β-Subunit 102–104 residues are crucial to confer FSH activity to equine LH/CG but are not sufficient to confer FSH activity to human CG

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Abstract

Horse LH/CG (eLH/CG) and donkey LH/CG (dk LH/CG) are strictly LH-specific in their respective homologous species. However, both bind to the FSH receptors from non-equid species, whereas the zebra hormone (zb LH/CG) does not. The FSH/LH ratio of eLH/CG and of the dkLHβζ hybrid is about tenfold higher than that of dLH/CG and of the αβdk hybrid, showing that the β subunit contains the structural features responsible for the high FSH activity of eLH/CG. Only six amino acid positions (51, 94, 95, 102, 103 and 106) are unique to the βζ subunit when compared with the βdk and βζβ subunits. The Gly-Pro and Val-Phe sequences in positions 102–103 of βdk and βζ respectively were swapped by site-directed mutations and the mutated β-subunits cDNAs were cotransfected in COS cells with either αζ or αζζ subunit cDNA. Other mutations were also introduced in 102–103 dk LH/CG βζ-subunit: Ala-Ala, Gly-Ala or Ala-Pro. These mutations with Ala-Ala, Gly-Ala or Ala-Pro in the 102–103 βζdk LH/CG subunit did not change the FSH/LH ratio of dLH/CG but the Gly102-Pro103→Val102-Phe103 mutation promoted a marked increase in the FSH/LH activity ratio. This was observed with the two heterodimers containing αζ or αζζ. Conversely, the Val102-Phe103 mutation in βζ led to a dramatic drop in FSH/LH activity ratio of eLH/CG, to a level similar to that of dk LH/CG. Since all FSHs possess a Gly residue at position 104, we introduced the Gly102-Pro103→Arg104→Val102-Phe103-Gly104 mutation in βζdk with the expectation that the increase in FSH activity observed with the Gly102-Pro103→Val102-Phe103 mutation could be potentiated. In fact, the additional Arg104,Gly104 mutation was found to abolish the increase in FSH activity observed with Gly102-Pro103→Val102-Phe103. Mutations Gly102-Pro103→Val102-Arg103 or Gly102-Pro103→Lys Gly104 also led to a drop in FSH/LH activity ratio of dk LH/CG subunit but the Gly102-Pro103→Val102-Arg103→Gly104 were introduced in human CGβ (hCGβ) to compare the impact of these amino acid changes in the well-studied gonadotrophin hCG. The βζCG mutants obtained, co-expressed either with the human or the horse αζ-subunit, did not display any FSH activity. In conclusion, the 102–104 sequence in eLH/CG βζ-subunits appears to be of utmost importance for their binding to FSH receptors. However, these results obtained with equid βζ-subunits are not transposable to other gonadotrophins as similar mutations in hCGβ did not lead to any increase in FSH activity.


Introduction

The glycoprotein hormone family consists of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone, which are secreted by the pituitary gland in all mammalian species, and chorionic gonadotrophin (CG), which is secreted by the placenta in primates and equids. These hormones are composed of a common α-subunit and a hormone-specific β-subunit (Pierce & Parsons, 1981) which are non-covalently associated.

Among all mammalian gonadotrophins, human CG (hCG) is the most studied. Until very recently it was the only gonadotrophin for which a crystallographic model had been resolved (Lapthorn et al. 1994, Wu et al. 1994) and all other gonadotrophins were thought to have the same global conformation based on the conserved cystines on each subunit: each subunit of hCG has a similar cystine knot architecture that divides it into three elongated loops. The 90–110 sequence of the β-subunit surrounds the α-subunit forming a ‘seabelt’ which is fastened by the 26–110 disulfide bond. This region is now recognised as influencing gonadotrophin specificity: the 93–100 region would influence LH specificity (Huang et al. 1993, Han et al. 1996) whereas the 100–110 region influences FSH specificity (Moyle et al. 1994). The mechanism by which
this region has an impact on specificity is not understood; either directly by binding with the receptor or indirectly by exerting a strong influence on the conformation of the heterodimer.

