The β104–109 sequence is essential for the secretion of correctly folded single-chain βα horse LH/CG and for its FSH activity

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Abstract

The dual LH and FSH activity of the equine LH (eLH)/equine chorionic gonadotropin (eCG) in heterologous species makes eLH/CG a good model to study structure/function relationships of gonadotropins. In order to bypass the problem of intracellular association of the heterodimer, a recombinant single-chain βαeLH/CG was used to identify sequences in the β-subunit involved in the secretion and activities of the hormone. The C-terminal region of the β-subunit was progressively truncated. All resulting truncated single-chains were secreted in the media as detected by an anti-βpeptide antibody in reducing conditions. However, using a conformation sensitive ELISA we show that the truncated single-chains were differently recognized: deletion of the last 40 amino acids of the β-subunit (β109αeLH/CG) resulted in a 90% decrease in the recognized correctly folded hormone compared with the full-length βαeLH/CG single-chain and no properly folded hormone was detected in the secretion medium when the last 46 amino acids of the β-subunit were deleted (β103αeLH/CG). We thus focused on the six amino acids sequence 104–109, which belongs to the seat-belt region. Mutation of the 104–109 sequence in alanines in the full-length βαeLH/CG (β104–109Alaβ) led to a 50% decrease in the production of properly folded hormone in COS-7 as well as in αT3 pituitary cells. Moreover, the FSH activity of this mutant was decreased by 70% whereas its LH activity remained intact. These data lead us to conclude that the 104–109 region of the β eLH/CG subunit is essential for the secretion of a fully folded βαeLH/CG and for its FSH activity but not for its LH activity.


Introduction

The LH, FSH, and chorionic gonadotropin (CG) belong to the glycoprotein hormone family and are involved in the control of gonadal function. LH and FSH are secreted by the pituitary gland in all mammalian species, whereas CG is secreted by the placenta only in primates and equids. All glycoprotein hormones consist of two noncovalently associated α- and β-subunits. Within a species, the α-subunit is identical for the different glycoprotein hormones and is encoded by a single gene. The β-subunit is specific for each glycoprotein hormone and confers their specific activity. Only the αβ dimer is active (Pierce & Parsons 1981, Combarnous 1992). The resolved structures of deglycosylated human CG (hCG; Lapthorn et al. 1994, Wu et al. 1994) and FSH (Fox et al. 2001) revealed that both subunits present a cystine-knot motif giving a similar structure composed of three disulfide-bonded loops (Avsian-Kretchmer & Hsueh 2004). Numerous studies have shown that the region between the 10th (Cys893) and 12th cysteine (Cysβ110) of the β-subunit is important for specific receptor recognition (Dias et al. 1994, Han et al. 1996, Grossmann et al. 1997) of hCG and human FSH (hFSH). This region, also called the ‘seat-belt region’, wraps around the α-subunit and is stabilized by β26–110 disulfide bond.

In the horse, equine CG (eCG) has the same amino acid sequence as equine LH (eLH) because they are encoded by the same common horse α gene and the same βLH/CG gene (Sherman et al. 1992). The recombinant hormone is thus referred as eLH/CG and is a good model for structure/function relationships of gonadotropins since it exhibits both LH and FSH activities in nonequid species, like its pituitary and placental counterparts (Stewart et al. 1977, Guillou & Combarnous 1983).

Interestingly, the β-subunits of hCG, eCG and eLH present an extension of about 30 amino acids carboxy-terminal peptide (CTP) that typically contains four (hCG) to up to 12 (eLH, eCG) clustered O-linked oligosaccharides (Bousfield & Butnev 2001), which confer prolonged circulatory survival of the hormones (Matzuk et al. 1990, Murphy & Martinuk 1991).

The hydrophilic CTP of hCG contains determinants that favor the secretion of recombinant hLH and hFSH when fused to the C-terminus of their β-subunits (Fares et al. 1992, Di Menza et al. 1998, Di Menza et al. 2000).
Muyan et al. 1996). We also showed that deletion of the CTP downstream of residue 111 in the dimeric eLH/CG (ζeβe111LH/CG) induces a 50% decrease in the secretion of the truncated hormone compared with the full-length ζeβeLH/CG (Galet et al. 2000) as detected by ELISA.

