Expression of an *in vitro* biologically active equine LH/CG without C-terminal peptide (CTP) and/or β 26–110 disulphide bridge

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Abstract

The C-terminal region of the β subunit of the human chorionic gonadotrophin (hCG) is implied in heterodimer stability (β 26–110 disulphide bridge), *in vitro* LH bioactivity (region β 102–110) and in *in vivo* LH bioactivity (β CTP). Like the hCG β , the equine eLH and eCG β subunits, also possess a C-terminal extension (CTP). But, in contrast to hCG, eLH and eCG bind to both LH and FSH receptors in species other than the horse. This allows investigation of the roles of the β subunit C-terminal region of a eLH/CG recombinant molecule on both LH and FSH activities. To do so, the CTP was deleted and/or the β 26–110 disulphide bond was mutated and the resulting mutated β subunits were transiently co-expressed with common α subunit in COS7 cells. These regions were also deleted in a β aeLH/CG single chain also expressed in COS7 cells. The hormones produced were characterized

by different ELISAs and *in vitro* LH and FSH bioassays. Mutation of the 26–110 disulphide bond and deletion of the β CTP led to a decrease in eLH/CG heterodimer production. Double mutation promoted an additive effect on production of the heterodimer and of the corresponding tethered eLH/CG. The elimination of the β 26–110 disulphide bond in the β a single chain had no effect on its production. However, neither the 26–110 disulphide bond nor the CTP mutations affected dimer stability and bioactivities of the secreted heterodimers and/or single chain molecules. Therefore, in contrast to hCG, the 26–110 S–S bond of the recombinant eLH/CG β subunit does not seem to be essential for eLH/CG dimer stability upon secretion and expressing LH and FSH bioactivities.

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Introduction

The glycoprotein hormone family consists of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH), which are secreted by the pituitary gland in all mammalian species, and chorionic gonadotrophin (CG), which is secreted by the placenta only in primates and equids. The gonadotrophins LH, FSH and CG are involved in the control of gonadal function. All glycoprotein hormones consist of two non-covalently associated α and β subunits. Within a species, the amino acid sequence of the α subunit is identical for all the glycoprotein hormones and is encoded by a single gene. Only the $\alpha\beta$ dimer is active and the biological specificity is conferred by the β subunits (Pierce & Parsons 1981, Combarnous 1992).

Human CG (hCG) is the only gonadotrophin whose crystallographic structure has been resolved (Laphorn *et al.* 1994), and all other glycoprotein hormones are thought to have the same global conformation based on the presence of conserved cysteines. Many studies have focused on the role of the hCG β subunit in intracellular folding, stability

of the $\alpha\beta$ dimer and biological activity. The CG β subunit is distinguished among other β subunits by the presence of a 30 amino acid extension called C-terminal peptide (CTP). The CTP of the hCG β subunit has been shown to be involved in the half-life of the hormone (Matzuk *et al.* 1990). It is not implicated in *in vitro* biological activity but is required for the correct intracellular folding of the dimer (Matzuk *et al.* 1989, Muyan *et al.* 1996, Muyan & Boime 1998).

Like hCG, eCG is secreted by the placenta and presents a CTP on its β subunit. Interestingly, eLH, secreted by the pituitary, has the same amino-acid sequence as eCG since its subunits are encoded by the same common α gene and the same β gene (Sherman *et al.* 1992). The recombinant hormone will thus be referred to as eLH/CG. Moreover, eLH/CG exhibits the peculiarity of binding to both LH and FSH receptors in species other than horses, including the donkey (Stewart *et al.* 1977). Previous biochemical studies on hypophyseal eLH (Bousfield *et al.* 1989) have shown that the chemical deletion of the CTP did not affect LH bioactivity of the hormone. By contrast, a more recent study (Min *et al.* 1996) has shown that elimination of the

Construction of truncated β subunit cDNAs Construction of $\beta\alpha$ single chain cDNAs

Figure 1 Schematic representation of wild-type, mutant subunits and eLH/CG single chains. A solid bar represents primary structure of β subunit, a hatched bar represents primary structure of α subunit, and Δ represents mutation of cysteines 26 and 110 in alanines by site-directed mutagenesis. Symbols in parentheses are those used in Fig. 2 for each heterodimer or single chain.