In an effort to better delineate which amino acids in the 100–110 region are responsible for the FSH specificity, we chose to work with the horse/donkey LH/CG model. Horse LH (eLH) and horse CG (eCG), which are identical apart from their oligosaccharide structures (they are encoded by the same gene (Sherman et al. 1992)), are basically LH molecules but exhibit both LH and FSH activities in species other than the horse (Stewart et al. 1977). Donkey LH (dkLH) and donkey CG (dkCG) behave similarly but have much less FSH activity than eLH and eCG (Aggarwal et al. 1980, Roser et al. 1984). Recombinant eLH/CG and dkLH/CG expressed in COS-7 cells exhibit the same characteristics as the natural hormones: if the FSH/LH ratio is set at 1 for eLH/CG it is only 0.1 for dkLH/CG (Chopineau et al. 1997a). In addition, zebra LH/CG (zbLH/CG) does not display any FSH activity (Chopineau et al. 1999).

Thus, although the amino acid sequences of eLH/CG, dkLH/CG and zbLH/CG are highly homologous (>92% identity between α-subunits and >93% identity between β-subunits (Chopineau et al. 1999)), they display different FSH/LH activity ratios. This makes these hormones of great interest to study structure–function relationships of gonadotrophins. As it is the β-subunit that determines the level of FSH activity as observed in heterologous expressions (αe/βdk displays an FSH/LH ratio similar to that of the dkLH/CG whereas αdk/βe displays an FSH/LH ratio equivalent to the eLH/CG (Chopineau et al. 1997b)), we were interested in amino acids of the β-subunit different between horse, donkey and zebra LH/CG. There are only six differences in amino acids between these three β-subunits in positions 51, 94, 95, 102, 103 and 106 (Chopineau et al. 1999), and within these six differences, two (positions 102–103) are particularly interesting: in addition to being located in the 100–110 region described above, they present similarities with LHS or FSHs. Indeed, zbLH/CG and dkLH/CG exhibit Gly Pro at positions 102–103 like all LHs, whereas eLH/CG possesses Val Phe at these positions, which are very homologous to Val Arg present in all FSHs (Combar nous, 1992).

In order to investigate the role of these two amino acids in the level of FSH activity of eLH/CGs, the Gly–Pro and Val–Phe sequences in positions 102–103 of βdk and βe respectively were swapped by site-directed mutations and unrelated amino acids were also introduced at these positions. Similar mutations were also introduced in the well-studied gonadotrophin hCG. These mutant hormones were then tested for their in vitro LH and FSH bioactivities.

### Materials and Methods

#### Mutant constructs

The cDNAs encoding the horse or the donkey LH/CG β-subunits inserted in pBluescript vectors (Stratagene, Cambridge, Cambs, UK) (Chopineau et al. 1995) were used as templates to construct the different equid mutants. The horse and donkey α-subunit cDNAs have already been described (Stewart et al. 1987, Chopineau & Stewart, 1996, Chopineau et al. 1997a). The cDNA encoding the human α-subunit was kindly given by Dr J J Rémy (INRA, Jouy-en-Josas, France), whereas the βCG cDNA without CTP (ending at residue 121), in pM2 vector was a gift from Dr I Boime (Washington University, St Louis, MO, USA). This last cDNA was reintroduced in pBluescript vector to serve as a template to construct hCG mutants. It has been demonstrated for a long time that absence of CTP does not modify *in vitro* bioactivity of hCG (Matzuk et al. 1990).

Specific base substitutions were introduced by extension of mutagenic oligonucleotides containing the desired mutations by using a site-directed mutagenesis kit (Quick-change™ site-directed mutagenesis kit; Stratagene).

Equid mutants were constructed in order to get the Gly<sup>102–Pro<sup>103</sup>→Val<sup>102–Phe<sup>103</sup></sub></sup> mutation in eLH/CG β-subunit, using primers A and B (Table 1). Conversely, the Gly<sup>102–Pro<sup>103</sup>→Val<sup>102–Phe<sup>103</sup></sub></sup> mutation in dkLH/CG β-subunit was performed using primers C and D (Table 1). The Gly<sup>102–Pro<sup>103</sup>→Ala<sup>102–Ala<sup>103</sup></sub></sup>, Ala<sup>102–Pro<sup>103</sup>→Gly<sup>102–Ala<sup>103</sup></sub></sup> or Gly<sup>102–Ala<sup>103</sup></sup> mutations were also introduced in dkLH/CG β-subunit using E–F, G–H or I–J primers respectively (Table 1). The Gly<sup>102–Pro<sup>103</sup>→Arg<sup>104</sup>→Ala<sup>102–Ala<sup>103</sup>→Gly<sup>104</sup></sup> mutations were also introduced in the dkβ-subunit using K–L and M–N primers respectively (Table 1).

