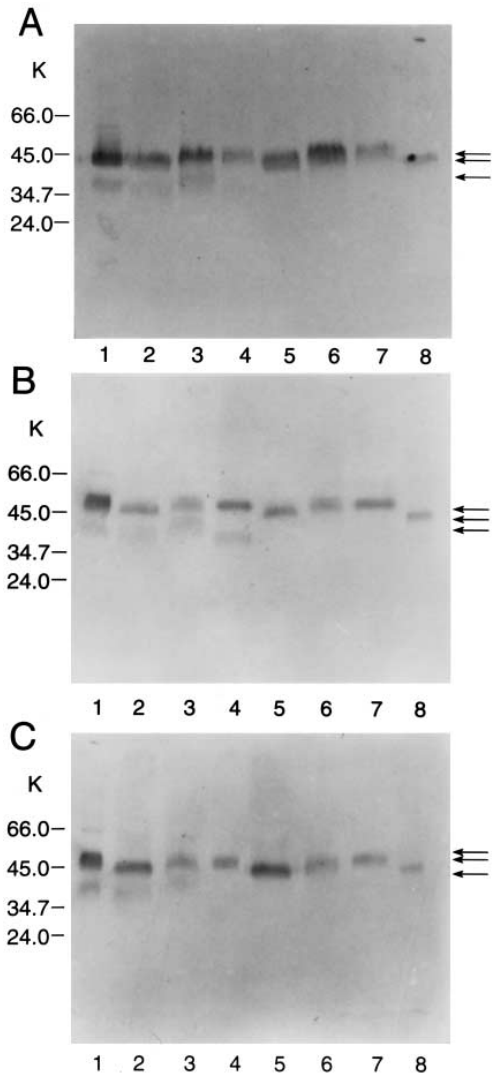


Fig. 2. Immunoblots of hCG mutants. Culture media (30 μl) before and after passing through an immunoaffinity column of hCGβ specific monoclonal antibody, B158, were run on SDS-PAGE in 12% polyacrylamide gel. After transferring to nitrocellulose membrane, the protein bands were visualized using rabbit anti-hCG and anti-hCGβ antibodies and goat anti-rabbit IgG-conjugated alkaline phosphatase. (A) lane 1, rehCG; lane 8, hCGβ; lanes 2, 3 and 4, hCG$\alpha_1\beta_1$, hCG$\alpha_1\beta_2$ and hCG$\alpha_1\beta_3$ before and lanes 5, 6 and 7, hCG$\alpha_1\beta_1$, hCG$\alpha_1\beta_2$ and hCG$\alpha_1\beta_3$ after passing through the immunoaffinity column respectively. (B) lane 1, rehCG; lane 8, hCGβ; lanes 2, 3 and 4, hCG$\alpha_2\beta_1$, hCG$\alpha_2\beta_2$ and hCG$\alpha_2\beta_3$ before and lanes 5, 6 and 7, hCG$\alpha_2\beta_1$, hCG$\alpha_2\beta_2$ and hCG$\alpha_2\beta_3$ after passing through the immunoaffinity column respectively. (C) lane 1, rehCG; lane 8, hCGβ; lanes 2, 3 and 4, hCG$\alpha_3\beta_1$, hCG$\alpha_3\beta_2$ and hCG$\alpha_3\beta_3$ before and lanes 5, 6 and 7, hCG$\alpha_3\beta_1$, hCG$\alpha_3\beta_2$ and hCG$\alpha_3\beta_3$ after passing through the immunoaffinity column respectively. Arrows from top to the bottom represent hCG, native hCGβ and rehCGβ.

