

Stability and biological activities of heterodimeric and single-chain equine LH/chorionic gonadotropin variants

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Abstract

Recombinant equine LH/chorionic gonadotropin (eLH/CG) was expressed in the baculovirus–Sf9 insect cell system either as a single-chain with the C-terminus of the β -subunit fused to the N-terminus of the α -subunit or as non-covalently linked heterodimers with or without a polyhistidine tag at various locations. All these non-covalently linked eLH/CG variants were secreted as stable heterodimers in the medium of infected Sf9 cells. To assess the influence of the presence and the position of polyhistidine tag on LH bioactivity, we expressed four non-covalently linked tagged heterodimeric eLH/CG variants that were secreted in threefold higher quantities than the single chain. Among them, only two exhibited full *in vitro* LH bioactivity, relative to untagged heterodimers, namely the one His-tagged at the N-terminus of α -subunit and the other at the C-terminus of the β -subunit both of which are amenable to nickel-affinity purification. Furthermore, single-chain eLH/CG was found to be *N*- and *O*-glycosylated but nevertheless less active in *in vitro* LH bioassays than natural eCG and heterodimeric recombinant eLH/CG. The thermal stability of natural and recombinant hormones was assessed by the initial rates of dissociation from 20 to 90 °C. Heterodimeric eLH/CG from Sf9 cells was found to be as stable as pituitary eLH and serum eCG ($T_{1/2}$, 74–77 °C). Although Sf9 cells only elaborated short immature-type carbohydrate side chains on glycoproteins, recombinant eLH/CG produced in these cells exhibited stabilities similar to that of pituitary eLH. In conclusion, recombinant heterodimeric eLH/CG exhibits the same thermal stability as natural pituitary LH and its advantages over the single-chain eLH/CG include higher secretion, higher *in vitro* bioactivity, and reduced potential risk of immunogenicity.

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Introduction

Glycoprotein hormones include placental chorionic gonadotropin (CG) and pituitary-derived luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH). They are heterodimeric proteins composed of a common α -subunit non-covalently associated with hormone-specific β -subunit. As an exception in equids, eCG and LH (eLH) are encoded by the same α and β genes (Sherman *et al.* 1992) and thus exhibit the same peptidic moiety. However, they strongly differ in their *N* and *O*-linked carbohydrate side chains (Smith *et al.* 1993, Matsui *et al.* 1994, Bousfield & Butnev 2001), conferring different *in vivo* biological potencies due to a prolonged eCG half-life as compared with eLH (Klett *et al.* 2003), as well as different thermal stabilities (Galet *et al.* 2004).

The establishment of intra-subunit disulfide bonds involved in the acquirement of the global protein conformation influences glycosylation (Moriwaki *et al.* 1997) and enables the formation of $\alpha \times \beta$ heterodimer with a stable quaternary structure supporting an efficient secretion (Furuhashi *et al.* 1996, Moriwaki

et al. 1997). Some authors have chosen to produce single-chain gonadotropins arguing that several heterodimeric gonadotropins (hLH, bLH,...) were not efficiently assembled in transfected heterologous cells (Corless *et al.* 1987, Matzuk *et al.* 1988, Keene *et al.* 1989, Jablonka-Shariff *et al.* 2007). It is noteworthy that hCG subunits assemble more efficiently than hLH subunits suggesting that the C-terminal peptide (CTP) of hCG plays a favorable role in assembly, secretion, or stability (Corless *et al.* 1987, Suzuki *et al.* 2000). In keeping with this view, we previously demonstrated the efficient formation and secretion of a stable heterodimeric eLH/CG in infected Sf9 cells (Legardinier *et al.* 2005a) and COS-7 cells (Chopineau *et al.* 1997a, Legardinier *et al.* 2005a).

In order to study more thoroughly the properties of heterodimeric and single-chain β - α eLH/CG, we expressed both of them in the highly efficient baculovirus insect cell protein expression system by using either the polyhedrin promoter (PH) of *Autographa californica* multiple nuclear polyhedrosis virus (*AcMNPV*)-derived baculovirus or the p10 promoter (P10) of *AcSLP10*-derived baculovirus.

For the sake of nickel-affinity purification, fusion of a single histidine tag at either the N- or C-terminus of the α - or β -subunit was performed. In the present work, we studied the bioactivity and thermal stability of tagged and untagged heterodimeric variants as well as single-chain β - α eLH/CG and compared them with pituitary eLH and serum eCG.

In brief, single-chain β - α eLH/CG was found to be less efficiently secreted and less bioactive *in vitro* than heterodimeric eLH/CG. In spite of the immature-type glycosylation, heterodimeric recombinant eLH/CG was efficiently secreted in Sf9 cells, exhibited a high thermal stability, and full *in vitro* bioactivity compared with natural eCG and eLH.