Human mutants were constructed in order to obtain Gly<sup>102–Pro<sup>103</sup>→Val<sup>102–Arg<sup>103</sup></sub></sup> in hCG β-subunit, using O–P primers, whereas Q–R primers were used to obtain the Gly<sup>102–Pro<sup>103</sup>→Lys<sup>104</sup>→Val<sup>102–Arg<sup>103</sup></sub></sup> mutation (Table 1).

Each construct was sequenced in both directions by the dideoxy chain termination method to ensure that correct mutations were introduced. Each cDNA was then subcloned into the eukaryotic expression vector pCDM8 (InVitrogen, Leek, Staffs, UK). The constructs were then amplified in MC1061/P3 (InVitrogen), purified using the Qia gen maxiprep plasmid kit (Coger, Paris, France) and the sequence was checked again.

#### Transient transfections of COS-7 cells

COS-7 monkey kidney cells (ATCC-CRL 1651), maintained at 37°C under 5% CO<sub>2</sub> in a humidified atmosphere in Dulbecco’s modified Eagle’s medium
supplemented with 10% foetal calf serum, were co-
transfected at 65% confluency in 6 cm diameter Petri
 dishes with 2 µg of each vector, one containing the
α-subunit (horse, donkey, or human) and the other the
β-subunit (wild-type or mutant), using the calcium phos-
phate precipitation procedure described by Chopineau
et al. (1997a). Control cells were transfected with the
expression vector containing no insert. After 48-h
incubation in serum-free Dulbecco’s modified Eagle’s
medium, the media were harvested, centrifuged at 200 g
for 10 min and the supernatants were stored at −20 °C
until assayed.

Estimations of activities

The recombinant equid hormones were quantified in a
sandwich enzyme-linked immunosorbent assay (ELISA)
using one monoclonal antibody against the eCG α-subunit
as first antibody and a rabbit polyclonal anti-eCG as second
antibody, and revelation with an anti-rabbit antibody
coupled to peroxidase and o-phenylenediamine as sub-
strate (Galet et al. 2000). The eCG used as a standard had
been previously purified in our laboratory (eCG 652, 5000
IU/mg). Quantitation of recombinant hCG was per-
fomed by ELISA specific for the heterodimer using a
polycional antibody raised in the rabbit against hCG (M P
Dubois, Nouzilly, France) using a previously published
protocol (Lecompte & Combarnous 1992).

The LH bioactivity of the recombinant hormones was
estimated by measuring their abilities to stimulate testos-
terone production in a rat Leydig cell bioassay (Guillou
et al. 1985), and the FSH bioactivity was assessed in an

Results

The bioactivities of the different equid β-subunit mutants
cotransfected with the horse α-subunit are presented in
Table 2, whereas the bioactivities of these mutants
cotransfected with the donkey α-subunit are presented in
Table 3. To simplify and homogenise the comparisons
between the mutants, the bioactivities of the recombinant
dimer obtained after co-transfection of the wild-type horse
β-subunit are taken as the reference in both Tables 2 and
3: thus αE/βeLH is the 100% reference in Table 2, and αdk/βeLH is the 100% reference in Table 3.

Bioactivities of the Gly102-Pro103 eLH/CG and Val102-Phe103 dkLH/CG mutants

The LH and FSH activities and FSH/LH ratios of the
Gly102-Pro103 βeLH/CG mutant and the Val102-Phe103
βdkLH/CG mutant cotransfected with the horse α-subunit are shown in Table 2A, whereas the bio-
activities of these mutants cotransfected with the donkey α-subunit are shown in Table 3A.