RIA using [^{125}I]hCG and anti-hCG, it was necessary to remove the β-subunit because of its cross-reactivity with anti-hCG. The mutant preparations were freed of the β-subunit by passing through an immunoaffinity column of hCGβ specific monoclonal antibody, B158, which had no significant cross-reactivity with intact hCG. The removal of the β-subunit was ascertained by Western immunoblotting as shown in Fig. 2A,B,C, lanes 5–7. The dose-response curves of all mutants except for hCG$\alpha_1\beta_2$ were parallel to that of rehCG indicating that they all shared the common epitopes (data not shown). HCG$\alpha_1\beta_2$ did not cross-react with anti-hCG as well as the other mutants and rehCG. The concentration of this mutant was measured by an RIA system using [^{125}I]hCG and anti-hCGβ. In this system, the dose-response curve of hCG$\alpha_1\beta_2$ was parallel to that of the rehCG (data not shown). It appears that the simultaneous mutations in the α_1 and β_2 loops caused a conformational change in the heterodimer thereby resulting in the alteration of the epitopes. The expression levels of the various hCG mutants ranged between 1 μg/ml and 6 μg/ml, and were considerably lower than that of the wild type hCG [25].

3.2. Effect of modification of the β-hairpin and long loops in the α- and β-subunits of hCG on its receptor binding and stimulation of cAMP and progesterone

The receptor binding activities of the mutants were determined by radioligand receptor assay (RRA) using [^{125}I]hCG and rat ovarian membrane suspension [30]. Two of the three mutants of α_1, hCG$\alpha_1\beta_1$ and hCG$\alpha_1\beta_3$ had an increase in their receptor binding activity relative to rehCG by 200% and 273% respectively (Fig. 3A). The receptor binding activity of the third mutant of α_1, hCG$\alpha_1\beta_2$ and of all the other mutants of hCG remained unaltered (Fig. 3A,B,C). Two to three fold enhancement in the receptor binding activity of hCG$\alpha_1\beta_1$ and hCG$\alpha_1\beta_3$ was probably the result of a conformational change caused by the mutation in the α_1 loop which rendered the receptor binding site of the hormone more accessible to the receptor. It appears that the conformational change caused by the mutation of αPhe18 in hCG$\alpha_1\beta_2$ was offset by the mutation in the β_2 loop. Therefore, unlike other mutants containing mutated α_1, it did not have enhanced receptor binding activity.

The stimulation of cAMP by the hCG mutants was measured by estimating the amount of cAMP produced by MA-10 Leydig cells as described [25]. The dose response curves of the hCG mutants with mutation in the α_1 loop showed that the mutants hCG$\alpha_1\beta_1$ and hCG$\alpha_1\beta_3$ had almost 200–300% higher cAMP stimulation activity as compared with the rehCG while hCG$\alpha_1\beta_2$ had no significant effect on the stimulation of cAMP consistent with its receptor binding activity (Fig. 4A). All other mutants also did not show any significant effect on the stimulation of cAMP (Fig. 4B,C). Thus, the data indicate that with the exception of the mutation in α_1, the mutations in the other loops had no significant effect on the ligand induced stimulation of cAMP.

The steroidogenic activity of hCG mutants was examined by measuring their ability to stimulate MA-10 cells to produce progesterone. The results are presented in Fig. 5. Just as in cAMP stimulation, the mutants hCG$\alpha_1\beta_1$ and hCG$\alpha_1\beta_3$ had almost 200–240% increase in the stimulation of progesterone as compared with rehCG (Fig. 5A). HCG$\alpha_1\beta_2$ and all other mutants did not show any alteration in the stimulation of progesterone (Fig. 5A,B,C). It appears from the data that the

