Single-chain human chorionic gonadotropin analogs containing the determinant loop of the β -subunit linked to the α -subunit

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Abstract

Human chorionic gonadotropin (hCG) is a member of the family of glycoprotein hormones containing a common α -subunit and distinct β -subunits that confer hormonal specificity. hCG binds to the relatively large ectodomain of the human luteinizing hormone receptor (hLHR), a member of the G protein-coupled receptor superfamily, leading to increased intracellular production of cAMP. Using protein engineering, two miniaturized versions of hCG β have been separately fused to the N-terminus of the α -subunit to give N-des[1–91]hCG β - α -C and N-des[1–91,110–114]hCG β - α -C, i.e. fusion proteins of the hCG β determinant loop (extended to include the complete seat belt and carboxy-terminal peptide) coupled to the α -subunit. Bioactivity of these single-chain gonadotropin analogs was assessed in two systems following transient transfections into HEK 293 cells and subsequent cAMP measurements. In one, each mini- β - α cDNA was fused to that of hLHR and transfected into cells to create yoked miniaturized hCG–hLHR complexes; in the other, the cDNA of each single chain mini- β - α was co-transfected with that of hLHR in an effort to produce non-covalent miniaturized hCG–hLHR complexes. Using yoked hCG–hLHR and hLHR as positive and negative controls respectively, expression of each mini-hCG–hLHR complex was confirmed using antibody and ligand binding assays. The two mini-hCGs led to minimal activation of hLHR, suggesting weak intrinsic activity of the mini- β - α fusion proteins. These results suggest that potent agonists and antagonists will require the presence of other portions of hCG β in addition to the determinant loop/seat belt.

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Introduction

The four human (h) glycoprotein hormones are heterodimers that share a common α -subunit, comprised of 92 amino acid residues, and a distinct β -subunit that confers hormonal specificity (Hearn & Gomme 2000). The β -subunit of human chorionic gonadotropin (hCG) contains 145 amino acid residues, including a 30 residue extension at its C-terminus, referred to as the C-terminal peptide (CTP). The four hormones act via three G protein-coupled receptors, with the β -subunits of hCG and luteinizing hormone (LH) being sufficiently similar that the two gonadotropins both bind to and activate the LH receptor (hLHR) (Dufau 1998, Ascoli *et al.* 2002).

hCG is the most comprehensively studied of the human glycoprotein hormones, and therefore there is an abundance of information available concerning the regions of the two subunits believed to be important for bioactivity. Using various techniques such as site-directed mutagenesis (Chen & Puett 1991*a,b*, Chen *et al.* 1991, 1992, Bielinski & Boime 1992, Liu *et al.* 1993, Yoo *et al.* 1993, Puett *et al.* 1994, Puett & Narayan 2000), protein chimeras (Campbell *et al.* 1991, Dias *et al.* 1994, Han *et al.* 1996, Grossmann *et al.* 1997), and synthetic peptides (Salesse *et al.* 1990, Keutmann 1992), many of the regions of hCG important for heterodimer formation and receptor binding and activation have been determined, as well as the suggestion that both subunits are required for full

activity. The combined findings from these studies concerning ligand–receptor interactions have indicated that the central portion of the α -subunit and the determinant loop (Moore *et al.* 1980) of hCG β (residues 93–100), i.e. the N-terminal portion of the β -seat belt (Laphorn *et al.* 1994, Wu *et al.* 1994), are required for LHR binding and activation, although whether the determinant loop directly contacts the receptor remains controversial (Han *et al.* 1996). The importance of this region in glycoprotein receptor discrimination confirms earlier predictions based upon amino acid sequence comparisons of the glycoprotein hormone β -subunits (Moore *et al.* 1980). The crystal structure of aglyco-hCG (Laphorn *et al.* 1994, Wu *et al.* 1994), followed by that of human follicle-stimulating hormone (hFSH) (Fox *et al.* 2001), demonstrated that the regions of α and β , delineated as putative receptor contact sites, are in relatively close proximity to one another.