First, it is noteworthy that the LH activities of the
different constructs reassociated with the horse α-subunit
are always two to three times higher than the LH
activity of αE/βeLH/CG taken as a reference of 100%
The Gly\textsuperscript{102}–Pro\textsuperscript{103} → Val\textsuperscript{102}–Phe\textsuperscript{103} mutation in the donkey β-subunit led to enhanced FSH activity. Co-transfection of the mutant β-subunits gave equivalent whatever the α-subunits used, horse or donkey, with an FSH/LH ratio of 0·65 for βdk Val\textsuperscript{102}Phe\textsuperscript{103} cotransfected with αe and of 0·72 for this mutant β-subunit cotransfected with adk (αe/βe and adk/βe being taken as reference 1). Figure 1 illustrates these effects

Table 2 Relative FSH and LH potencies of the mutant equid LH/CG β-subunits co-expressed with the horse α-subunit and corresponding FSH/LH ratios

<table>
<thead>
<tr>
<th>Construct</th>
<th>Relative FSH potency</th>
<th>Relative LH potency</th>
<th>FSH/LH ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αe/βe</td>
<td>100</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>αe/βdk (n = 4)</td>
<td>28 ± 9</td>
<td>215 ± 51</td>
<td>0·14 ± 0·05</td>
</tr>
<tr>
<td>αe/βeG\textsuperscript{102}P\textsuperscript{103} (n = 4)</td>
<td>15 ± 6±8</td>
<td>207 ± 51</td>
<td>0·08 ± 0·04</td>
</tr>
<tr>
<td>αe/βdkV\textsuperscript{102}F\textsuperscript{103} (n = 4)</td>
<td>145 ± 60</td>
<td>244 ± 141</td>
<td>0·65 ± 0·18</td>
</tr>
<tr>
<td>(B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αe/βdkA\textsuperscript{102}A\textsuperscript{103} (n = 3)</td>
<td>30 ± 21</td>
<td>175 ± 110</td>
<td>0·15 ± 0·02</td>
</tr>
<tr>
<td>αe/βdkA\textsuperscript{102}P\textsuperscript{103} (n = 3)</td>
<td>23 ± 12</td>
<td>220 ± 41</td>
<td>0·1 ± 0·03</td>
</tr>
<tr>
<td>αe/βdkG\textsuperscript{102}A\textsuperscript{103} (n = 3)</td>
<td>34 ± 16</td>
<td>412 ± 37</td>
<td>0·08 ± 0·04</td>
</tr>
<tr>
<td>(C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αe/βdkA\textsuperscript{102}A\textsuperscript{103}G\textsuperscript{104} (n = 2)</td>
<td>29 ± 17</td>
<td>243 ± 127</td>
<td>0·14 ± 0·03</td>
</tr>
<tr>
<td>αe/βdkV\textsuperscript{102}F\textsuperscript{103}G\textsuperscript{104} (n = 2)</td>
<td>30 ± 15</td>
<td>319 ± 176</td>
<td>0·09 ± 0·01</td>
</tr>
</tbody>
</table>

Each construct was tested according to an initial quantification using the equine specific sandwich ELISA described in the text. The αe/βeLH was given a reference activity of 100% in both assays. The activities of the other recombinant hormones were calculated according to their ED\textsubscript{50} values compared with αe/βeLH ED\textsubscript{50}. Values are averages ± S.E.M. of at least two different expression experiments and in many cases more (n = at least 2). The FSH/LH ratio was determined by dividing the FSH potency by the LH potency.

Table 3 Relative FSH and LH potencies of the mutant equid LH/CG β-subunits co-expressed with the donkey α-subunit and corresponding FSH/LH ratios