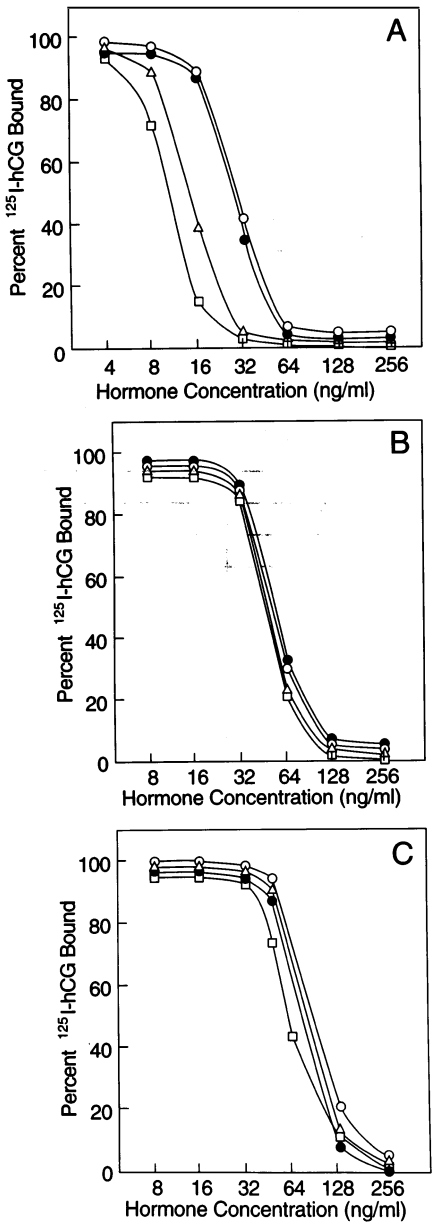


Fig. 3. Radioligand receptor assay of hCG mutants. About 100 ng of the crude LH/CG receptor from rat ovary was incubated with 30 000 cpm of [^{125}I]hCG with varying concentrations of rehCG or its mutants overnight at 4°C. The bound [^{125}I]hCG was precipitated with PEG-8000 and the radioactivity was counted by a Beckman gamma counter. (A) rehCG (●), hCG$\alpha_1\beta_1$ (△), hCG$\alpha_1\beta_2$ (○) and hCG$\alpha_1\beta_3$ (□). (B) rehCG (●), hCG$\alpha_2\beta_1$ (△), hCG$\alpha_2\beta_2$ (○) and hCG$\alpha_2\beta_3$ (□). (C) rehCG (●), hCG$\alpha_3\beta_1$ (△), hCG$\alpha_3\beta_2$ (○) and hCG$\alpha_3\beta_3$ (□).

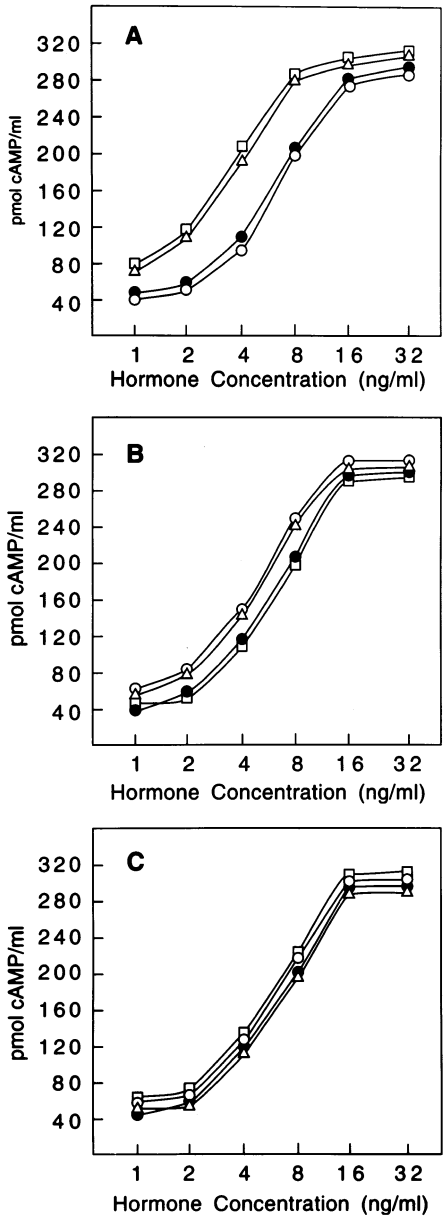


Fig. 4. Stimulation of cAMP by hCG mutants in MA-10 Leydig cells. The concentration dependent stimulation of cAMP in MA-10 cells by rehCG or its mutants was determined by incubating the cells with various concentrations of hCG or its mutants at 37°C for 1 h. The cAMP produced was measured by protein binding assay. (A) rehCG (●), hCG$\alpha_1\beta_1$ (△), hCG$\alpha_1\beta_2$ (○) and hCG$\alpha_1\beta_3$ (□). (B) rehCG (●), hCG$\alpha_2\beta_1$ (△), hCG$\alpha_2\beta_2$ (○) and hCG$\alpha_2\beta_3$ (□). C. rehCG (●), hCG$\alpha_3\beta_1$ (△), hCG$\alpha_3\beta_2$ (○) and hCG$\alpha_3\beta_3$ (□).