CTP in recombinant eLH/CG induced a tenfold increase in FSH bioactivity. So, the CTP could be crucial for correct folding of the β eLH/CG C-terminal region (90–110 sequence) just upstream of CTP which is thought to contain biological determinants as hCG β (Moyle *et al.* 1994, Han *et al.* 1996).

All β subunits possess 12 cysteines at conserved positions, forming six intrachain disulphide bonds. According to the literature (Huth *et al.* 1993), the 26–110 disulphide bond in hCG is formed after $\alpha\beta$ dimer formation and plays an essential role in its stability and in the presentation of the β 90–110 sequence involved in the hCG biological activity (Moyle *et al.* 1994). The β 26–110 disulphide bridge fastens the so-called 'seat belt' formed by the 90–110 sequence of the β subunit, which wraps around the α subunit. However, this S–S bond in the hLH β subunit which has no CTP would be formed before the interaction of α and β subunits (Muyan *et al.* 1998), indicating that the folding stabilized by the β 26–110 disulphide bridge fastening could be different in LH and hCG and could be important for the production, stability and bioactivity of gonadotrophins.

In order to investigate the roles of CTP and the β 26–110 disulphide bridge, we studied the effect of

deletion of CTP and/or the 26–110 disulphide bond in the eLH/CG β subunit on the production, stability and bioactivities of recombinant $\alpha\beta$ dimer and $\beta\alpha$ single chain produced in COS7 cells cultures. We show that deleted recombinant eLH/CG retains its full LH and FSH bioactivities compared with its wild-type counterpart. In contrast to hCG, the stability of the eLH/CG heterodimer in the production media appears independent of the 26–110 disulphide bond and of CTP.

Materials and Methods

Construction of mutant eLH/CG β subunit cDNAs and eLH/CG single chains

The cDNA encoding the horse LH/CG β subunit (Chopineau *et al.* 1995) inserted in the expression vector CDM8 was used as a template to construct the truncated β subunit (Fig. 1).

Two truncated β subunits were obtained by PCR using the primers A and B or C (Table 1), each containing a XbaI restriction site for cloning.

In the first truncated β subunit (β 109), the cysteine 110 was replaced by a stop codon (TTA instead of ACA) by

Table 1 Oligonucleotide sequence of all the primers used for the construction of cDNA for wild type, mutants and single chains. They are given with their names and single-letter abbreviation used in text

	Primer name	Primer sequence
A	5'β subunit	5'-GCTCTAGAACCAAGGATGGAGATGCTCGAGGGACT-3' <small>XbaI site</small>
B	β 109stop	5'-CGTCTAGATTAGGCCAAGGGCTGGTCTC-3' <small>stop aa109</small>
C	β 111stop	5'-CGTCTAGATTAGGCACAGGCCAAGGGCTG-3' <small>aa111</small>
D	C26A	5'-GCCTGCCCCATCGCCATCACCTTCACC-3' <small>cys → ala</small>
E	C26A reverse	5'-GGTGGAGGTGATGGCGATGGGGCAGGC-3'
F	C110A	5'-CCAGCCCTTGGCCGCTGCCCCCAGGCC-3'
G	C110A reverse	5'-GGCCTGGGGGCGAGCGGCCAAGGGCTGG-3'
H	β 5' end	5'-GCTCTAGAACCAAGGATGGAGATGCTCCAGGGACT-3' <small>Met</small>
I	β 149 3' end	5'-CGGAATTCAGAAGTCTTTATTGGGAGGGGA-3' <small>EcoRI Site aaβ149</small>
J	α 5' end	5'-CGGAATTCGCTCCTGATGTGCAGGATTGCC-3'
K	α 3' end	5'-GCTCTAGACCTTTAAATCTTGTGGTGAT-3' <small>Stop</small>
L	β 109 3' end	5'-CGGAATTCGGCCAAGGGCTGGTCTCTGAAAACCCC-3' <small>aaβ109</small>

PCR using the primers A and B. Thus, the resulting β subunit lacked both CTP and the 26–110 disulphide bond.