When the hormone was further truncated downstream of residue 109 (ζeβe109LH/CG) thus eliminating cysteine 110, a significant decrease in its secretion by COS-7 cells was of the truncated hormone compared with the full-length eLH/CG (Galet et al. 2000). It was thus hypothesized that residues upstream of the βCTP, i.e. belonging to the ‘seat-belt’ region, were also involved in the folding and secretion of gonadotropins.

Since the deletions and/or mutations of the β-subunit upstream of CTP involved in the seat-belt, we used ζeβeLH/CG single-chain mutants in order to ensure stable association of ζ- and β-subunits. We found that substitution of the six residues in the 104–109 sequence by Ala residues led to diminished production of conformational antibody recognized hormone in COS-7 cells as well in αT3 pituitary cell line. Moreover, FSH activity of the mutated hormone was strongly affected in contrast to its LH activity.

### Materials and Methods

#### Construction of mutants’ eLH/CG single-chains

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) as previously described (Galet et al. 2000). Briefly, the β eLH/CG cDNA (Chopineau et al. 1995) was amplified by PCR using the A and B primers (Table 1). The α-subunit cDNA (Chopineau et al. 1997) was amplified by PCR using the C and D primers. After amplification β and α eLH/CG cDNAs were fused via the EcoR1 site. The signal sequence of α-subunit had been deleted from the single-chain and the signal sequence of β-subunit was used to direct production of tethered eLH/CG (Fig. 1).

The C-terminal region of the β-subunit was progressively deleted in the βα eLH/CG. First, the 110–149 sequence was deleted from the β-subunit by PCR using the A and E primers. The β109α cDNA was then fused to the αβ cDNA (β109α single-chain) as described above. Using the same strategy, two other truncated single-chains, β103α and β96α single-chains, were constructed using, the A and F; and A and G primers respectively to amplify the truncated β-subunit (Table 1).

Another single-chain was constructed in which the sequence 104–109 of the β-subunit was substituted by alanines (Fig. 1) using a site-directed mutagenesis kit (Quick-change site-directed Mutagenesis kit (Stratagene, Cambridge, UK)). The sequences of the primers used to obtain the mutation are described in Table 1 (primers H and I).

Truncated and mutant βζeLH/CG 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) and purified using the Qiagen maxiprep plasmid kit (Coger). The sequences of all resulting constructs were verified by double-stranded DNA sequencing.

#### Transient transfections of cells

COS-7 monkey kidney cells (ATCC-CRL 1651) and αT3, a mouse gonadotrope cell line, kindly provided by Dr P Mellon (University of California, San Diego, USA) were used. Cells were transfected at 65% confluence in 6 cm diameter Petri dishes with 2 μg pCDM8 containing wild-type or mutated βζeLH/CG using either a calcium phosphate

### Table 1 Sequence of the primers

<table>
<thead>
<tr>
<th>Primer names</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>A</td>
<td>β 5′ extremity</td>
</tr>
<tr>
<td>B</td>
<td>β 3′ extremity</td>
</tr>
<tr>
<td>C</td>
<td>α 5′ extremity</td>
</tr>
<tr>
<td>D</td>
<td>α 3′ extremity</td>
</tr>
<tr>
<td>E</td>
<td>β109 3′ extremity</td>
</tr>
<tr>
<td>F</td>
<td>β103 3′ extremity</td>
</tr>
<tr>
<td>G</td>
<td>β96 3′ extremity</td>
</tr>
<tr>
<td>H</td>
<td>X104–109A</td>
</tr>
<tr>
<td>I</td>
<td>X104–109A reverse</td>
</tr>
</tbody>
</table>

In bold are restriction sites, Meth, Stop and Ala mutations. In italics are last aa of the β-subunit.
A previously described sandwich ELISA (Galet et al. 2000) was used to quantify single-chain eLH/CGs in the cell lysates and in the media. Briefly, the first antibody (89A2, mAb raised against native eCG and showing 63% w/w of cross-reaction with free horse CG α-subunit, and no cross-reaction with free eCG β-subunit, Chopineau et al. 1993) was coated on to the microtitration plate. This antibody is horse specific (it does not recognize α-subunit from other species) and conformational since it does not recognize reduced and carboxymethylated α-subunit neither in ELISA nor in western blot. After washing and saturation, increasing concentrations of standard eCG (eCG FL652 (5000 IU/mg), purified in our laboratory) 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 was added (Lecompte & Combarnous 1992). This antibody also recognizes a conformational epitope as it only reveals hormone in native conditions. After 1 h at 37 °C and washes, the plate was incubated with a 1/1000 dilution of a HRP-linked anti-rabbit antibody for 1 h at 37 °C. The plates were washed again and the TMB substrate added; the reaction was stopped after 15 min with H2SO4 and the absorbance measured at 450 nm.