We and others have shown that biologically active fusion proteins of hCG could be constructed in the manner N-hCG β - α -C (Narayan *et al.* 1995, Sugahara *et al.* 1995, Heikoop *et al.* 1997, Schubert *et al.* 2003) and N- α -CTP-hCG β -C (Narayan *et al.* 2000a, Sen Gupta & Dighe 2000, Ben-Menahem *et al.* 2001). These studies were expanded to include yoked hCG–rat LHR complexes (YHR) of the form, N-hCG β - α -CTP-LHR-C (Wu *et al.* 1996) and N- α -hCG β -LHR-C (Narayan *et al.* 2000a), resulting in ligand-mediated constitutive receptor activation. The goal of the current investigation is the design and characterization of miniaturized hCG hormones, a continuation of work from this and other laboratories focused on N-terminal and C-terminal deletion mutants of hCG β that retained the ability to form heterodimers and activate LHR (El-Deiry *et al.* 1989, Matzuk *et al.* 1990, Chen & Bahl 1991, Chen & Puett 1991a, Huang *et al.* 1993a, Slaughter *et al.* 1995). Currently, the shortest hCG β fragment retaining full activity, i.e. capable of holoprotein formation and subsequent LHR activation with equipotency to wild-type hCG, is *des*[1–7,111–145]hCG β , while *des*[1–7,101–145]hCG associates poorly with α ; however, the resulting heterodimer exhibits some activity (Huang *et al.* 1993a). Only three hCG β C-terminal deletion fragments have been described in β - α single chain gonadotropins, all involving deletion of portions of the CTP, *des*[124–145] (Wu *et al.* 1996)

and *des*[103–145] and *des*[112–145] (Heikoop *et al.* 1997).

We have created single-chain mini-gonadotropins in which a large N-terminal portion of hCG β has been removed (residues 1–91) and the remainder of the subunit, containing the determinant loop/seat belt region and the CTP, was fused to the N-terminus of the full-length α -subunit to create YMG1. YMG2 incorporates a further deletion of hCG β , residues 110–114, eliminating a portion of the C-terminal region of the seat belt that includes Cys-110, which pairs with Cys-26 in native hCG (Laphorn *et al.* 1994, Wu *et al.* 1994). These deletions were chosen in order to retain the β -seat belt, deemed important for hormone binding and receptor activation via the determinant loop, as well as the CTP to serve as a linker. However, difficulty was experienced in expressing sufficient quantities of the mini-gonadotropins in mammalian cells for dose–response curves. Based on this limitation and anticipating low affinity interaction of these hCG analogs with LHR, we have used the yoked hormone–receptor system and co-transfection studies to evaluate the activity of the two miniaturized yoked hCG hormones. It was found that each mini-gonadotropin activates hLHR, but only to a very small degree.

Materials and methods

Construction of yoked mini-gonadotropins and yoked mini-gonadotropin–receptor complexes

MG1 and MG2 were created using PCRs to generate the hCG β fragments in the following manner: MG1: 5'-BamHI restriction site/hCG β signal sequence/flag tag/hCG β residues 92–145/EcoRI-3', and MG2: 5'-BamHI restriction site/hCG β signal sequence/flag tag/hCG β residues 92–109, 114–145/EcoRI-3'.

In order to create the miniaturized hCG fusion proteins, the N-terminal deletion cDNAs of hCG β were ligated in place of full length hCG β in N-hCG β - α -C to give N-MG1- α -C (YMG1) and N-MG2- α -C (YMG2).

A BamHI/EcoRI double digestion was used to excise the miniaturized hCG β subunits from YMG1 and YMG2. The full length hCG β was then removed from the existing N-hCG β - α -LHR-C construct by BamHI/EcoRI digestion. The miniaturized hCG β subunits were then ligated

in place of full length hCG β to produce Y(MG1/LHR) and Y(MG2/LHR).

Cell culture and transient transfections

HEK 293 cells were grown in monolayer culture in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% (v/v) newborn calf serum, 10 mM HEPES buffer, 50 U/ml penicillin, 50 μ g/ml streptomycin, 50 μ g/ml Fungizone, and 0.125 μ g/ml amphotericin B (Schubert *et al.* 2003). The cDNAs were transfected into HEK 293 cells using lipofectamine as recommended by Invitrogen Life Technologies (5 μ g of each cDNA for each fusion protein; 5 μ g of each cDNA for each co-transfection for a total of 10 μ g).