In the second (β111) the stop codon was inserted just after amino acid 111 (TTA instead of CTG) by PCR using the primers A and C. Thus, the resulting β subunit only lacked the CTP but retained its 26–110 disulphide bond.

Mutagenesis of cysteines 26 and 110 of the horse wild-type β LH/CG subunit, subcloned into pBluescript vector (Stratagene, Cambridge, UK) via their XbaI sites, was performed by extension of mutagenic oligonucleotides containing the mutation of cysteine 26 and/or 110 for an alanine. This was carried out using a site-directed mutagenesis kit (Quick-change site-directed mutagenesis kit, Stratagene) (Fig. 1). The sequences of the primers used to obtain the mutation in position 26 are given in Table 1 (primers D and E). The primers F and G were used to obtain the mutation in 110 using the same methods.

eLH/CG single chain was constructed using an EcoRI restriction site to join the 3' extremity of β subunit cDNA to the 5' extremity of α subunit cDNA (βα single chain). First, the β eLH/CG cDNA was amplified by PCR using the primers H and I (Table 1). The α subunit cDNA was amplified by PCR using the primers J and K.

After amplification, β and α eLH/CG cDNAs were fused via the EcoRI site then the βα single-chain was subcloned into the CDM8 vector via its XbaI sites. The signal sequence of the α subunit has been deleted from the

single chain and the signal sequence of the β subunit is used to direct production of tethered eLH/CG.

In this single chain, the 110–149 region was deleted from the β subunit by PCR using the primer H and the primer L. The β109 cDNA was then fused to the α cDNA (β109α single chain) as described above. The cysteines 26 and 110 of the β subunit were also mutated in tethered eLH/CG. This was carried out as described above for the β subunit.

Truncated and mutant β eLH/CG and single chain cDNAs were subcloned into the expression vector pCDM8 via their XbaI restriction sites. All constructs were amplified in MC1061/P3 bacteria (Invitrogen BV, Groningen, The Netherlands) and purified using the Qiagen maxiprep plasmid kit (Coger, Paris, France).

The sequences of all resulting constructs were checked by double-stranded DNA sequencing.

Transient transfections of COS7 cells

COS7 monkey kidney cells (ATCC–CRL 1651) were co-transfected at 65% confluence in 6 cm diameter Petri dishes with 2 μg pCDM8α-eLH and 2 μg pCDM8β-eLH or with 2 μg of one of the three mutated β subunit constructs using a calcium phosphate precipitation procedure as previously described (Chopineau *et al.* 1997). For single-chain chimeras, COS7 cells were transfected with 4 μg pCDM8 βαeLH/CG. The cells were incubated

with 3 ml serum-free DMEM for 48 h. The media were then harvested, centrifuged and the supernatants stored at -20°C until analysis.

The supernatants from transfected COS7 cells were quantified using the ELISA described below before being assayed in the *in vitro* bioassays. Supernatants were concentrated four times with Centricon YM 10 (Amicon, Millipore Corporation, Bedford, MA, USA) when the secreted amounts of recombinant hormone were too low to permit accurate measurement of the activities.

Quantitation of expressed hormones

Three different ELISAs were used to quantify the expressed hormones.

First, the quantity of recombinant wild-type, mutant and single chain horse LH/CG present in the harvested media was estimated in a dimer-specific sandwich ELISA using two monoclonal antibodies (mAbs) directed against eCG (one anti α , 89A2 and one anti β , 24A3) as previously described (Chopineau *et al.* 1993, 1997) and eCG FL652 (5000 UI/mg), purified in our laboratory as the reference preparation. As this assay is dimer-specific, none of the free subunits cross-react, allowing only the detection of assembled subunits.