Materials and methods

Materials

Hormones

All the natural hormones used in this study were purified in our laboratory: eCG NZY-01 (1500 IU/mg; Lecompte *et al.* 1998), eLH CY1781 (11.4 \times NIH LH S1; Guillou & Combarous 1983, Hofferer *et al.* 1993), and oLH CY1083 (3.1 \times NIH LH S1; unpublished). The reference preparation eCG NZY-01 was chosen in spite of its low specific activity because in contrast to the highly purified eCG preparation (10–12 000 IU/mg), it contains all or most of the eCG isoforms and warrants activity estimates that are consistent between *in vitro* and *in vivo* assays (Cahoreau & Combarous 1987). In contrast, highly purified eCG preparations contain various limited sets of isoforms that exhibit very different potencies in *in vitro* and *in vivo* assays (Lecompte *et al.* 1998).

Antibodies

Three primary antibodies were used in this study. The first was a mouse monoclonal anti-eCG α antibody (α mAb 89A2), which recognized the native α -chain as either single subunit or as eCG heterodimer (Chopineau *et al.* 1993). The second was a rabbit polyclonal antibody raised against a synthetic LH/CG β 1–9 peptide (β 1–9 Ab). This peptide sequence is conserved in LH and CG β -subunits from numerous species and the antibody is capable of detecting reduced subunits (Legardinier *et al.* 2005b). The third was a rabbit anti-eCG polyclonal antibody (eCG Ab; Cahoreau & Combarous 1987). Two secondary antibodies were peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch, Interchim, Montluçon, France).

Products

Plasmid constructs were amplified and purified using the Wizard Plus SV Minipreps DNA purification system kit (Promega) and Nucleobond AX kit (Macherey Nagel, Hoerd, France). PCRs were carried out using Taq or plaque forming unit (PFU) DNA polymerase (Promega) and the sequences of all the amplified products were checked by the dideoxy chain termination method (Genome Express, Meylan, France). Restriction enzymes were purchased from Roche and oligonucleotides from Eurogentec (Seraing, Belgium).

Methods

Introduction of a cassette encoding an N-terminal signal peptide (SP) and a hexahistidine sequence in the baculovirus transfer vector pGmAc115T

The pGmAc115T baculovirus transfer vector contains the very late promoter and polyadenylation signal of the PH protein, which are flanked with specific PH gene sequences allowing homologous recombination with wild-type *AcMNPV* DNA. We introduced a new fragment encoding the insect cell UDP-glucosyltransferase SP followed by six histidine residues and two new cloning sites, Xba I and Sma I, into Bgl II and Asp718 I cloning sites (Fig. 1). Briefly, the pGmAc115T baculovirus transfer vector was digested using Bgl II and Asp718 I restriction enzymes and ligated with the purified prehybridized oligonucleotides from A to H (Table 1). About 1 μ g of each oligonucleotide was heated separately for 10 min at 80 °C. Equal amounts of A–H

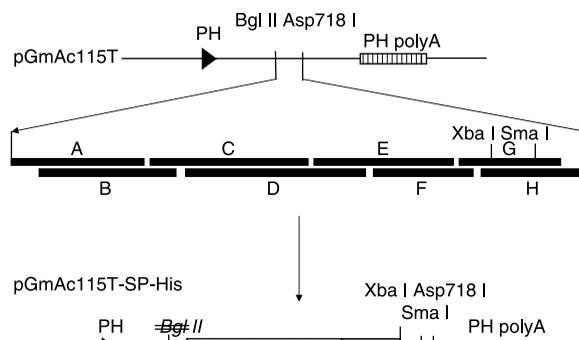


Figure 1 Construction of a modified baculovirus transfer vector by hybridization strategy (pGmAc115T-SP-His). The pGmAc115T baculovirus transfer vector contains the very late promoter and polyadenylation signal of the polyhedrin protein (PH). An additional DNA sequence encoding the signal peptide (SP) of insect cell UDP-glucosyltransferase gene, six histidine residues (6 \times His) and new cloning sites Xba I and Sma I was inserted between Bgl II and Asp718 I restriction cloning sites of pGmAc115T transfer vector by using prehybridized (A–H) overlapping primers (listed in Table 1) to obtain the modified pGmAc115T-SP-His baculovirus transfer vector lacking the Bgl II restriction site.