<table>
<thead>
<tr>
<th>Construct</th>
<th>Relative FSH potency</th>
<th>Relative LH potency</th>
<th>FSH/LH ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>udk/βe</td>
<td>100</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>udk/βdk (n = 5)</td>
<td>30·4 ± 7·5</td>
<td>427·5 ± 183</td>
<td>0·08 ± 0·02</td>
</tr>
<tr>
<td>udk/βeG\textsuperscript{102}P\textsuperscript{103} (n = 3)</td>
<td>116 ± 6±5</td>
<td>344 ± 203</td>
<td>0·05 ± 0·04</td>
</tr>
<tr>
<td>udk/βdkV\textsuperscript{102}F\textsuperscript{103} (n = 4)</td>
<td>119 ± 23</td>
<td>167 ± 21</td>
<td>0·72 ± 0·15</td>
</tr>
<tr>
<td>(B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>udk/βdkA\textsuperscript{102}A\textsuperscript{103} (n = 4)</td>
<td>33 ± 16</td>
<td>273 ± 136</td>
<td>0·15 ± 0·09</td>
</tr>
<tr>
<td>udk/βdkA\textsuperscript{102}P\textsuperscript{103} (n = 2)</td>
<td>15 ± 2</td>
<td>295 ± 35</td>
<td>0·05 ± 0·01</td>
</tr>
<tr>
<td>udk/βdkG\textsuperscript{102}A\textsuperscript{103} (n = 2)</td>
<td>9 ± 8</td>
<td>215 ± 21</td>
<td>0·04 ± 0·04</td>
</tr>
<tr>
<td>(C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>udk/βdkA\textsuperscript{102}A\textsuperscript{103}G\textsuperscript{104} (n = 3)</td>
<td>17 ± 10</td>
<td>162 ± 49</td>
<td>0·12 ± 0·09</td>
</tr>
<tr>
<td>udk/βdkV\textsuperscript{102}F\textsuperscript{103}G\textsuperscript{104} (n = 3)</td>
<td>15 ± 7</td>
<td>182 ± 98</td>
<td>0·11 ± 0·07</td>
</tr>
</tbody>
</table>

Each construct was tested according to an initial quantification using the equine specific sandwich ELISA described in the text. The udk/βeLH was given a reference activity of 100% in both assays. The activities of the other recombinant hormones were calculated according to their ED\textsubscript{50} values compared with udk/βeLH ED\textsubscript{50}. Values are averages ± S.E.M. of at least two different expression experiments and in many cases more (n = at least 2). The FSH/LH ratio was determined by dividing the FSH potency by the LH potency.
when the β-subunits are cotransfected with the donkey α-subunit.

**Bioactivities of the Gly<sup>102</sup>-Pro<sup>103</sup>→Ala<sup>102</sup>-Ala<sup>103</sup>, Gly<sup>102</sup>-Ala<sup>103</sup> and Ala<sup>102</sup>-Pro<sup>103</sup> dkLH/CG mutants**

The LH and FSH activities and FSH/LH ratios of the βdkAla<sup>102</sup>-Ala<sup>103</sup>, βdkAla<sup>102</sup>-Pro<sup>103</sup> and βdkGly<sup>102</sup>-Ala<sup>103</sup> mutants cotransfected with the horse α-subunit are shown in Table 2B, whereas the bioactivities of these mutants cotransfected with the donkey α-subunit are given in Table 3B.

As previously, LH activities of the different constructs reassocciated with the horse α-subunit or the donkey α-subunit were always superior to those of the references (α/e/βLH/CG and αdk/βLH/CG respectively). However, these LH activities are comparable with those of αe/βdkLH/CG and αdk/βdkLH/CG respectively. The FSH activities were equivalent to the FSH activities of αdkβdkLH/CG, giving an FSH/LH ratio which was always equivalent to the FSH/LH ratio of the donkey LH/CG. This result was the same whatever the horse or donkey α-subunit used (Tables 2B and 3B).

**Bioactivities of the Gly<sup>102</sup>-Pro<sup>103</sup>-Arg<sup>104</sup>→Ala<sup>102</sup>-Ala<sup>103</sup>-Gly<sup>104</sup> and Val<sup>102</sup>-Phe<sup>103</sup>-Gly<sup>104</sup> dkLH/CG mutants**

The LH and FSH activities and FSH/LH ratios of the βdk Ala<sup>102</sup>-Ala<sup>103</sup>-Gly<sup>104</sup> and βdk Val<sup>102</sup>-Phe<sup>103</sup>-Gly<sup>104</sup>
mutants cotransfected with the horse α-subunit are shown in Table 2C and the bioactivities of these mutants cotransfected with the donkey α-subunit are shown in Table 3C.

Again, LH activities of the different constructs re-associated with the horse α-subunit or the donkey α-subunit were always superior to those of the references (αe/βeLH/CG and αdk/βeLH/CG respectively). Moreover, FSH activities were equivalent to those of dkLH/CG, giving again an FSH/LH ratio equivalent to that of dkLH/CG.