A second sandwich ELISA was used to quantify single chains and to detect the dimer and free α subunit in the supernatant from COS7 co-transfected with α and β subunit cDNAs. Briefly, the first antibody (anti- α 89A2) was coated on to the microtitration plate. After washing and saturation, increasing concentrations of standard eCG or the sample were added. After 1 h at 37°C , the excess of hormone was washed and a rabbit polyclonal antibody directed against eCG (Cahoreau & Combarnous 1987), which also recognizes free subunit in our ELISA system, was added. After 1 h at 37°C and washing, the plate was incubated with a 1/1000th dilution of a horseradish peroxidase linked anti-rabbit antibody for 1 h at 37°C . The plates were washed again and the substrate (*o*-phenylenediamine) added (50 $\mu\text{g}/100\ \mu\text{l}$ 100 mM sodium acetate pH 5.6 and 10 μl H_2O_2) and the absorbance measured at 492 nm after 15 min at room temperature. This assay detects free α subunit at a level of 35% (w/w), in addition to the heterodimer. α subunit expressed alone in COS7 cells is correctly secreted and detected in the medium by this sandwich ELISA at a range of 20% compared with the wild-type heterodimer. The difference between these two sandwich ELISAs allows the measurement of the free α subunit.

As all the results are based on the estimation of the production by immunoassay, a third ELISA was used to confirm the estimated quantities by the two sandwiches. This is a competitive ELISA using the anti- α mAb 89A2 detecting total α , free plus combined. As previously described by Chopineau *et al.* (1993), this mAb presents 63% w/w of cross-reaction with free α subunit. As this

mAb is anti- α , as we never mutated the α subunit and as this mAb recognizes free and combined α subunits, we can reasonably assume that its epitope is always intact on all the recombinant products.

Estimation of LH bioactivity

LH bioactivity of recombinant wild type and mutant LH/CG was estimated by measuring the testosterone production in a rat Leydig cell bioassay (Guillou *et al.* 1985). Briefly, Leydig cells from 52-day-old Wistar rats were dispersed with collagenase and incubated for 4 h at 34°C in Leibowitz L15 medium, with the samples of recombinant hormones being tested at different concentrations based on ELISA estimates. After incubation, the media were harvested, centrifuged and supernatants were stored at -20°C until testosterone assay in a specific RIA.

Estimation of FSH bioactivity

FSH bioactivity was assessed in an *in vitro* progesterone stimulation assay using Y1 cell line from a mouse adrenal cortex tumour stably expressing the human FSH receptor (a kind gift from Ares Serono, Geneva, Switzerland) (Kelton *et al.* 1992). Approximately 150 000 cells were incubated in 1 ml growth medium (Ham's F10, 15% horse serum (HS), 2.5% foetal bovine serum (FBS), 1% Gln, 80 $\mu\text{g}/\text{ml}$ Geneticin (G418) and 2.5 $\mu\text{g}/\text{ml}$ fungizone) in 24-well plates for 2 days. The medium was changed and the cells incubated for another 2 days and then incubated with 1 ml medium containing less serum (Ham's F10, 5% HS, 0.8% FBS, 1% Gln, 80 $\mu\text{g}/\text{ml}$ G418 and 2.5 $\mu\text{g}/\text{ml}$ fungizone) for 24 h before the final 4-h incubation in 800 μl of stimulation medium (Ham's F10, 1 mg/ml BSA, 1% Gln, 80 $\mu\text{g}/\text{ml}$ G418) plus 200 μl COS7 medium containing recombinant hormones. Finally, the media were harvested, boiled for 10 min, centrifuged and the supernatants were collected in glass-heated tubes and assayed for progesterone using a specific RIA (Saumande *et al.* 1985).

Statistical analysis

Five expressions of each mutant were achieved. All immunoassays and bioassays were done once or twice for each expression. Statistical analyses were carried out using a paired *t*-test.