Table 1 Oligonucleotide sequences

Name	Nucleotide sequence 5'–3'
A=AM01	5'-GATCCATGACTATTCTCTGCTG-3'
B=AM05	5'-CAAGCCAGCAGAGAATAGTCATG-3'
C=AM02	5'-GCTTGCACTGCTGTCTACGCTTACTG-3'
D=AM06	5'-CATTTACAGCAGTAAGCGTAGACAGCAGTG-3'
E=CC-01	5'-CTGTAATGCGCACCATCATCAC-3'
F=CC-03	5'-GTGGTGATGATGGTGCG-3'
G=CC-02	5'-CACCATCTAGAACCCGG-3'
H=CC-04	5'-GTACCCGGGTTCTAGATG-3'
I= α_{P10} -F	5'-CATCGGGCCAGATCTTCTAGAC-3'
J= α_{P10} -R	5'-CTTGGTGAAGCTTTAAATCTTG-3'
K= β_{PH} -F	5'-CTAAGATCTAGAACCAAGGATGGAG-3'
L= β_{PH} -R	5'-CAAAGCTTGGTACCTCAAGAAGTC-3'
M=His- α -F	5'-GTTCTAGACTTTCCTGATGGAGAGTTT-3'
N=His- α -R	5'-GGGTACCTCTACATTAATCTTGTGGTG-3'
O=His- β -F	5'-CGGGTCTAGAAATCCAGGGGG-3'
P=His- β -R	5'-GCTTGGTACCTCAAGAAGTCTT-3'

Oligonucleotides used to construct the modified baculovirus transfer vector pGmAc115T-SP-His (oligonucleotides A–H) and primers to amplify by PCR cDNA encoding the α - (I–J and M–N) and β - (K–L and O–P) subunits with (I–L) or without (M–P) signal peptide. F, forward; R, reverse. Restriction enzyme recognition sites are underlined.

oligonucleotides were mixed and incubated for 10 min at 80 °C just before overnight hybridization that involved a progressive decrease in temperature from 80 to 25 °C. About 50 ng double-digested pGmAc115T transfer vector was ligated with 500 ng pre-hybridized A–H oligonucleotides overnight at 4 °C using 5 units of T4 DNA ligase. The resulting vector is referred to as pGmAc115T-SP-His.

Cloning of equine α - and LH/CG β -subunit cDNAs into the baculovirus transfer vectors

Transfer vector pGmAc116T. The specific transfer vector pGmAc116T- α contains chimeric full-length cDNA encoding a genuine equine α -subunit placed downstream of the PH promoter, as described previously (Legardinier *et al.* 2005a). Full-length cDNA for the equine LH/CG β -subunit previously cloned into baculovirus transfer vector p119 (Legardinier *et al.* 2005a) was used as a template for PCR using K and L primers (Table 1) to generate modified cDNA ends with the conserved Bgl II site at the 5'-end and a new Asp718 I site adjacent to the stop codon at the 3'-end. The amplified fragment was subcloned into pGEM-T (Promega) and then directly cloned into the transfer vector pGmAc116T to generate the specific transfer vector pGmAc116T- β (Table 2).

Transfer vector p119. Chimeric full-length cDNA for the equine α -subunit previously cloned into pGmAc116T (Legardinier *et al.* 2005a) was used as a template for PCR using I and J primers (Table 1) to generate modified cDNA ends with the conserved Bgl II site at the 5'-end and a new Hind III site at the 3'-end, which overlaps the stop codon.

Table 2 Names of transfer vectors, recombinant viruses and proteins

Transfer vector	Promoter	Recombinant virus	Recombinant protein
pGmAc116T	PH	NPV- α NPV- β NPV- $\beta\alpha$	α_{PH} β_{PH} β - α_{PH}
pGmAc115T-SP-His	PH	NPV-His α	His- α_{PH}
p119	P10	NPV-His β AcSLP10- α	His- β_{PH} α_{P10}
p119-His	P10	AcSLP10- β AcSLP10- α His AcSLP10- β His	β_{P10} α -His $_{P10}$ β -His $_{P10}$

The amplified fragment was subcloned into pGEM-T and then directly cloned into the baculovirus transfer vector p119 (Chaabihi *et al.* 1993) downstream of the p10 protein promoter (P10) to generate the specific transfer vector p119- α . The specific transfer vector p119- β contains full-length cDNA encoding the equine LH/CG β -subunit placed downstream of the p10 protein promoter (P10), as described previously (Legardinier *et al.* 2005a).

Modified transfer vector p119-His. The specific transfer vectors p119- α His and p119- β His containing full-length cDNAs encoding the equine α - and LH/CG β -subunit respectively, have been described previously (Legardinier *et al.* 2005b). These cDNAs were cloned downstream of the p10 protein promoter (P10) in the modified transfer vector p119-His using the Bgl II and Hind III cloning sites.