Bioactivities of the hCG mutants

The LH and FSH activities and FSH/LH ratios of the βhCG Val<sup>102</sup>-Arg<sup>103</sup> and βhCG Val<sup>102</sup>-Arg<sup>103</sup>-Gly<sup>104</sup> mutants cotransfected with the human α-subunit are shown in Table 4A, using αh/βhCG as reference, whereas the bioactivities of these mutants cotransfected with the horse α-subunit are given in Table 4B, using αe/βhCG as reference.

LH activities were equivalent for all these expressed heterodimers compared with the reference 100%. It is to be noted that the LH activities of the mutants cotransfected with the horse α-subunit were estimated without initial quantification; however, whatever the α-subunit used (human or horse), in no case was any detectable FSH activity seen.

Discussion

All the different mutants have been compared at equivalent concentrations as estimated by the sandwich ELISA. This initial quantification of equid hormones is not a critical point as we took care to use a polyclonal antibody as the second antibody in the sandwich ELISA in this study, whereas in the previous studies we used a monoclonal antibody specific for the β-subunit (24A3) (Chopineau et al. 1997a,b); the epitope of which has not been exactly defined. However, we used these two different ELISAs in some expressions and they gave identical results.

The mutations affected a region known to be implicated in FSH specificity. Therefore we used the LH activity of eLH/CG as an internal measure of the quantity of biologically active hormone in the medium and the FSH/LH ratio as a measure of the variations in FSH activity (Chopineau et al. 1997b).

We noticed that the LH activities of the different equid constructs reassociated with the horse α-subunit are always superior to the LH activity of αe/βeLH/CG (Table 2). We have previously observed that αdk/βdkLH/CG displayed an LH activity about twice that of eLH/CG (Chopineau et al. 1997a). It can be hypothesised that either dkLH/CG has a higher LH bioactivity than the horse hormone, due to differences in their respective β-subunits, or the quantities of donkey mutants are slightly underestimated in the ELISA since the antibodies are produced against eCG. However, as already mentioned above, this has no implication when using FSH/LH ratios.

The LH bioactivities of the different mutants cotransfected with the donkey α-subunit are also higher than that of αdk/βeLH/CG (Table 3). We have previously observed that αdk/βdkLH/CG shows an LH activity half that of αe/βeLH/CG (Chopineau et al. 1997b) and, as αdk/βdkLH/CG is about twice as active as αe/βeLH/CG, it seems logical to obtain an LH activity four times more active for αdk/βdkLH/CG compared with αdk/βeLH/CG when taken as a reference 100%. This is in agreement with Bousfield et al. (1985) who observed that LH hybrids prepared with βeLH and αLH of different species (ovine (o), porcine (p) and human) were very inactive in rat radioligand and Leydig cell in vitro bioassay.

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### Table 4 Relative FSH and LH potencies of the mutant human CG β-subunits co-expressed with the human or horse α-subunit and corresponding FSH/LH ratios

<table>
<thead>
<tr>
<th></th>
<th>Relative FSH potency</th>
<th>Relative LH potency</th>
<th>FSH/LH ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) αh/βhCG</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>αh/βhCG&lt;sup&gt;V102R103&lt;/sup&gt; (n=2)</td>
<td>0</td>
<td>88:5±3:5</td>
<td>0</td>
</tr>
<tr>
<td>αh/βhCG&lt;sup&gt;V102R103G104&lt;/sup&gt; (n=2)</td>
<td>0</td>
<td>75 ± 5</td>
<td>0</td>
</tr>
<tr>
<td>(B) αe/βhCG</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>αe/βhCG&lt;sup&gt;V102R103&lt;/sup&gt;</td>
<td>0</td>
<td>161</td>
<td>0</td>
</tr>
<tr>
<td>αe/βhCG&lt;sup&gt;V102R103G104&lt;/sup&gt;</td>
<td>0</td>
<td>148</td>
<td>0</td>
</tr>
</tbody>
</table>