Results

Elimination of the 26–110 disulphide bond and CTP of β subunit influence the production of the mutated hormones

Deletion of the CTP leads to a 50% diminution in the detection of mutant $\alpha\beta$ 111 heterodimer which can not be ascribed to dissociation since no more free α subunit could

Table 2 Production of all constructs estimated by the three different ELISA systems: (1) specific dimer $\alpha\beta$ ELISA, (2) free α and $\alpha\beta$ sandwich ELISA and (3) free and combined α -specific ELISA. For each construction, three to five expressions were carried out. The quantity of each mutant is given as a percentage $\pm$ S.E.M. of the wild-type $\alpha\beta$

Constructions	Specific dimer ELISA (%)	Free $\alpha + \alpha\beta$ sandwich ELISA (%)	Free and combined α displacement ELISA (%)
$\alpha\beta$	100	100	100
$\alpha\beta$ 109	4.6 ± 1.7	8.6 ± 4.3	16.75 ± 9.3
$\alpha\beta$ 111	49 ± 18.2	55 ± 18.9	77.3 ± 8.1
$\alpha\beta$ C26–110A	30.8 ± 8.1	29.8 ± 7.4	30 ± 9
Single chain $\beta\alpha$	97 ± 20	105 ± 31.4	ND
Single chain β 109 α	5	9.2 ± 2.4	ND
Single chain β C26–110A α	ND	85 ± 28.4	ND

ND: not determined.

be demonstrated for the mutated dimer compared with the wild type (Table 2), even if in the third ELISA, the level of α subunit seems overestimated. A drastic decrease in the production of the mutant $\alpha\beta$ 109 in which both the CTP and the 26–110 disulphide bond were deleted was shown by the different ELISAs (Table 2). The analysis of the three ELISAs shows that there is no more free α subunit in the $\alpha\beta$ 109 medium than in the wild-type medium. Quantitation of corresponding single-chain shows that full-length $\beta\alpha$ was produced at the same level as wild-type $\alpha\beta$ dimer, whereas the production of the β 109 α single chain was only tenfold less than the $\alpha\beta$ 109 dimer (Table 2).

To determine precisely the role of the 26–110 disulphide bond on the formation of recombinant eLH/CG, cysteines 26 and 110 of the wild-type β subunit were mutated into alanine by site-directed mutagenesis (β C26–110A). This disulphide bridge was also eliminated in the $\beta\alpha$ single chain hormone (β C26–110A α). The elimination of β 26–110 disulphide bond in β ($\alpha\beta$ C26–110A dimer) led to diminished detection (30%) in the dimer-specific ELISA compared with the wild-type $\alpha\beta$ (Table 2). In contrast, the deletion of the bridge did not affect the production of the β C26–110A α single chain hormone (85% compared with the $\alpha\beta$ wild type). Since there was no increase in free α subunit in the media, the low detection rate can again be attributed to a lower production of the $\alpha\beta$ C26–110A dimer compared with the wild-type $\alpha\beta$.

The CTP and ‘the fastening of the seat belt’ by the β 26–110 disulphide bond are not required for the bioactivities of recombinant eLH/CG

The LH and FSH bioactivities of eLH/CG were unaffected by the deletion of CTP as observed in Fig. 2A and B ($\alpha\beta$ 111 heterodimer). The FSH/LH ratio was 2.46 ± 0.64 fold compared with the wild type (Table 3). However, this difference was not significant since the FSH

bioactivity estimated for this mutant was not significantly different from wild-type FSH activity. As for $\alpha\beta$ 111, the LH and FSH activities of the $\alpha\beta$ 109 heterodimer (Fig. 2A and B), and of the full-length $\beta\alpha$ and β 109 α single chain (Fig. 2C and D, Table 3) were detected and were identical to those of the wild type. Therefore, the bioactivities of eLH/CG were not modified by the elimination of both the CTP and the 26–110 disulphide bond. Concerning the role of the 26–110 S–S bond strictly, as for previous mutants, LH and FSH activities of the $\alpha\beta$ C26–110A dimer and the β C26–110A α single chain were similar to those of wild-type $\alpha\beta$ (Fig. 2, Table 3).