Modified pGmAc115T-SP-His (Fig. 1). Full-length cDNAs encoding the equine α - and LH/CG β -subunits previously cloned into baculovirus transfer vectors pGmAc116T and p119 respectively (Legardinier *et al.* 2005a) were used as templates for PCR using the M–N primers for the α cDNA or the O–P primers for the β cDNA (Table 1). This introduces a new Xba I site at the 5'-end, just upstream of the codon encoding the first residue of the mature protein and an Asp718 I site at the 3'-end after the stop codon. The amplified fragments were subcloned into pGEM-T and then directly cloned into the modified baculovirus transfer vector pGmAc115T-SP-His to generate the specific transfer vectors pGmAc115T-SP-His α and pGmAc115T-SP-His β .

Cloning of equine LH/CG β - α single-chain cDNA into the baculovirus transfer vector pGmAc116T

cDNA encoding the β - α single-chain protein of eLH/CG (Galet *et al.* 2001) was inserted downstream of the PH promoter in the modified pGmAc116T baculovirus transfer vector (Poul *et al.* 1995) at a unique Xba I site previously added between cloning sites Bgl II

and Kpn I (Legardinier *et al.* 2005a) to obtain the baculovirus transfer vector.

Construction of recombinant baculoviruses

Sf9 cells were co-transfected with 5 µg transfer vector and 500 ng purified viral DNA using a lipofection method (DOTAP; Roche). Two distinct viral DNA preparations were used. They are the following: i) viral DNA was extracted from the wild-type AcMNPV when PH-specific transfer vectors, such as pGmAc116T- α (Legardinier *et al.* 2005a), pGmAc116T- β , pGmAc116T- β - α , pGmAc115T-SP-His α , or pGmAc115T-SP-His β were used and ii) viral DNA was extracted from the baculovirus AcSLP10 (Chaabihi *et al.* 1993), which possesses only the very late P10 promoter, driving the expression of the PH gene when the P10-specific transfer vectors, such as p119- β (Legardinier *et al.* 2005a), p119- α His, p119- β His (Legardinier *et al.* 2005b), or p119- α were used. Recombinant baculoviruses were selected by plaque assay and distinguished from the wild-type progeny by their occlusion body-negative phenotype. The screening and purification of the new recombinant baculoviruses NPV- β NPV- β - α , NPV-His α , NPV-His β , and AcSLP10- α were carried out as previously described (Summers & Smith 1987). Recombinant baculoviruses NPV- α , AcSLP10- β , AcSLP10- α His, and AcSLP10- β His were obtained earlier (Legardinier *et al.* 2005a,b).

Cells and viral infections

Sf9 cells from *Spodoptera frugiperda* Sf21 cells (Vaughn *et al.* 1977) were maintained at 28 °C in a supplemented TC-100 growth medium containing 5% heat-inactivated fetal bovine serum, 50 units/ml penicillin G, and 50 µg/ml streptomycin. They were maintained as adherent cells at a density of 2.0–2.5 × 10⁶ cells per ml in 25 cm² flasks (Falcon, VWR, France). The viruses were propagated in Sf9 lines and recovered as described previously (Summers & Smith 1987). The cells were infected with recombinant baculoviruses at a multiplicity of infection (MOI) ranging from 5–10 Pfu/cell in 25 or 75 cm² flasks. After 45 min incubation with viral suspensions, 4 or 12 ml of fresh culture medium was added and cells were incubated at 28 °C until day 4 post-infection. The viral titers were determined by plaque assay (Summers & Smith 1987).

Quantitation of secreted recombinant heterodimeric variants of eLH/CG and monomers

The concentrations of the recombinant eLH/CG proteins produced in Sf9 insect cells culture media were determined employing a sandwich ELISA as described previously (Legardinier *et al.* 2005a) using a

monoclonal anti-eCG α antibody (α mAb 89A2, 1 µg/ml; Chopineau *et al.* 1993) coated on the microtiter plate and a rabbit polyclonal anti-eCG antibody (dilution 1:100 000; Cahoreau & Combarous 1987). The absorbance was measured at 450 nm using a SpectraCount (Packard, Downers Grove, IL, USA) spectrophotometer and data were analyzed by the I-SMART software (Packard). The eCG NZY-01 reference preparation exhibits a potency of only 1500 IU/mg but quantitations of the hormones in the media were based on a specific activity of 10 000 IU/mg for pure eCG.

The concentrations of recombinant α - and β -monomers were determined by competitive ELISA, using specific anti-eCG α -subunit (α mAb 89A2, 1 µg/ml) and anti- β subunit peptide (β 1–9) antibodies respectively, as described previously (Legardinier *et al.* 2005b).