Hormone binding

Approximately 16–18 h after transfection, the cells were resuspended in growth media and added to tissue culture plates coated with 0.1% gelatin in PBS. Approximately 24 h later the cells were assayed for 125 I-hCG binding as described earlier (Schubert *et al.* 2003). Briefly, for competitive binding, increasing concentrations of unlabeled hCG (purchased from Dr Albert Parlow (Torrance, CA, USA) and the NIDDK (Bethesda, MD, USA) in Waymouth's media with 0.1% bovine serum albumin (BSA) (w/v) and 50 pM 125 I-hCG were incubated with the cells at 37 °C for 6 h. Nonspecific binding was determined by addition of 1 μ g/ml unlabeled hormone. The cells were washed with PBS, lysed with 1 M NaOH, and counted in a gamma counter. All assays were performed in duplicate, and mock transfected cells were used as a control.

Anti-LHR antibody binding assay

Approximately 16–18 h after transfection, the cells were processed as described above and the cells assayed for binding of an anti-rat LHR polyclonal antibody (raised against a synthetic peptide to residues 15–38 and kindly provided by Dr Patrick Roche, Mayo Medical School, Rochester, MN, USA) that was detected by secondary 125 I-anti-rabbit antibody. The cells were incubated in a 1:4000 dilution of the primary antibody in Waymouth's media and 0.3% (w/v) BSA for

4 h while shaking. After washing twice with Waymouth's media and 0.3% (w/v) BSA, the cells were incubated with the 125 I-anti-rabbit secondary antibody (400 000 c.p.m./well) in Waymouth's media and 0.3% BSA for 1 h with shaking. After the second incubation period, the cells were washed with PBS and then removed from the plates as described above and counted. Each experiment was performed in duplicate and all steps were performed at room temperature.

cAMP assay

About 16–18 h after transfection, the cells were replated and washed as described above, then incubated in Waymouth's media with 0.1% (w/v) BSA and 0.8 mM isobutylmethylxanthine (IBMX) for 15 min at 37 °C. Following removal of the medium, the cells were incubated with increasing concentrations of hCG for 30 min at 37 °C in Waymouth's media containing 0.1% (w/v) BSA and 0.8 mM IBMX. After removal of the incubation medium, the cells were lysed in 100% ethanol at –20 °C overnight, the extract collected, dried under vacuum, and cAMP concentrations were determined by RIA (Schubert *et al.* 2003). The results are presented in two units for basal cAMP: pmol/ml and pmol/fmol bound 125 I-hCG, the latter to correct for receptor expression differences. (The correction for receptor expression was based on B_o , i.e. the specific binding of 50 pM 125 I-hCG in which c.p.m. bound was converted to fmol bound. However, since the K_d values of the constructs are the same as that of LHR, this serves as a relative correction for receptor densities. Since no correction was made for transfection efficiency or B_{max} , the values cannot be considered as absolute pmol cAMP produced per fmol bound hCG in 30 min. On the other hand, transfection efficiencies are comparable within a given assay, and B_o is directly proportional to B_{max} under these conditions. Thus, the relative corrections are valid for comparative purposes.) Each experiment was performed in duplicate.

Data analysis

Competitive binding and cAMP data were analyzed by non-linear regression using the Prism software program (Graph Pad Software, San Diego, CA, USA). The results are given as

Figure 1 Schematic representation of yoked mini-gonadotropins and yoked hCG–hLHR complexes. (A) YMG1 and YMG2 compared with YhCG with the CTP. YMG1 contains amino acid residues 92–145 of hCG β , i.e. residues 92–114 plus the CTP (residues 115–145, indicated by vertical stripes), fused to the full length α -subunit (92 amino acid residues). YMG2 contains a further deletion of hCG β residues 110–114, which removes Cys-110 that is present in YMG1. (B) Y(MG1/LHR) and Y(MG2/LHR) compared with Y(CG/LHR). The CTP is again denoted by vertical stripes. The two sites of N-glycosylation on each of the intact subunits and the four sites of O-glycosylation on the CTP are not shown.

means $\pm$ S.E.M., based on 3–7 independent transfections, and significance ($P < 0.05$) was determined by Student's *t*-test. The figures showing competitive binding and hCG-mediated cAMP production are based on overall averages from replicate transfections, and the IC₅₀s and ED₅₀s in Table 1 refer to

the average of each individual parameter from each transfection. The IC₅₀ and ED₅₀ values are given in Table 1 in nM units; by convention, the dose–response curves are presented in ng/ml units. To convert the latter to nM, simply multiply by 0.027.