Detection of expressed hormones by western blot

When necessary, media samples were concentrated using Microcon Ultracell YM-10 (Millipore, Molsheim, France; 10 kDa cut-off). Equal volume of media and equal protein amounts of cell lysates were subjected to 12% SDS-PAGE after addition of 2× Laemmli sample buffer (125 mM Tris pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.001% bromophenol blue), and 5 min of boiling. After electrophoresis, the gels were blotted onto a nitrocellulose membrane (Protran, Schleicher and Schuell, Biosciences, Mantes La Ville, France) for 2 h at 4 °C to 250 mA using 25 mM Tris, 192 mM glycine, pH 8.3 containing 20% methanol. The membranes were then incubated overnight at 4 °C in blocking buffer (5% nonfat dry milk/TBS/0.1% Tween). The membranes were then incubated for 2 h at RT with a 1/4000 dilution in TBS Tween with 1% nonfat dry milk of a rabbit polyclonal anti-peptide antiserum (497β) raised against a synthetic peptide corresponding to the nine N-terminal amino acids of the horse βLH/CG subunit. The membranes were then washed thrice in TBS Tween and incubated with a 1/10 000 dilution of a goat anti-rabbit IgG HRP conjugate (Bio-Rad) in TBS Tween with 1% nonfat dry milk for 1 h at RT followed by three washes in TBS. Finally, the nitrocellulose membranes were revealed using ECL luminal substrate (Super Signal West Pico chemiluminescent, Pierce, PerbioSciences, Brebieres, France) and exposed to X-ray films (Hyperfilm ECL, Amersham). The films were scanned and densitometric analyses of the signals were performed with Image J (http://rsb.info.nih.gov/ij/).

Detection of expressed hormones by ELISA

Bioactivities

LH bioactivity LH bioactivity was assessed in an in vitro progesterone stimulation assay using MLTC-1 cell line from a mouse Leydig tumor (Rebois 1982). About 150 000 cells per well were incubated at 37 °C in 0.5 ml supplemented RPMI growth medium (10% FBS, 50 μg/ml gentamicin, 10 units/ml penicillin and 10 μg/ml streptomycin) in 48-well plates for 2 days. One hour before stimulation, media were replaced with serum-free RPMI media and then stimulated.
for 3 h with 400 μl serum-free RMPI media plus 200 μl COS-7 or αT3 media containing recombinant hormones. The supernatants were harvested and stored at −20 °C until assayed for progesterone using a specific RIA (Saumande et al. 1985).

**FSH bioactivity** FSH bioactivity was assessed in an *in vitro* progesterone stimulation assay using Y1 cell line from a mouse adrenal cortex tumor stably expressing the hFSH receptor (kindly provided by Ares Serono, Geneva, Switzerland) as already described (Chopineau et al. 1997).

**Data analysis**

All of the assays were analyzed with GraphPad Prism2 (GraphPad Software, San Diego, CA, USA). For bioactivities studies, the ligand concentrations that induced half-maximal (GraphPad Software, San Diego, CA, USA). For bioactivities studies, the ligand concentrations that induced half-maximal stimulation (i.e. EC₅₀ values) were calculated by fitting the data to the sigmoidal dose–response curves. Statistical comparisons were performed on the log (EC₅₀) values using one-way ANOVA, followed by the Bonferroni test. A *P* value of <0.05 was considered statistically significant.

**Results**

**Single-chain βαεLH/CG with shortened β are all secreted by COS-7 cells, but at different levels**

To assess the importance of the C-terminal region of β-subunit in the production of the eLH/CG, a βαε single-chain was constructed to bypass any effects linked to the intracellular association of the α- and β-subunits. The C-terminal region of the β-subunit was progressively deleted in single-chain mutants. The expression of recombinant hormones in total cell lysate extracts as well as in the secretion media was assessed by western blotting using an anti-βpeptide. It is important to note that in western blot this anti-βpeptide antibody only recognizes the protein under reducing conditions and not under nonreducing conditions (data not shown). Therefore, western blot analysis under reducing conditions allows us to assess the expression of all forms of recombinant βαεLH/CG single-chains.