Western blot analysis of expressed recombinant heterodimeric and single chain protein variants of eLH/CG

Sf9 insect cells were seeded in 25 cm² flasks and co-infected with recombinant baculoviruses to generate different tagged/untagged heterodimeric variants of eLH/CG or mono-infected with baculovirus expressing eLH/CG β - α single chain. The supernatants were recovered by centrifugation at 100 g for 5 min and the aliquots (30 µl) were diluted in Laemmli's 4× buffer (Laemmli 1970) under non-reducing conditions. The samples were electrophoresed in 10 or 12% running gels, electrotransferred overnight at 4 °C to nitrocellulose membranes (Schleicher and Schuell, Ecqueville, France), and incubated with rabbit polyclonal anti-eCG antibody (dilution 1:50 000) as described previously (Legardinier *et al.* 2005a). The membranes were developed using a chemiluminescent substrate (Super-Signal West Dura from Pierce, Interchim, Montluçon, France).

Glycosylation analyses

Lectin analysis of glycosylation. A panel of biotinylated plant lectins (Vector Laboratories, AbCys, Paris, France) with various specificities were used to analyse non-denatured glycoproteins in culture medium. We used an enzyme-linked lectin immunoassay as described previously (Legardinier *et al.* 2005b). The microtiter plates were coated overnight at 4 °C with 100 µl per well of α mAb 89A2 (1 µg/ml) in 0.1 M sodium carbonate/bicarbonate buffer (pH 9.6). After extensive washing, non-specific sites were saturated for 1 h at 4 °C with TBS-T (0.05% Tween20 Tris buffer saline: 25 mM Tris (pH 7.4), 140 mM NaCl, 3 mM KCl) containing 2% w/v polyvinylpyrrolidone K30 (Fluka, Sigma). About 100 ng recombinant dimeric eLH/CG (100 µl supernatant per well) were incubated 1 h at 4 °C in the saturation buffer. Similar quantities of

serum-derived eCG reference preparation NZY-01, and recombinant eLH/CG β -subunit were used. After washing, the microtiter plates were incubated with different concentrations of biotinylated lectins (0.4 $\mu\text{g}/\text{ml}$ peanut agglutinin (PNA) or 0.2 $\mu\text{g}/\text{ml}$ Galanthus nivalis agglutinin (GNA) for 1 h at 4 °C in TBS-T with 1 mM MgCl_2 , 1 mM MnCl_2 , and 1 mM CaCl_2 . After rinsing, 1 ng (100 μl) peroxidase-labeled NeutrAvidin (Pierce Interchim) was added for 1 h at 4 °C. After the addition of SureBlue TMB peroxidase substrate (KPL, Eurobio, Les Ulis, France), the reaction was stopped and the absorbance was measured at 450 nm as described above.

Radioreceptor assay

Fifty-two-day-old Wistar rats were used for the preparation of binding fraction used in the radioreceptor assay (RRA). All these assays were performed as described previously (Combarrous *et al.* 1986), except that the assay buffer used (10 mM Tris-HCl, pH 7.4) was complemented with the TG3 insect cell medium as for the *in vitro* bioassays. Standard eCG preparation, NZY-01, or recombinant hormones were initially diluted in assay buffer to which 50 μl of 36 mM CaCl_2 were added. Samples were incubated with a rat testis receptor preparation in presence of iodinated ovine LH (^{125}I -oLH, oLH CY1083) prepared using chloramin T as the oxidant. LH receptor-binding relative potencies were determined for recombinant hormones using the eCG reference preparation NZY-01 as the standard. The relative affinities of the hormones were determined from their concentrations giving half-maximal inhibition of ^{125}I -oLH binding.

In vitro LH bioassays

LH bioactivities of recombinant hormones were estimated by the *in vitro* stimulation of progesterone production in mouse Leydig Tumor Cell line, MLTC-1 (ATCC-CRL 2065). As described previously (Legardinier *et al.* 2005a), about 1.5×10^5 MLTC-1 cells per well were incubated at 37 °C in 0.5 ml supplemented RPMI growth medium (10% fetal bovine serum, 50 $\mu\text{g}/\text{ml}$ gentamicin, 10 units/ml penicillin, and 10 $\mu\text{g}/\text{ml}$ streptomycin) in 48-well plates until 80 percent confluence. Just before stimulation, the cells were incubated for 2 h in 0.5 ml serum-free RPMI medium and then stimulated for 2 h with 0.5 ml of a serum-free RPMI medium containing samples of recombinant hormones at different concentrations as previously determined by specific ELISA. The supernatants were recovered and stored at -20 °C until progesterone content was measured. The secreted progesterone recovered in the media from MLTC-1 was measured by a specific RIA (Saumande & Batra 1985). The LH

potencies for the different hormone preparations were calculated on the basis of ED50 values.