Discussion

In the present study, it was demonstrated that the absence of CTP and/or the 26–110 disulphide bond in the eLH/CG β subunit does not impede its association to α subunit and does not reduce its LH and FSH bioactivities. Nevertheless, it severely reduces its production without affecting its quaternary structure upon secretion.

These results on eLH/CG are partially in agreement with those concerning hCG and allow comparison of these two gonadotrophins. Many studies have addressed the role of hCG CTP but, though having about the same length (≈ 30 amino acids) and the same characteristics as that of eLH/CG (several O-linked sugars), it exhibits an amino acid sequence which largely differs from that of eLH/CG. As observed here for eLH/CG, deletion of hCG β CTP has no effect on heterodimer formation and *in vitro* bioactivity (El Deiry *et al.* 1989). However, the *in vivo* activity of this hormone was much reduced, in accordance with the fact that the O-linked oligosaccharides present on the CTP extension are important for the half-life of the hormone (Matzuk *et al.* 1987, 1990). The fusion of a β CTP to hFSH β subunit had no effect on its *in vitro* activity but enhanced its *in vivo* half-life and consequently its *in vivo* activity. Interestingly, it also increased its

Figure 2 LH and FSH bioactivities of each eLH/CG mutant and single chain. Estimation of LH bioactivity was carried out by stimulation of rat Leydig cells. For each heterodimer (A) and eLH/CG single chain (C), the production of testosterone was represented as a function of quantities of each one estimated by ELISA. Each point is the mean $\pm$ S.E.M. from one representative experiment. The estimation of FSH bioactivity of heterodimer (B) and tethered eLH/CG (D) was carried out by stimulation of Y1 cells. After stimulation, progesterone was assayed with a specific RIA and expressed as a function of quantities of each mutant or wild-type recombinant eCG estimated by ELISA. Each point is the mean $\pm$ S.E.M. from one representative experiment. $\alpha\beta e$ ■; $\alpha\beta 111$ ◇; $\alpha\beta 109$ ●; $\alpha\beta C26-110A$ ▼; $\beta\alpha$ single chain □; $\beta 109\alpha$ ○; $\beta C26-110A\alpha$ ▽.

production rate (Fares *et al.* 1992). Elimination of the β CTP in a tethered $\beta\alpha$ hCG did not affect its *in vitro* bioactivity but lowered its production by fivefold compared with full-length tethered $\beta\alpha$ hCG (Narayan *et al.* 1995).

In the present study, the elimination of the CTP had no effect on *in vitro* LH and FSH bioactivities of the eLH/CG dimer. This agrees with a study of Bousfield *et al.* (1989) on pituitary eLH, in which the CTP was removed by selective acid hydrolysis (eLH des 121–149) and which exhibited the same *in vitro* LH activity as native eLH (its FSH activity was not determined). In a more recent study

(Min *et al.* 1996), the elimination of β CTP in recombinant eLH/CG (des 115–149= $\alpha\beta 114$) was shown to increase its FSH activity by tenfold compared with wild-type hormone without changing its LH activity. The authors suggested a negative role for O-linked oligosaccharides in the FSH activity of eLH/CG. In the present study, elimination of the $\beta 112-149$ region had no effect on LH bioactivity of eLH/CG (Table 2, Fig. 2) but did not significantly increase its FSH bioactivity. Since the mutations do not match exactly in the two studies, one interpretation would be that residues 112–114 might be important for FSH activity. Another explanation is that the

Table 3 LH and FSH bioactivities of mutated recombinant eLH/CG. Estimated LH and FSH activities represent the hormone concentration (ng/ml) that yields half-maximal stimulation of testosterone production for LH bioactivity and/or progesterone for FSH bioactivity. The LH and FSH estimates are from four to seven experiments (mean $\pm$ S.E.M.) each conducted in duplicate