β-hairpin loops of hCG were not directly involved in the post receptor signaling events including cAMP and progesterone stimulation.

3.3. *Affinity between α- and β-subunits in hCG mutants containing mutated α_1 loop*

It has been previously reported [34] that a conformational change could result in reducing the affinity be-

tween the α- and β-subunit in hCG mutants. In order to study if there were any conformational changes as a result of mutation involving the replacement of α18Phe with Thr in α_1 loop that resulted in the increased receptor binding and post-receptor signaling activities, the dissociation kinetics of the mutants at low pH, 3.0 were investigated. The dissociation studies of the mutants hCG$\alpha_1\beta_1$, hCG$\alpha_1\beta_2$ and hCG$\alpha_1\beta_3$ were carried out at 23°C at pH 3.0. The rate of dissociation was

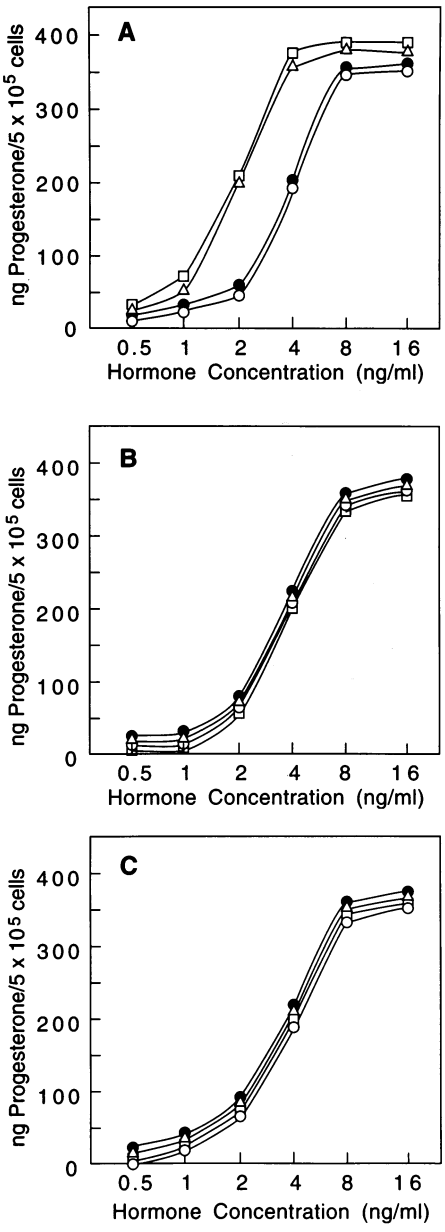


Fig. 5. Effect of hCG mutants on the progesterone stimulation in MA-10 Leydig cells. MA-10 cells were exposed to varying concentrations of rehCG or its mutants at 37° for 4 h. The progesterone produced was estimated by radioimmunoassay using [^{125}I]progesterone and anti-progesterone antibody. (A) rehCG (●), hCG$\alpha_1\beta_1$ (△), hCG$\alpha_1\beta_2$ (○) and hCG$\alpha_1\beta_3$ (□). (B) rehCG (●), hCG$\alpha_2\beta_1$ (△), hCG$\alpha_2\beta_2$ (○) and hCG$\alpha_2\beta_3$ (□). (C) rehCG (●), hCG$\alpha_3\beta_1$ (△) hCG$\alpha_3\beta_2$ (○) and hCG$\alpha_3\beta_3$ (□).