Nickel-affinity trapping

Sf9 insect cells were seeded in 75 cm^2 flasks and co-infected with NPV- α and AcSLP10- β His recombinant baculoviruses at a MOI of 5–10 Pfu per cell in 12 ml final volume. After four days incubation at 28 °C, 80–100 ml supernatants were recovered by centrifugation at 100 *g* and then at 1000 *g* for 10 min. Desalination was carried out by using five PD10 gel filtration columns in parallel (Amersham); the columns were loaded for each run with 2.5 ml sample and exchanged with 3.5 ml binding buffer (25 mM Tris-HCl (pH 8.0), 500 mM NaCl and 5 mM imidazole). Pooled desalted samples were loaded on a 2.5 ml Ni-NTA agarose (Qiagen) column pre-equilibrated with the same buffer. After extensive washing in binding buffer, non-specific proteins were removed from column with washing buffer (25 mM Tris-HCl (pH 8.0), 500 mM NaCl, 25 mM imidazole). The bound proteins were eluted with an elution buffer (25 mM Tris-HCl (pH 8.0), 500 mM NaCl, 500 mM imidazole) at a rate of 50 ml/h. One milliliter fractions were collected and stored at 4 °C until use.

Thermal stability of recombinant eLH/CG

Sf9 insect cells were seeded in 25 cm^2 flasks and co-infected with recombinant baculoviruses to generate different tagged/untagged heterodimeric variants of eLH/CG or mono-infected with baculovirus expressing eLH/CG β - α single chain. The supernatants were recovered by centrifugation at 100 *g* for 5 min and the aliquots (100 μl) were prepared in sealed 'oil-free' tubes for thermocyclers (Mo Bi Tec, VWR, Strasbourg, France). Natural eCG and eLH were prepared at the same concentration (4 $\mu\text{g}/\text{ml}$) in TG3 insect cell medium and aliquoted in the same way. The tubes were incubated in a water bath (WB7 Memmert) for 5 min at different temperatures between 65 and 90 °C. The reaction was stopped by cooling the tubes in ice and the percentage of the remaining dimers were measured by the sandwich ELISA as described above. The values are means of three experiments. The sandwich ELISA dose-response data were analyzed by plotting absorbance as a function of dilution of the samples; the quantities of the remaining dimers were derived from the slope of the curves so obtained by linear regression. These percentages of dimer were then plotted as a function of incubation temperature and the $T_{1/2}$ values were derived from the temperatures at which 50% of the hormones remained as dimers.

Results

Construction of a baculovirus transfer vector for expression of N-terminal polyhistidine-labeled proteins

DNA sequencing confirmed that the baculovirus transfer vector pGmAc115T contained the very late promoter and polyadenylation signal (Fig. 1) of the PH protein. The modified vector was used in this study for cloning of PCR-amplified equine α - and LH/CG β -subunit cDNAs. Downstream of the PH promoter, the modified baculovirus transfer vector pGmAc115T-SP-His was extended with ABCDEFGH-prehybridized oligonucleotides (Table 1) containing the insect cell UDP-glucosyltransferase gene SP sequence (MTILCW-LALLSTLTAVNA) in frame with the sequence encoding six histidine residues ($6\times\text{His}$), followed by Xba I, Sma I, and Asp718 I unique cloning sites. The unique Bgl II cloning site was degenerated so that it cannot be used anymore.

Production of monomeric equine α -subunit and LH/CG β -subunit

Sf9 cells were mono-infected with recombinant baculoviruses NPV- α or AcSLP10- α for α -subunit and recombinant baculoviruses NPV- β or AcSLP10- β for the β -subunit. The supernatants from infected cells were recovered by centrifugation at 100 g for 5 min at 4 days pi and assayed by specific competitive ELISAs. The maximal production of α -subunit from Sf9 cells was found to be 3.5 $\mu\text{g/ml}$ when expressed under the PH promoter (AcMNPV-derived baculovirus) and 5.5 $\mu\text{g/ml}$ when expressed under the P10 promoter (AcSLP10-derived baculovirus). More β -subunit was secreted when expressed under the control of the P10 promoter (6 $\mu\text{g/ml}$) than when expressed under the PH promoter (1.5–2 $\mu\text{g/ml}$).

Production of recombinant eLH/CGs

Sf9 insect cells were co-infected with appropriate recombinant baculoviruses (Fig. 2). The supernatants from infected cells were recovered by centrifugation at 100 g for 5 min, 4 days pi and assayed by a specific sandwich ELISA.