Figure 2 Crystal structure of deglycosylated hCG. The α -subunit from residues 5–89 and the β -subunit from residues 2–111 are shown. The other amino acid residues did not appear in the electron density maps (Lapthorn *et al.* 1994, Wu *et al.* 1994), presumably due to their flexible nature. The region of the miniaturized β -subunit from residues 92–111 is highlighted in a darker tone.

Results

Structures of the yoked mini-hCGs and yoked mini-hCG–hLHR complexes

Schematics of the two yoked mini-gonadotropins, YMG1 and YMG2, are shown in Fig. 1A and compared with YhCG. YMG1 lacks the N-terminal 91 amino acid residues of hCG β , and thus both sites of N-glycosylation and the Cys-knot cluster, but retains the remainder of the β -subunit, including the determinant loop-containing seat belt and the CTP with its four sites of O-glycosylation, that is fused to α . YMG2 is similar but has an additional five amino acid residue sequence

Figure 3 Cell surface expression of hLHR and the yoked ligand–receptor complexes, Y(CG/LHR), Y(MG1/LHR), and Y(MG2/LHR). (A) Specific binding following addition of 50 pM ^{125}I -hCG to HEK 293 cells expressing hLHR and three yoked ligand–receptor complexes. The levels of exogenous ligand binding to hLHR, Y(MG1/LHR), and Y(MG2/LHR) are similar, while Y(CG/LHR), as expected (Wu *et al.* 1996, Narayan *et al.* 2000a, 2002), binds negligible amounts of ^{125}I -hCG. (B) Expression of hLHR, free and in yoked complexes, as monitored by a rabbit anti-rat LHR antibody, followed by binding of ^{125}I -anti rabbit secondary antibody. The data are corrected for binding to mock-transfected cells, and there are no significant differences between the receptor levels.

deleted, 110–114, hence removing Cys-110 that pairs with Cys-26 in hCG β . The two yoked mini-gonadotropin–hLHR structures are given

Figure 4 Competitive binding and cAMP dose-response curves with HEK 293 cells expressing hLHR and fusion proteins. (A) Competitive binding with 50 pM ^{125}I -hCG and various concentrations of unlabeled hCG for hLHR and the two yoked mini-gonadotropin-receptor complexes. (B) cAMP production of mock-transfected (MT) cells and cells expressing hLHR and the three yoked ligand-receptor complexes. The IC_{50} s and EC_{50} s are given in Table 1.

schematically in Fig. 1B and compared with that of yoked hCG-hLHR (Y(CG/LHR)). The crystal structure of aglyco-hCG (Lapthorn *et al.* 1994, Wu *et al.* 1994) is shown in Fig. 2 with the seat belt of hCG β preserved in the mini-gonadotropins highlighted.

Anticipating that any discernible binding of the miniaturized hCG β s, fused to α , would be of low affinity, experimental conditions were chosen to maximize association with hLHR. One paradigm, based on protein engineering to produce fusion

proteins, was developed by our laboratory to give constitutive ligand-mediated receptor activation and involved yoking a single chain hCG to the full length receptor (Wu *et al.* 1996, Puett & Narayan 2000, 2002, Narayan *et al.* 2000a,b, Schubert *et al.* 2003) and to the receptor ectodomain (Fralish *et al.* 2001). The former approach was used in the present study with full length hLHR in order to permit an evaluation of receptor activation in transfected HEK 293 cells. Another paradigm involved co-transfection of the hLHR cDNA