Table 2 shows that all constructs were detected in the media but secretion of the β109α and β103α single-chain mutants is decreased to 30% that of the βαεLH/CG single-chain. Secreted βαεLH/CG single-chain was detected as a doublet of ~46 and 44 kDa (Fig. 2) whereas secreted β109α and β103α single-chains were detected at ~40 and 38 kDa respectively. Secreted β96α exhibited a doublet at ~38 and 36 kDa (Fig. 2).

All constructs were present in the cellular lysates and were expressed at similar levels (Table 2), but exhibited different molecular weights: single-chain βαεLH/CG appeared as a doublet at 40 and 37 kDa and other minor forms including one at 25 kDa (Fig. 2), whereas truncated β109α as well as β103α single-chains also appeared as doublets: at 36 and 34 kDa (plus a band around 22 kDa). Intracellular β96α also showed a doublet at 34 and 32 kDa (plus a band around 20 kDa).

### Table 2 Relative quantification of the production of the different constructs, estimated by western blot under reducing conditions with anti-peptide 497β, in media and cell lysates.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Media</th>
<th>Lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>βαεLH/CG single-chain</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>β109α single-chain</td>
<td>39 ± 23</td>
<td>102 ± 21</td>
</tr>
<tr>
<td>β103α single-chain</td>
<td>26 ± 7</td>
<td>98 ± 20</td>
</tr>
<tr>
<td>β96α single-chain</td>
<td>120 ± 10</td>
<td>151 ± 11</td>
</tr>
<tr>
<td>104–109Alaβα single-chain</td>
<td>111 ± 32</td>
<td>96 ± 31</td>
</tr>
</tbody>
</table>

**Deletion of the C-terminal region of β eLH/CG subunit affects the production and secretion of the correctly folded βαεLH/CG single-chain hormone in COS-7 cells**

The use of a conformation sensitive ELISA (89A2/polyclonal ELISA) allowed us to determine the level of secretion of the correctly folded βαεLH/CG single-chains. As shown in Table 3, the deletion of the 97–149 or the 104–149 sequence were the most drastic since no correctly folded hormone.
Table 3 Production of the different constructs estimated by the conformation sensitive sandwich ELISA in the media and the cell lysates. The quantity of hormone produced is given as a percentage ± S.E.M. of the wild-type β104−109AlaεLH/CG single-chain taken as 100%. For each construction, nine to 13 transfections were carried out in COS-7 cells and six in αT3 cells

<table>
<thead>
<tr>
<th>Construct</th>
<th>Medium (%)</th>
<th>Lysate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β104−109AlaεLH/CG single-chain</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>β109εLH/CG single-chain</td>
<td>9.1±1.5</td>
<td>15.7±4.2</td>
</tr>
<tr>
<td>β103εLH/CG single-chain</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>β96εLH/CG single-chain</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>104–109AlaβεLH/CG single-chain</td>
<td>54±6.5</td>
<td>53.1±6.4</td>
</tr>
<tr>
<td>αT3</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>104–109AlaβεLH/CG single-chain</td>
<td>37.7±7.1</td>
<td>43.2±8.8</td>
</tr>
</tbody>
</table>

ND, not detectable.

was detected in the culture media after 48 h of expression. The deletion of the 110–149 region was less drastic and allowed secretion of 10% correctly folded β109εLH/CG compared with wild-type βεLH/CG (Table 3). The hormone levels detected by this conformation sensitive ELISA in media and in cell extracts are always closely correlated indicating that correct folding is a prerequisite for secretion.

Identification of a C-terminal β-subunit sequence involved in βεLH/CG production and secretion in COS-7 cells

The lower production of correctly folded β103εLH/CG (0%) and β109εLH/CG (10%) single-chain mutant hormones compared with βεLH/CG (Table 3) suggested that the β104−109 sequence could play a role in the production of correctly folded hormone. Since truncation of this region leads to a shortening of the linker between subunits that can drastically affect their relative orientations and correct folding, we studied this issue by Ala replacement of all residues in the β104−109 sequence by site-directed mutagenesis. As a result, the overall production of the X104−109A mutated βεLH/CG in COS-7 cells was identical to that of the wild-type single-chain as detected by western blot using the anti-βpeptide (Table 2). The production of correctly folded hormone was 50% that of the wild-type βεLH/CG as detected by the conformation sensitive ELISA (Table 3).