(A) Each construct was tested according to an initial quantification using the ELISA described in the text. The αh/βhCG was given a reference activity of 100%. Values are averages ± S.E.M. (B) Each construct was tested without initial quantification, but according to dilution of the expressed medium. The αe/βhCG was given a reference activity of 100%. Only one expression was performed. The activities of the other recombinant hormones were calculated according to their ED<sub>50</sub> values compared with the reference ED<sub>50</sub>. No FSH activity could be detected.
comparing with eLH. However, this decrease in activity was much more dramatic (less than 2% of the activity for these three hybrids compared with αe/βeLH/CG) than for our expressed αdk/βeLH/CG (50% compared with αe/βeLH/CG). This can be interpreted by the fact that the amino acid sequence of αdk is much closer to that of αe than to those of αh, αp or αt. This reinforces the view that α-subunits play a role in bioactivity. However, it is noteworthy that αdk/βeGly102-Pro103 also showed an enhanced LH bioactivity compared with αdk/βe as αe/βeGly102-Pro103 compared with αe/βe, suggesting that these two amino acids might also influence LH activity in equeid hormones. It is notable that there is one report in the literature (Liao et al. 1998) of a natural mutation of Gly102 into Ser in LHβ identified in 4% of the infertile women studied, suggesting that this missense mutation might be implicated in endometriosis-associated infertility in women. Moreover, one very recent communication (Liao et al. 2000) showed that this mutant expressed in Chinese hamster ovary cells had lower LH biopotency.

Cotransfection of the different equid β-subunit mutants with horse or donkey α-subunit gave identical results in terms of FSH/LH ratios (Table 2 compared with Table 3). The introduction of the motif Gly102-Pro103 instead of Val102-Phe103 in the horse β-subunit diminishes its FSH activity down to a level identical to the low FSH activity of dkLH/CG. Conversely, introduction of Val102-Phe103 in the donkey β-subunit enhances its FSH activity, up to an FSH/LH ratio around 0·7, showing that these two amino acids are implicated in equine FSH specificity.

This equine FSH specificity seems to be due to the presence of Val102-Phe103 rather than to the absence of Gly102-Pro103. Indeed, the introduction of unrelated amino acids in the two positions (Ala102-Ala103) or in only one (Gly102-Ala103 or Ala102-Pro103) did not confer any augmentation of the FSH activity of dkLH/CG.

Additional mutation of dkLH/CG at position 104: Gly102-Pro103→Arg104→Val102→Arg103→Gly104, did not further increase FSH activity as expected on the basis of the presence of a Gly residue in position 104 of all FSHs. In contrast, this mutation abolished the gain in FSH activity observed after the Gly102-Pro103→Val102→Arg103 mutation. The importance of the residue in position 104 for FSH activity was further substantiated by the observation that the replacement of 104–109 residues in one-chain β-α eLH/CG by Ala residues led to a tenfold decrease in FSH/LH activity ratio (data not shown). This clearly indicates that amino acid present in position 104 is important for FSH bioactivity.

It is to be noted that the Val102→Phe103→Gly102→Pro103 mutation abolished the high FSH activity of eLH/CG whereas the mutation Gly102→Pro103→Val102→Phe103 in the dkLH/CG β-subunit did not confer exactly the high FSH activity present in eLH/CG (FSH/LH ratio of 0·6–0·7 instead of 1) showing that other amino acids in eLH/CG β-subunit must participate in FSH specificity. It is interesting to note that eLH/CG β-subunit possesses another difference from dkLH/CG β-subunit between Cys100 and 110; position 106, which is a Gln in horse and an His in donkey (Table 5). It will be interesting to check whether this specific amino acid is also involved in the FSH specificity of eLH/CG.

Introduction of Val102→Arg103 in hCGβ does not confer any FSH activity to the molecule, whether it is cotransfected with a human or an equine α-subunit. We cotransfected these mutants with a horse α-subunit as we have previously shown that FSH activity of equid LH requires an equid α-subunit (Chopineau et al. 1997b), suggesting that some equine α-residues are implicated in FSH specificity of equeid hormones. However, the hCGβ mutant dimers obtained did not show an FSH activity. We also produced a Val102→Arg103→Gly104 hCGβ mutant because we reasoned that the presence of two neighbouring basic amino acids (Arg103 and Lys104) could not be adequate, but no FSH activity could be shown.