Constructions	LH (ng/ml)	FSH (ng/ml)	FSH/LH
$\alpha\epsilon\beta\epsilon$	20.12 $\pm$ 11.3	26 $\pm$ 11.7	1.3 $\pm$ 0.35
$\alpha\epsilon\beta$ 109	21.9 $\pm$ 14.6	23.02 $\pm$ 13.3	1.05 $\pm$ 0.13
$\alpha\epsilon\beta$ 111	15.1 $\pm$ 4.6	37.11 $\pm$ 6.2	2.46 $\pm$ 0.64
$\alpha\epsilon\beta\epsilon$ C26-110A	15.9 $\pm$ 7.7	19.72 $\pm$ 6.4	1.3 $\pm$ 0.53
Single chain $\beta\alpha$	25.9 $\pm$ 11.7	37.3 $\pm$ 2.34	1.44 $\pm$ 0.3
Single chain β 109 α	14.46 $\pm$ 4.8	28.73 $\pm$ 15	1.92 $\pm$ 0.54
Single chain β C26-110A α	31.3 $\pm$ 8.65	42.05 $\pm$ 4.95	1.34 $\pm$ 0.04

two molecules differ because they were expressed in CHO cells in Min *et al.* (1996) vs COS7 cells in the present study, leading to different *N*-saccharide chains. Finally, the difference could also originate from the fact that the FSH bioassays used were different. The CTP of eLH/CG could possibly interfere with FSH binding in the primary rat granulosa cell system used by Min *et al.* (1996) but not in the Y1 cell line expressing the human FSH receptor that we used.

As in the case of CTP, the absence of a 26–110 disulphide bridge had no effect on eLH/CG bioactivities. Most studies did not investigate the bioactivity of 26–110 C→A mutants because the amount recovered in the medium was too small. However, tethered gonadotrophins have recently been constructed to bypass the problem of assembly, and a single chain hCG $\beta\alpha$ mutated in 26–110 was shown to exhibit full LH activity *in vitro* compared with the one-chain molecule with an intact 26–110 disulphide bridge (Ben-Menahem *et al.* 1997). Our data confirm that this bridge is not required for LH bioactivity but is also not indispensable for the FSH bioactivity of eLH/CG. More surprisingly, this result indicates that the fastening of the 26–110 bridge is not mandatory for the assembly of α eLH/CG and β eLH/CG which is required for the expression of its LH and FSH activities. By contrast, the disruption of this bond in hTSH abolished its bioactivity (Medeiros-Neto *et al.* 1996).

A recent paper (Jackson *et al.* 1999) proposed that all the quaternary structure of hCG was not required for its *in vitro* bioactivity. The present data would, at least in part, support this view. Nevertheless, elimination of CTP and/or the 26–110 S–S bond leads to a decreased production of the heterodimer of both mutated dimers ($\alpha\beta$ 109, $\alpha\beta$ 111, $\alpha\beta$ C26–110A) and truncated one-chain gonadotrophin (β 109 α). This decrease could not be ascribed to a defect in heterodimer assembly since no free α subunit was detected in the culture medium by the different ELISA systems used (ELISAs 2 and 3, Table 2), keeping in mind that α subunit expressed alone in COS7 cells is well

secreted and detected by these immunoassays at a range of 20% (data not shown). Moreover, the different ELISAs used an anti- α mAb and this subunit was never mutated, so we could reasonably assume that its epitope is always intact. It is now well established that the CTP and the 26–110 disulphide bridge of the hCG β subunit are involved in the intracellular trafficking and secretion of the hCG heterodimer (Suganuma *et al.* 1989, Huth *et al.* 1993, Muyan *et al.* 1996, Ruddon *et al.* 1996). It seems to be the same for eLH/CG, particularly for the 26–110 disulphide bridge since its deletion induced a severe decrease in the heterodimer production but no decrease in the $\beta\alpha$ single chain production (Table 1). Our hypothesis based on previous data (Feng *et al.* 1996, Ruddon & Bedows 1997) is that in the case of the 26–110 mutated heterodimer, the majority of the hormone is not correctly folded for secretion, whereas the 26–110 mutated single chain takes the correct conformation required for secretion. Studies are now under way to determine how CTP and/or the 26–110 disulphide bridge influence synthesis and/or secretion of the hormone.

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