Bioactivities of βεLH/CG mutants secreted in COS-7 cells

LH and FSH activities of truncated and Ala-mutated βεLH/CG single-chains were measured as described in Materials and Methods, and results are given according to initial quantification of the constructs by the conformation sensitive ELISA (Table 4). The secretion media from COS-7 cells expressing truncated β96εLH/CG exhibited no LH and no FSH activities, even when concentrated.

The LH bioactivity of both secreted full-length β104−109AlaεLH/CG single-chain and truncated β109εLH/CG single-chain is similar to that of wild-type single-chain βεLH/CG (Table 4 and Fig. 3). In contrast, the FSH activity of truncated β109εLH/CG single-chain is similar to that of wild-type βεLH/CG single-chain whereas the full-length β104−109AlaεLH/CG single-chain exhibited only 25% FSH activity relative to the wild-type βεLH/CG single-chain (Table 4 and Fig. 3).

Table 4 LH and FSH bioactivities of the different constructs expressed in the COS-7 and αT3 cell lines. Estimated LH and FSH activities represent the hormone concentration (ng/ml), estimated by the conformation sensitive ELISA, that yields to half-maximal stimulation in each bioassay (EC50). Results from four different experiments (mean ± S.E.M.) each conducted in duplicate

<table>
<thead>
<tr>
<th>Construct</th>
<th>LH (ng/ml)</th>
<th>FSH (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>βεLH/CG</td>
<td>29.4±7.3*</td>
<td>33.9±3.5*</td>
</tr>
<tr>
<td>β109εLH/CG</td>
<td>14.46±4.8*</td>
<td>28.73±15*</td>
</tr>
<tr>
<td>β103εLH/CG</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>β96εLH/CG</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>β104−109AlaβεLH/CG</td>
<td>32.9±9*</td>
<td>134.7±17.2*</td>
</tr>
<tr>
<td>αT3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>βεLH/CG</td>
<td>22.9±1.8*</td>
<td>47.9±6.6*</td>
</tr>
<tr>
<td>β104−109AlaβεLH/CG</td>
<td>23.3±1.7*</td>
<td>132.7±27.4*</td>
</tr>
</tbody>
</table>

ND, not detectable. Within columns, values with different superscripts differ significantly (P < 0.001).

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Figure 3 LH and FSH bioactivities of the wild-type βεLH/CG and β104−109AlaεLH/CG single-chains along with purified eCG652 taken as a standard. The bioactivities of the recombinant hormones expressed in COS-7 cells and αT3 cells are shown. The LH activity was assayed on MLTC-1 cells, whereas the FSH activity was measured by stimulation of Y1 cells stably transfected with the human FSH receptor. After stimulation, progesterone was assayed with a specific RIA and expressed as a function of quantities of each construct estimated by conformation sensitive ELISA. Each point is the mean ± S.E.M. from one representative experiment.

- eCG652; ▼ βεLH/CG single-chain; ● β104−109AlaεLH/CG single-chain.


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The βαeLH/CG single-chains expression is identical in COS-7 and in αT3 cells

Although the production of the βαeLH/CG single-chain is four to five times less in αT3 cells than in COS-7 cells, the effect of the progressive truncation of the β-subunit C-terminal tail on the secretion of the resulting βαeLH/CG single-chains is similar to those in COS-7 cells. Using conformation-sensitive ELISA, no hormone was detected in the culture media from cells expressing β103α single-chain. Mutation of the 104–109 sequence into alanine residues (β104–109Ala) led to a 60% decrease in secretion compared with the wt βαeLH/CG single-chain. Similarly, the effect of the progressive truncation of the β-subunit C-terminal tail on the LH and FSH bioactivities of the resulting βαeLH/CG single-chains was identical in αT3 cells and COS-7 cells. The β104–109Ala single-chain expressed in αT3 cells exhibited a 65% decrease in FSH activity compared with wt βαeLH/CG single-chain, whereas LH activity remained unchanged (Table 4 and Fig. 3). Mock-transfected cell media tested as negative controls did not present any LH or FSH bioactivities.