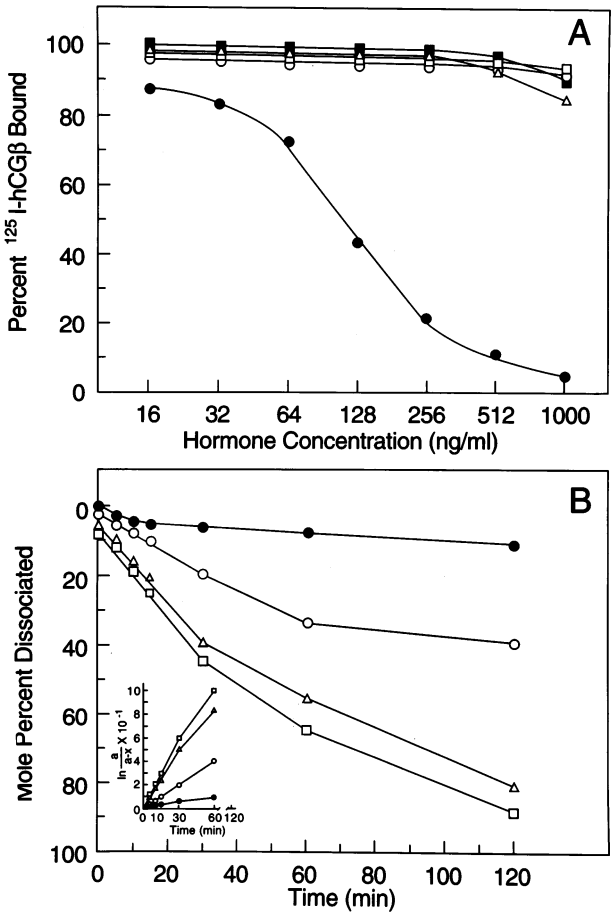


Fig. 6. (A) Radioimmunoassay of hCGβ subunit, rehCG and its mutants, hCG$\alpha_1\beta_1$, hCG$\alpha_1\beta_2$ and hCG$\alpha_1\beta_3$. Varying concentrations of hCGβ (●), rehCG (■), hCG$\alpha_1\beta_1$ (△), hCG$\alpha_1\beta_2$ (○) or hCG$\alpha_1\beta_3$ (□) was incubated overnight at 4°C with 30,000 cpm of [^{125}I]hCGβ and the specific monoclonal anti-hCGβ antibody, B158. in 300 μl of 50 mM PBS, pH 7.4. The bound and free tracer were separated by precipitation with 10% PEG-8000. (B) Dissociation of rehCG (●) and its mutants, hCG$\alpha_1\beta_1$ (△), hCG$\alpha_1\beta_2$ (○) and hCG$\alpha_1\beta_3$ (□) into its subunits at pH 3.0 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 In a/a-x and time (min) where 'a' is the initial concentration and 'x' is the amount dissociated at a certain time.

followed by monitoring the release of the β-subunit and/or the disappearance of the heterodimer by Western immunoblotting using anti-hCGβ and anti-hCG antibodies. Similar studies were carried out with the rehCG as a control [25]. As shown by the immunoblots (data not shown), the rates of dissociation of the mutants were greater than that of rehCG. 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. 6A shows the standard curve for hCGβ. The rehCG as well as the mutants did not show any cross-reactivity with the antibody. Fig. 6B is the mole percent dissociation of the specific mutant and rehCG as measured by the release of the β-subunit. In 2 h, about 80% hCG$\alpha_1\beta_1$,

35% hCG$\alpha_1\beta_2$ and 90% hCG$\alpha_1\beta_3$ were dissociated, while in the same period only about 10% of the rehCG was dissociated. The dissociation rate constants of hCG$\alpha_1\beta_1$, hCG$\alpha_1\beta_2$ and hCG$\alpha_1\beta_3$ were, 3.7×10^{-2} min^{-1}, 1.4×10^{-2} min^{-1} and 4.6×10^{-2} min^{-1} relative to rehCG with the dissociation constant of 4.7×10^{-3} min^{-1} and were one order of magnitude higher than that of the rehCG, respectively. It is clear from the data that the affinity between the subunits of all the mutants containing mutation in the α_1 loop was reduced.