For heterodimeric eLH/CGs, the mean production in Sf9 insect cells ranged from 3.2 ± 1.4 $\mu\text{g/ml}$ for $\alpha_{\text{PH}}\times\beta_{\text{PH}}$ to 4.4 ± 0.7 $\mu\text{g/ml}$ for $\alpha_{\text{P10}}\times\beta_{\text{P10}}$ 4 days pi ($n=4$; Table 3), as already shown for $\alpha_{\text{PH}}\times\beta_{\text{P10}}$ heterodimeric eLH/CG (Legardinier *et al.* 2005a). Polyhistidine-tagged variants of eLH/CG possessed a short sequence of six histidine residues (His) at the

N- or C-terminal end of the α - or β -subunit. As indicated in Table 3, the mean production of polyhistidine-tagged eLH/CG variants was close to and even slightly higher than that found for untagged heterodimers with maximal values ranging from 4.5 to 5.3 $\mu\text{g/ml}$. All possible combinations of His-tagged α - or β -subunits with untagged complementary subunits to form polyhistidine-tagged heterodimers were expressed and mean productions were found to be equivalent. However, the β - α_{PH} single-chain eLH/CG production (1.5 ± 0.1 $\mu\text{g/ml}$) by infected Sf9 insect cells was twofold less than that of the heterodimeric variant obtained when production of both the subunits was driven by the same (PH) promoter (3.2 ± 1.4 $\mu\text{g/ml}$). When the P10 promoter was used for one or both the subunits, the heterodimer production was slightly higher (3.9–4.4 $\mu\text{g/ml}$).

Immunological characterization of eLH/CG heterodimers secreted in the supernatant of infected Sf9 insect cells

As shown in Fig. 2, Sf9 cells co-infected with the appropriate recombinant baculoviruses should express recombinant heterodimeric eLH/CG ($\alpha\times\beta$), polyhistidine-tagged variants of eLH/CG with the histidine sequence either on α -subunit at the N-terminus (His- α - β) or the C-terminus (α -His- β) or on the LH/CG β -subunit at the N-terminus ($\alpha\times$ His- β) or the C-terminus ($\alpha\times\beta$ -His). Sf9 insect cells were also infected to express β - α single chain. The supernatants from infected cells were recovered by centrifugation at 100 g for 5 min 4 days pi and analyzed by western blotting using rabbit anti-eCG polyclonal antibody (eCG Ab; Fig. 3A).

Standard eCG (Fig. 3A, lane 1) exhibited a complex range of bands between 80 and 100 kDa, while the recombinant eLH/CG variants expressed in Sf9 insect cells appear at ~ 45 kDa for β - α (Fig. 3A, lane 5) and as doublets for heterodimeric eLH/CG (Fig. 3A, lanes 2–3) with an upper band at 45 kDa and a lower one at ~ 38 –40 kDa. However, the doublets for recombinant heterodimers are more apparent than those shown in a preceding paper (Legardinier *et al.* 2005a) because of stronger signals. Same doublets with identical apparent molecular weights were detected for the four variants with their His tag at the N- and C-termini of α - and β -subunits respectively (Fig. 3A, lanes 7–10).

Trapping of polyhistidine-tagged eLH/CG to Ni-NTA agarose

The $\alpha\times\beta$ -His heterodimer used as a sample (S) was produced in the medium of infected Sf9 insect cells and was desalted on a PD10 column (DS) before loading on a Ni-NTA agarose column. It was totally retained by Ni-NTA