Discussion

Like hCG, eLH and eCG possess a CTP in their β-subunits (amino acids 122–149) that are highly О-glycosylated (Bousfield & Butnev 2001). In hCG, the CTP has been shown to be important for the correct intracellular folding of the hormone and consequently for its efficient secretion (Matzuk et al. 1989, Muyan et al. 1996, Muyan & Boime 1998). Moreover, the addition of the hCG CTP to single-chain glycoprotein hormones such as βαzLH, βαzFSH, and βαzTSH improved their secretion compared with the ‘linkerless’ heterodimeric variants (Sugahara et al. 1996, García-Campayo et al. 1997, Fares et al. 1998). Recently, it has been demonstrated that homologous and heterologous CTP linker sequences enhance the secretion of bioactive single-chain bovine LH analogs by about threefold (Nakav et al. 2006). Using the same conformation-specific ELISA as the one used in the present study, our laboratory previously showed that the deletion of residues 112–145 in eLH/CG β-subunit induced a 50% decrease in the secretion of the resulting dimeric eLH/CG (Galet et al. 2000).

In the present study, deletion of residues 104–145 in βαeLH/CG single-chain (β103α) led to complete abolition of secretion by COS-7 cells of correctly folded hormone as determined with the conformation-sensitive ELISA in the culture medium. Deletion of residues 110–145 (β109α) resulted in a decrease in the secretion of β109α single-chain, however, 10% of the hormone was secreted in the culture media as detected by the conformation sensitive ELISA, leading us to focus on the role of the 104–109 sequence. Interestingly, using western blot and a nonconformational antibody (anti–peptide 497β), we found that β103α single-chain was readily secreted in the media, at the same level as β109α, but three to four times less than full-length βα single-chain. In the conformation-sensitive ELISA, two conformation-specific antibodies were used: a coated monoclonal anti-eCG (cross-reacting with α) as capture antibody and a polyclonal anti-eCG (cross-reacting with β) as detector antibody rendering this assay specific for correctly folded hormones. In contrast, the polyclonal anti-peptide 497β antibody used in western blots only detects the hormones in denaturing conditions, enabling to detect all forms of the hormones. Hence, based on conformation sensitive ELISA and western blot analyses, we can speculate that β103α single-chain is indeed efficiently secreted but in a misfolded completely inactive form, whereas β109α single-chain is less efficiently secreted but about 25% of it is in an immunologically well-folded and biologically active form.

As shown by western blot analysis (Fig. 2) all single-chain mutants are secreted in the media as a doublet reflecting the presence of several forms of the single-chains, which may present different N-glycosylation status: for all, an upward MW shift was observed between secreted molecules compared with extracted molecules: ~6–7 kDa for full-length βα and 104–109Ala eLH/CG single-chains, and ~3–4 kDa for β109α, β103α, and β96α eLH/CG single-chains. The extent of these shifts is consistent with the processing of the N-linked glycans attached to the subunits (two on α-subunits and one on β-subunits) and with the addition of O-linked carbohydrates to the CTP in the Golgi prior to secretion, for full-length βαeLH/CG and 104–109Ala mutated eLH/CG only. We also observed the presence of intracellular forms of lower molecular weight (~25 kDa for βα wt and 104–109Ala eLH/CG single-chains, ~21 kDa for β109α and β103α eLH/CG single-chains, ~20 kDa for β96α), which most likely represent nonglycosylated forms of single-chain eLH/CG mutants.

Ananyhow β109α and β103α are secreted at a lower level than βα full-length single-chain in the media, whereas β96α is secreted in the medium as efficiently as full-length single-chain. The lower level of secreted β109α and β103α single-chain compared with βα full-length single-chain is not due to diminished synthesis as demonstrated by western blot analyses in cell lysates (Table 2). Therefore, it is reasonable to assume that there is a blockade in the secretion process of β109α and β103α single-chains. In contrast, the secretion of 104–109Ala mutant single-chain is identical to that of full-length βα single-chain as detected by western blot using the anti–βpeptide. These data suggest that it is the shortening of β109α and β103α single-chains rather than the β104–109 sequence that is responsible for the defect in secretion. Using the conformation sensitive ELISA, the detected quantity of β104–109Ala α single-chain is 50% of that of full-length βα, suggesting that half of this secreted single-chain is not properly folded. Collectively, the data show that the eLH/CGβ104–109 sequence is important for the secretion of a correctly folded hormone. The secreted β109α single-chain