4. Discussion

According to the three dimensional structure of hCG as determined by X-ray diffraction, the α and β subunits share a similar core structure around which the structure of each subunit is organized [19,20]. This core structure is a cysteine knot motif in which one disulfide bridge between β_1 and β_3 strands passes through an eight-residue circle formed by a pair of bridges joining two other strands, β_2 and β_4 [19,20]. The three hairpin loops emanate in each subunit from the core cysteine knot motif. Similar cysteine knot motifs and hairpin loops have been found to occur in NGF, PDGFBB, TGFβ and TNFβ [19,20]. Although the inter-cysteine 'determinant' loop, residues 93–100, in the β-subunit of the heterodimer has been shown by protein engineering [14] to be involved in the receptor binding, it is surprising that such a variant sequence would have hormone binding specificity. It has been postulated that it is not the sequence but the charge which is responsible for the specificity, positive charge for hCG or LH and negative for FSH activity [13]. Therefore, we decided to examine all the loops both in the α- and β-subunits to determine if one or more of them were also involved in the receptor binding and/or signal transduction. Previously, we reported the role of individual loops in the function of the hormone by introducing mutations by site directed mutagenesis [25]. The present communication is a continuation of the earlier work and deals with the receptor binding and signaling properties of the heterodimers containing simultaneous mutations in both the α and β loops. Various hCG mutants with mutations in the loops in both α- and β-subunits were expressed in insect cells. The mutations were confined to single residue mutations (α-subunit) or to substitutions by analogous hFSHβ sequences in order to avoid misfolding of the subunits (Table 1, Fig. 1).

All the heterodimers containing mutated αl loop, hCG$\alpha_1\beta_1$ and hCG$\alpha_1\beta_3$ with the exception of hCG$\alpha_1\beta_2$ showed significantly enhanced effect on the receptor binding and signal transduction activities relative to hCG. The receptor binding activities of these heterodimers increased 200–270% (Fig. 3) relative to rehCG. A similar increase was also observed (Figs. 4 and 5) in the post-receptor signaling events such as stimulation of cAMP and progesterone. These results are consistent with the ones previously reported [25] on the heterodimer, hCG$\alpha_1\beta$ comprised of mutated α_1 loop and the wild type β-subunit. It seems that it is the mutation of α18Phe to Thr in the α_1 loop which is responsible for the enhancement of hormone function since the mutations in the β_1 and β_3 loops do not affect the receptor binding and signaling activity as was previously shown in the mutants, hCG$\alpha\beta_1$ and hCG$\alpha\beta_3$ [25]. Obviously, since the α_1 loop and β_1 and β_3 loops are far removed from each other in hCG structure [19,20], the mutation in the α_1 loop is not affected by mutation in the β_1 or β_3 loop. However, this is not the case with the heterodimer hCG$\alpha_1\beta_2$ in which the α_1 loop is closely situated to the β_2 loop [19,20]. Although the mutant hCGα_1 β_2 contained the mutated α_1 loop, its receptor binding or signal transduction activity remained unaltered. It is conceivable that any change due to the mutation in the α_1 loop might have been offset by the mutation in the proximal β_2 loop of the β-subunit. Clearly, the mutation in the α_1 loop in which the hydrophobic residue α18Phe was replaced by Thr, had an activating effect on both the receptor binding and signal transduction sites. This effect is believed to be due to a conformational change as indicated by weaker affinity between the subunits as shown by the increased rates of dissociation of hCG$\alpha_1\beta_1$ and hCG$\alpha_1\beta_3$ at pH 3.0 relative to the rehCG. This result can be reconciled by the crystallographic structure of hCG [19,20]. The α and β subunits each has a hydrophobic core which is reported to stabilize the hairpin loops. The α-subunit core consists of α17Phe, α18Phe, α25Ile, α68Val, α70Val, α71Met, α74Phe and α76Val. The β subunit core includes residues β16Leu, β18Val, β29Val, β67Ile, β69Leu, β80Val and β82Tyr [19]. All three phenylalanines in the α-core are clustered together and are partially exposed to the surface [19]. There are hydrophobic contacts between the triplet phenylalanine cluster and the β-subunit residues, β44Val and β45Leu [19]. 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 the enhanced receptor binding and signaling activity is due to a short range conformational change affecting the receptor binding site probably located in close proximity to α18Phe. Moyle et al. [31,32] have proposed, by epitope mapping of the hormone when it is complexed with the receptor, that the receptor binding site is located in the loops one and three of the β-subunit in the groove created between the two subunits of the hormone [31,32]. This proposal