Human gonadotrophins have been largely used in the literature to study structure/function relationships of gonadotrophins, and particularly hCG. Many mutations in the 100–110 area of these human gonadotrophins have been described. The Lys104→Glu104 mutation of hCGβ, thus changing a positively charged amino acid into a

### Table 5 Comparison of the different 100–110 β-subunit LH or FSH sequences described in the literature aligned with their LH and FSH bioactivities (eLH has only one fifth of FSH activity compared with a real FSH)

<table>
<thead>
<tr>
<th>β-subunit</th>
<th>LH</th>
<th>FSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>eLH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eLH Gly102-Pro103 (present study)</td>
<td>CGVFRDQPLAC</td>
<td>1</td>
</tr>
<tr>
<td>dkLH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dkLH Val102 Phe103 (present study)</td>
<td>CGGPRDHPLAC</td>
<td>2</td>
</tr>
<tr>
<td>dkLH Val102 Phe103 Gly104 (present study)</td>
<td>CGVFRDQPLAC</td>
<td>0</td>
</tr>
<tr>
<td>hCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hCG Gly102 Pro103 (present study)</td>
<td>CTVRGKDHPTC</td>
<td>1</td>
</tr>
<tr>
<td>hCG Val102 Arg103 (present study)</td>
<td>CTVRGLGPSYC</td>
<td>0</td>
</tr>
<tr>
<td>hCG Val102 Arg103 Gly104 (present study)</td>
<td>CTVRGLGPSYC</td>
<td>0</td>
</tr>
</tbody>
</table>

Underlining corresponds to the mutated amino acids.
negatively charged one, produced a mutant hCG whose binding and bioactivity was reduced twofold, suggesting that Lys in position 104 might be implicated in LH binding (Xia et al. 1993).

However, most mutations in the 100–110 region showed that this area is implicated in FSH specificity: after the interchange of the amino acids present in hCG β-subunit between Cys 100 and Cys 110 with the corresponding amino acids present in hFSH β-subunit (between Cys 94 and 104), the resulting mutant hCG dimer displayed 30% FSH activity compared with 100% FSH and conserved 83% of LH activity (Moyle et al. 1994) (Table 5). Conversely, Dias et al. (1994) introduced the first six amino acids of hCG β-subunit after Cys 94 in hFSH β-subunit, and abolished its FSH activity (Table 5). Thus, these six residues are important to determine FSH specificity in the human gonadotrophins. In equine gonadotrophins we restricted this area to three amino acids.

By comparing the sequences between different LH β-subunits it is interesting to note that chicken LH β-subunits also present a sequence Val102–Gln103–Gly104 which is closer to FSHs than to other LHs. Interestingly, a recent report suggests that this chicken LH binds to rat ovarian follitropin receptor (Iwasawa et al. 1998).

Up to very recently, hCG was the only gonadotrophin for which a crystallographic model had been resolved (Lapthorn et al. 1994, Wu et al. 1994), and all other gonadotrophins were thought to have the same global conformation based on the conservation of the disulfide bonds. However, a very recent communication indicates that a crystal structure of hFSH has been obtained (Dias et al. 2000) and, although the overall structure closely resembles hCG, several differences in the β-subunits were revealed, including residues 94–104, proving again that this region is of utmost importance for gonadotrophin specificity. Interestingly, the conformation of the α-subunit, at its C-terminus, seems also to differ between hCG and hFSH, suggesting that this α-subunit might also play a role in FSH specificity.

The study presented here shows that amino acids 102–104 are implicated in equal FSH specificity. The Val102–Phe103–Arg104 sequence determines significant FSH activity in eLHβ and the basic amino acid in position 104 seems to mimic the role of the one in position 103 of all FSHs. How these amino acids determine FSH specificity is still unknown: either directly by binding to the receptor or indirectly by influencing the conformation of the molecule via the seatbelt. The fact that βhCG Val102–Arg103, even when co-expressed with a horse α-subunit, did not display any FSH activity whereas βdCG Val102–Phe103 and βe displayed an FSH activity, shows that other amino acids present in the horse and the donkey β-subunit must participate in the expression of FSH activity of the equid gonadotrophins, in co-operation with specific equid α amino acids.

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