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mutant partially retains correct folding permitting recognition in ELISA, whereas β103α single-chain must present a distorted conformation since it is not recognized in this assay. Possibly, the linkage between the β- and α-subunits becomes too short and induces a constraint disturbing the conformation of the hormone. A more important fact is that in β103α, the deletion of cysteine β110 leaves cysteine residue β26 unpaired so that it can possibly establish alternative noncorrect disulfide bridges. Interestingly, β96α single-chain that lacks both cysteine residues β100 and β110 is secreted in the medium to the same extent as the full-length β2 as detected by western blot analysis (Table 2), although it is not recognized in the conformation sensitive ELISA (Table 3). The cysteine residues in porcine LH corresponding to the two missing cysteines in β96α single-chain (β100 and β110) have been previously shown to establish a transient disulfide bridge due to an interchange between the disulfide bridges β26–110 and β93–100 (Belghazi et al. 2006) consistent with the data observed with the hCGCys93Ala in which a β100–110 bridge is also observed (Xing et al. 2004). This transient interchange allows the opening of the β-subunit seat-belt embracing its α partner and permits the association–dissociation of the α- and β-subunits. It can be hypothesized that the cysteine residues β26 and β93 can form a stable disulfide bridge giving to the β96α single-chain molecule a folded conformation that allows its transit through the ER–Golgi quality control and its secretion. However, this putative stable β26–93 bridge does not allow correct interaction with the α-subunit and therefore explains its low potency in the conformation sensitive ELISA as well as in in vitro LH and FSH bioassays.

COS-7 cells are often used for transient transfections of recombinant glycoproteins but in order to validate our findings in a more physiological model, we also used the gonadotrope αT3 cell line, which is derived from a pituitary tumor in transgenic mouse and synthesizes only free α-subunit. All data with mutants expressed in αT3 cells were similar to those previously obtained in COS-7 cells. Using conformation sensitive ELISA, β103α single-chain was undetectable in the secretion media of αT3 cells and β104–109 Ala α was detected at 40% compared with the wild-type βαeLH/CG single-chain. Thus, the defect in secretion of correctly folded hormones after mutation of these sequences is probably due to a common mechanism. In αT3 cells, there is some murine α-subunit produced (not detectable by our ELISA) but there is no evidence for an interaction of this free subunit with our constructs.

One important finding in this study concerns bioactivities. Indeed, a very close correlation is observed between ELISA data and LH bioactivity data confirming that the sandwich ELISA used in this study is highly specific for the biologically active forms of recombinant eLH/CG. As previously reported (Chopineau et al. 1997), recombinant eLH/CG molecules exhibit higher in vitro bioactivities than natural eCG. This is due to the fact that the very high carbohydrate content of the natural hormone comprising a high proportion of sialic acid residues has a strong unfavorable effect on its binding to the LH or FSH receptors that themselves also contain negatively charged sugar residues.

The β96α and β103α mutants exhibit neither LH nor FSH activities. These two shortened single-chain βα eLH/CG are thus produced in an ill-folded nonactive conformation.

Surprisingly, correctly folded β109αeLH/CG shows LH and FSH activities similar to wild-type βαeLH/CG single-chain whereas β104–109 Ala α shows a 65–75% decrease in FSH activity when expressed in αT3 cells and COS-7 cells respectively. This result is in agreement with previous findings with dimeric horse and donkey LH/CGs showing that amino acids 102–104 are important for FSH activity of these hormones. In fact, Argβ104 of eLH/CG has been shown to be required for full FSH activity (Chopineau et al. 2001). It must also be kept in mind that FSH activity of equidae’s LHs and CGs is observed exclusively in heterologous systems and that these hormones exhibit only LH activity in their own species.

In brief, the present study has pinpointed the β104–109 sequence belonging to the seat-belt region in eLH/CG as an important region for its efficient secretion and full FSH activity. Interestingly, the absence of cysteine β110 leaving cysteine β26 unpaired leads to disorganization of folding and dramatic drop in hormone secretion but full secretion is restored when cysteine β100 is also absent. This suggests that the two natural-unpaired cysteines (β26 and β93) are able to form a disulfide bridge that stabilizes a folded structure that is able to be fully secreted by the cells but that is not the properly folded structure exhibiting LH and FSH activities.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this work.

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