can only be reconciled by our data if one assumes that the mutation in the α_1 loop results in a long rather than a short range conformational effect on the receptor binding site and that there is only a single binding site in 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, showed a conformational change as indicated by alteration in its cross-reactivity with anti-hCG. In this mutant β45 Leu in hCGβ was replaced with Tyr. Tyrosine, being less hydrophobic than Leu because of the presence of phenolic hydroxyl, might cause a weakening of the affinity between the two subunits. It is surprising that the replacement of α74Phe to Thr in α_3 loop which is also a part of the phenylalanine triplet did not affect the receptor binding or signaling activity of the hormone. The mutants comprised of α subunit with mutations in the α_2 and α_3 loops and the β subunit, with mutations in the β_1, β_2 and β_3 loops with the corresponding hFSH sequences did not show any change in the receptor binding or signal transduction activity. Thus, only the mutants, hCG$\alpha_1\beta_1$ and hCG$\alpha_1\beta_3$ containing the mutated α_1 loop showed a significant enhancement in the receptor binding and post-receptor signaling activities. The increase was due to the mutation in the α_1 loop without any detectable contribution from the mutation in the β_1 or β_3 loop. The mutation in α_2 loop of α45Lys to Asp did not affect receptor binding or function of the hormone although chemical cross-linking studies had previously suggested the involvement of α45Lys in receptor binding [24]. Keutman and colleagues showed the inhibition of [^{125}I]hCG to rat ovarian membranes by peptide fragment 26–44 indicating that α45Lys was not involved in receptor binding. It may be noted that while α44Lys was essential in the receptor binding activity of the peptide, α45Lys had no detectable effect [33]. Similar conclusion was reached by site-directed mutagenesis studies in which replacement of α44Lys to Ala caused a drastic reduction in the receptor binding and signal transduction activity [18].

The sequence of the long β_2 loop ^{45}Leu Gln Gly Val Leu Pro Ala Leu Pro^{53} was replaced by the hFSH sequence Tyr Lys Asn Pro Ala Arg Pro Leu Ile. The leucine residue at position 52 was not substituted by the corresponding Lys in FSH. The receptor binding and signaling activities of the hormone were not affected although this sequence is a part of the β38–57 sequence which was previously reported by Keutman et al. [16] to be active in receptor binding and steroid stimulation in their studies with the synthetic peptides. Our data is in agreement with that of Moyle et al. [14] who replaced the entire loop (β38–57) sequence with the corresponding hFSH sequence and did not find any loss of receptor binding activity of the hormone. In the β_1 loop, β22Gly, β24Pro and β25Val were replaced by Glu, Arg and Phe respectively. The residue α23Cys was not mutated. Although two of these mutations were radical, they did not affect the biological properties of the hormone. Finally, the replacement β73 Pro, β74Arg and β75Gly in loop 3 by Ala, His, His respectively also did not affect the receptor binding and signal transduction. Thus, it appears that neither are β_1 and β_3 loops near or in the receptor and subunit binding sites nor do they affect the conformation of these sites.

In summary, unlike NGF and TNF, the loops with the possible exception of α_1 loop in each of the α and β subunits were not directly involved in receptor binding and signal transduction. The heterodimers containing the mutated α_1 (α18Phe to Thr) including hCG$\alpha_1\beta_1$ and hCG$\alpha_1\beta_3$ had 200–270 percent enhanced receptor binding and signaling activities such as cAMP and progesterone stimulation. Since hCGβ with mutated β_1 and β_3 loops alone with wild type α did not affect the properties of the heterodimer, the enhancement in the activity of the hormone was probably due to the replacement of Phe. Obviously, mutation in the β_1 or β_3 loop did not significantly affect the mutation in the α_1 loop. α18Phe probably located in or in the vicinity of the receptor binding site either affected the binding site directly or indirectly by changing its conformation. This is one of the few cases where the mutation results in the activation rather than inhibition of the receptor binding site. Further studies are in progress to elucidate the role of the α_1 loop region in the function and conformation of the hormone.

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