Table 1 Summary of binding and signaling parameters of YMGs and hLHR

	Binding	Signaling			
	IC ₅₀ (nM) (n)	ED ₅₀ (nM) (n)	Basal cAMP ^a (pmol/fmol) (n)	Basal cAMP ^a (pmol/ml) (n)	Maximal cAMP (pmol/ml) (n)
Fusion proteins of YMGs and hLHR					
Y(MG1/LHR)	0.46±0.11 (7)	0.36±0.09 (7)	7.5±0.9 ^b (7)	4.8±0.8 (7)	198±24 (7)
Y(MG2/LHR)	1.02±0.55 (7)	0.33±0.11 (6)	7.4±0.9 ^b (7)	5.0±0.55 ^b (7)	229±32 (6)
hLHR	0.56±0.15 (5)	0.26±0.04 (7)	4.9±0.7 (7)	3.2±0.4 (7)	201±28 (7)
Y(CG/LHR)	— ^c	— ^c	— ^c	107±9.2 ^b (7)	110±12.5 ^b (6)
Co-expression of YMGs and hLHR					
YMG1 & hLHR	0.88±0.48 (3)	0.19±0.01 (3)	11.6±3.6 (3)	5.8±1.3 (3)	338±91 (3)
YMG2 & hLHR	2.24±1.50 (4)	0.23±0.06 (4)	10.8±1.5 ^d (4)	6.0±0.7 ^d (4)	307±40 (4)
pcDNA3 & hLHR	0.75±0.21 (4)	0.21±0.03 (4)	7.2±0.7 (4)	4.0±0.4 (4)	265±70 (4)
YhCG & hLHR	— ^c	— ^c	— ^c	114±21 ^b (4)	173±63 (4)

HEK 293 cells were transiently transfected with cDNAs to YMG1, YMG2, Y(MG1/LHR), and Y(MG2/LHR), with LHR serving as negative control and Y(CG/LHR) and YhCG & LHR serving as positive controls. Dose–response curves were conducted with hCG, and competitive binding also included 50 pM ¹²⁵I-hCG. The maximal cAMP levels refer to maximum production at 250 ng/ml hCG with no correction for basal cAMP levels.

^aBasal cAMP values are presented in two units: pmol/fmol of bound ¹²⁵I-hCG to correct for expression levels, and pmol/ml. The former is relative and based on values of B₀ (see Materials and methods).

^bSignificantly different from LHR (*P*<0.05).

^cExpression of yoked hCG-hLHR and co-expression of yoked hCG and hLHR results in minimal binding of ¹²⁵I-hCG and in elevated levels of basal cAMP, compared with LHR, that are not further increased upon addition of exogenous hCG.

^d*P*<0.051 when compared with LHR.

and cDNAs to the yoked mini-gonadotropins to evaluate non-covalent association at the relatively high intracellular concentrations of each component. In each experimental paradigm, negative controls consisted of just hLHR expression or mock transfection, while positive controls involved Y(CG/LHR) expression and co-expression of YhCG and hLHR.

Yoked mini-hCG–hLHR complexes: expression of a single chain ligand–receptor complex

Addition of 50 pM ¹²⁵I-hCG to cells expressing Y(MG1/LHR) and Y(MG2/LHR) led to specific binding similar to that of hLHR (Fig. 3A), presumably reflecting the low affinity of YMG1 and YMG2 to LHR. In contrast, cells expressing Y(CG/LHR) bound negligible ¹²⁵I-hCG as expected (Wu *et al.* 1996, Narayan *et al.* 2000a,

2002, Schubert *et al.* 2003), attributable to the nearly constant high-affinity association of single-chain hCG fused to hLHR. There was no significant specific binding of ¹²⁵I-hCG to mock-transfected cells (data not shown), consistent with earlier reports (Wu *et al.* 1996, Narayan *et al.* 2002) and with data presented below. Transfected cells were incubated with an anti-LHR antibody, and receptor densities were then estimated by binding an ¹²⁵I-labeled secondary antibody to an anti-LHR antibody. As shown in Fig. 3B, there is no significant difference in expression levels of hLHR, whether free or fused to YMG1, YMG2, or YhCG.

Competition binding curves, using ¹²⁵I-hCG and hCG, are presented in Fig. 4A for cells expressing Y(MG1/LHR), Y(MG2/LHR), or hLHR. The IC₅₀s obtained from the three competition curves are essentially identical (Table 1). cAMP dose–response curves were determined at various

Figure 5 Basal cAMP production of HEK 293 cells expressing Y(MG1/LHR) and Y(MG2/LHR) and co-expressing YMG1 and hLHR and YMG2 and hLHR. (A) Results from cells expressing Y(MG1/LHR) and Y(MG2/LHR); $P < 0.05$ for each YMG fused to LHR. (B) Results from cells expressing YMG1 and hLHR and YMG2 and hLHR; $P < 0.051$ for YMG2 and hLHR only. The results are an average of each individual experiment, shown as fold-increase over that of hLHR production. Averages were made from values expressed in pmol of cAMP produced/fmol bound ^{125}I -hCG, which is a relative correction for protein expression levels. Values are presented in Table 1.

concentrations of hCG, and the ED_{50} s for Y(MG1/LHR), Y(MG2/LHR), and hLHR are also similar (Fig. 4B, Table 1). As expected from earlier studies (Wu *et al.* 1996, Narayan *et al.* 2000a, 2002, Schubert *et al.* 2003), Y(CG/LHR) results in a high level of basal cAMP that is not further increased at high concentrations of exogenous hormone, and mock-transfected HEK 293 cells do

not respond to exogenous hCG (Fig. 4B), consistent with the absence of LHR. Interestingly, the basal cAMP values of Y(MG1/LHR) and Y(MG2/LHR) are slightly elevated with reference to that of hLHR when corrected for expression levels, although only that for Y(MG2/LHR) is significantly different from hLHR if these corrections are not made (Fig. 5A, Table 1).

Yoked mini-hCGs and hLHR: co-expression of ligand and receptor

Binding measurements with ^{125}I -hCG suggested comparable receptor densities in cells expressing hLHR only and hLHR with each of the mini-hCGs (Fig. 6). Competition binding and cAMP activation curves show that IC_{50} s and EC_{50} s are similar in the hLHR control and when co-expressed with the YMGs. Co-expression of YhCG and hLHR results in a high basal level of cAMP that is not increased upon addition of hCG, analogous to that found with Y(CG/LHR). These results are summarized in Fig. 7 and Table 1. Compared with hLHR and corrected for expression levels, the fold-increases in basal cAMP values are slightly greater in cells co-expressing hLHR and each of the mini-gonadotropins, but not significantly so (Fig. 5B). (In this paradigm, YMG2 co-expressed with hLHR differs from just expressed hLHR with a P value of < 0.051 whether the data are corrected for expression levels or not.)

Discussion

This work has shown that minimal activity is obtained from hCG β fragments, consisting of amino acid residues 92–145 and (92–109)–(115–145), fused via the C-terminus to the N-terminus of the full-length α -subunit when corrected for relative expression levels of the receptor. The low potencies of the analogs is emphasized by comparing the ratios of basal cellular cAMP concentrations in the expressed yoked ligand-hLHR and co-expressed ligand and hLHR systems to that of the hLHR control: the fold-increases were 1.5–1.6 for the two mini-gonadotropins and roughly 30 for single chain $\beta\alpha$, whether yoked to or co-expressed with hLHR.

Nonetheless, the putative weak potencies of MG1 and MG2 may reflect specific effects of α and the

Figure 6 Cell surface expression of hLHR when co-expressed with yoked ligand–receptor complexes. Specific binding after addition of ¹²⁵I-hCG to HEK 293 cells expressing hLHR or co-expressing hLHR and YMG1, YMG2, and YhCG. There is insignificant binding of ¹²⁵I-hCG to mock-transfected (MT) cells, as expected (Wu *et al.* 1996, Narayan *et al.* 2002).

fused β fragments. For example, recent studies from our laboratory found no evidence of intrinsic activity of full length α or hCG β subunits when yoked to or co-expressed with rat LHR (Narayan *et al.* 2002). Controls were also run with prolactin and CTP yoked to rat LHR, and minimal increases in basal cAMP were observed; however, the limited numbers used in the experiment prohibited a statistical evaluation of these data. Clearly, potent hCG-based agonists and antagonists of the LH receptor will require a different protein engineering strategy, probably with the inclusion of different portions of the β -subunit rather than just the determinant loop-containing seat belt. Also, both subunits of hCG appear to contribute, either directly or indirectly, to LHR binding (Chen *et al.* 1991, 1992, Bielinski & Boime 1992, Liu *et al.* 1993, Yoo *et al.* 1993, Puett *et al.* 1994); thus, portions of α

and β subunits will probably be required for potent single chain LHR agonists and antagonists.

Previous studies with heterodimeric hCG have shown that C-terminal fragments of hCG β , including *des*[122–145] β (El-Deiry *et al.* 1989), *des*[115–145] β (Matzuk *et al.* 1990, Chen & Bahl 1991), and *des*[111–145] β (Huang *et al.* 1993a), associate well with α and the resulting heterodimers are equipotent to wild-type hCG in *in vitro* measurements. *des*[101–145] β also bound to α , but the heterodimer exhibited reduced potency; *des*[93–145] β , on the other hand, failed to associate with α (Chen & Puett 1991a). Two N-terminal deletion mutants of hCG β have been described, *des*[1–7] β (Huang *et al.* 1993a) and *des*[2–8] β (Slaughter *et al.* 1995). Although holoprotein formation was impaired in the latter, both deletion fragments bound α , and the resulting heterodimers were

Figure 7 Competitive binding and cAMP dose-response curves with HEK 293 expressing hLHR only or co-expressing hLHR with the yoked ligand-receptor complexes. (A) Competitive binding with 50 pM ^{125}I -hCG and various concentrations of unlabeled hCG for hLHR alone and hLHR co-expressed with YMG1 and YMG2. (B) cAMP production in mock-transfected (MT) cells, in cells expressing hLHR only (hLHR and pcDNA3), and in cells co-expressing hLHR with YhCG, YMG1, and YMG2. The IC_{50} s and EC_{50} s are listed in Table 1.

equipotent to wild-type hCG. We also reported that a combined N-terminal and C-terminal deletion fragment of hCG β , $\text{des}[1-7,111-145]\beta$, bound to α , resulting in a heterodimer equipotent to wild-type hCG, while $\text{des}[1-7,101-145]\beta$ associated poorly with α , but some activity was present in the heterodimer (Huang *et al.* 1993a). A single chain hCG in the same configuration described here, i.e.

N- β - α -C, but with a shortened CTP, $\text{des}[124-145]\beta$ was shown to be active (Narayan *et al.* 1995), as were fusion proteins of the form, $\text{des}[103-145]\beta$ -(Ser-Gly) $_x$ - α and $\text{des}[112-145]\beta$ -(Ser-Gly) $_x$ - α , where $x=4, 5$, or 6 (Heikoop *et al.* 1997).

The above studies, based on heterodimeric and single chain hCG derivatives, establish that the CTP does not contribute significantly to α

association or receptor binding. It was, however, retained in our characterization of the mini-hCGs to serve as a spacer between the β seat belt and the α subunit, as well as a linker between α and LHR in the yoked MG–LHR complexes. Prior to the present study, the shortest fragment of hCG β that yielded some activity *in vitro*, in combination with α , was *des*[1–7,101–145] β (Huang *et al.* 1993a). The retention of the determinant loop was based on studies using synthetic peptides (Keutmann *et al.* 1989, Keutmann 1992), site-directed mutagenesis (Chen & Bahl 1991, Chen & Puett 1991b, Huang *et al.* 1993b), and protein chimeras (Campbell *et al.* 1991, Dias *et al.* 1994, Grossmann *et al.* 1997) suggesting this region of hCG β to be important in receptor binding and activation, either directly or indirectly. While we have retained the determinant loop as part of the β seat belt, we have, of course, no assurance that the Cys-93–Cys-100 disulfide forms in the MGs. Ben-Menahem *et al.* (1997) showed that engineered mutations of Cys $\rightarrow$ Ala at each of positions β 93 and β 100 in N-hCG β – α -C led to an hCG analog that was slightly less effective than wild-type single chain hCG. In this case, the determinant loop may be constrained in a native-like conformation.

Much less work has been carried out with deletions of the 92-amino acid residue α -subunit. The C-terminal Ser can be removed without greatly impairing association with hCG β and subsequent LHR binding and activation; however, progressive deletion of residues 91–92, 90–92, and 89–92 results in reduced signaling, while not eliminating receptor binding (Yoo *et al.* 1993). The N-terminal fragment, *des*[88–92] α , associates with hCG β but has reduced LHR binding and minimal signaling (Chen *et al.* 1992, Yoo *et al.* 1993). In contrast to these observations, the fusion protein, N-hCG β –*des*[88–92] α -C, was found to bind to LHR with the same affinity as the full-length control, but signaling was diminished (Sen Gupta & Dighe 2000).

The suggestive finding of activity, albeit low, in the fusion proteins, N-*des*[1–91]hCG β – α -C and N-*des*[1–91,110–114]hCG β – α -C, offers promise for the design of mini-gonadotropins. Our data argue strongly, however, that potent hCG analogs, e.g. serving as agonists and antagonists, will require more of the β -subunit than just the determinant loop/seat belt. The results reported herein contribute to our understanding of hCG structure–

function relationships and demonstrate the power of using fusion proteins to probe weak biological interactions.

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