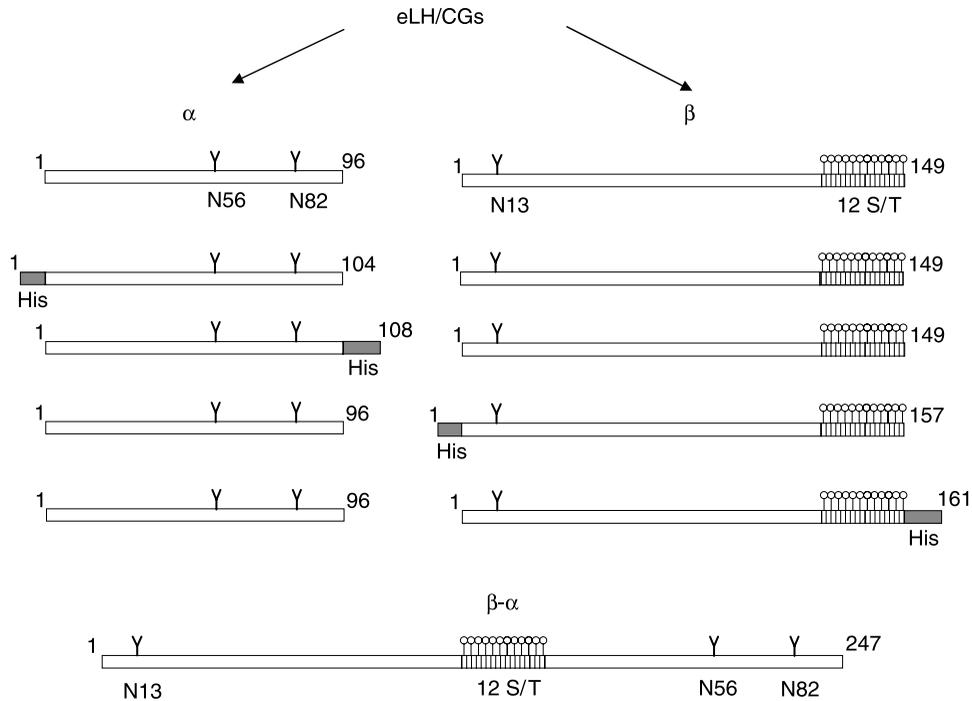


Figure 2 Schematic representation of recombinant untagged or polyhistidine-tagged heterodimeric variants of eLH/CG and β - α single-chain. Equine LH/CG is composed of two non-covalently associated α - and β -subunits. The 96-residue α -subunit bears two N-linked oligosaccharide chains (N56, N82, asparagine residues 56 and 82) and the 149-residue β -subunit bears only 1 N-glycan (N13) and 12 potential O-linked oligosaccharide chains on serine/threonine residues (12S/T) of the C-terminal peptide (CTP) (hatched box). N- and O-glycans are represented with specific symbols (Y for N-glycans and O for O-glycans).

agarose since no hormone was detected in the two fractions obtained by washing with 10 and 25 mM imidazole (W1, W2 respectively). The $\alpha \times \beta$ -His heterodimer was eluted in fractions E1–E4 with an elution buffer

Table 3 Mean production \pm s.e.m. ($n=4$) of recombinant untagged or polyhistidine-tagged heterodimeric and single-chain variants of equine luteinizing hormone (eLH)/chorionic gonadotropin (CG)

	Production ^b ($\mu\text{g/ml}$)
Recombinant protein^a	
Dimeric eLH/CG	
$\alpha_{\text{PH}} \times \beta_{\text{PH}}$	3.2 ± 1.4
$\alpha_{\text{PH}} \times \beta_{\text{P10}}$	3.9 ± 0.7
$\alpha_{\text{P10}} \times \beta_{\text{PH}}$	4.4 ± 1.0
$\alpha_{\text{P10}} \times \beta_{\text{P10}}$	4.4 ± 0.7
His-tagged dimeric eLH/CG	
His- $\alpha_{\text{PH}} \times \beta_{\text{P10}}$	4.7 ± 0.3
α -His _{P10} \times β_{PH}	4.7 ± 0.7
α_{P10} \times His- β_{PH}	4.5 ± 0.7
$\alpha_{\text{PH}} \times \beta$ -His _{P10}	5.3 ± 0.4
Single-chain eLH/CG	
β - α_{PH}	1.5 ± 0.1

^aPH and P10 subscripts refer to the promoter driving the expression of each subunit in heterodimeric recombinant proteins. The expression of eLH/CG single chain depends on the control of PH promoter.

^bIn terms of pure eCG (10 000 IU/mg) by sandwich ELISA.

containing 500 mM imidazole, as shown in the western blot using eCG Ab (Fig. 3B, left panel). The silver-stained electrophoresis of E1 fraction clearly shows a band corresponding to eLH/CG but it is contaminated by high MW proteins (Fig. 3B, right panel). The three other His-tagged eLH/CGs were also totally trapped by Ni-NTA (not shown) indicating that the position of the tag did not influence either binding to Ni-NTA or detection by the antibody. Therefore, the heterodimer can be trapped and detected irrespective of the position of the His tag at the N- or C-terminus of any subunit. Excess free α -subunit can be found in a few cases (Fig. 3A, lane 8; Fig. 3B, S or DS), which, as expected, is not retained by the Ni-NTA column.

Carbohydrate side-chain analysis of eLH/CG heterodimers by lectin ELISA

Recombinant heterodimeric hormone preparations were compared in the same assays to natural counterpart eCG for their ability to bind to *Galanthus nivalis* (GNA) and *Arachis hypogaea* (PNA) plant lectins (Table 4). GNA and PNA specifically recognized α -linked mannose on N-glycans (Shibuya *et al.* 1988) and Gal β 1-3GalNAc on O-glycans (Goldstein & Hayes 1978) respectively. Microtiter plates were coated with

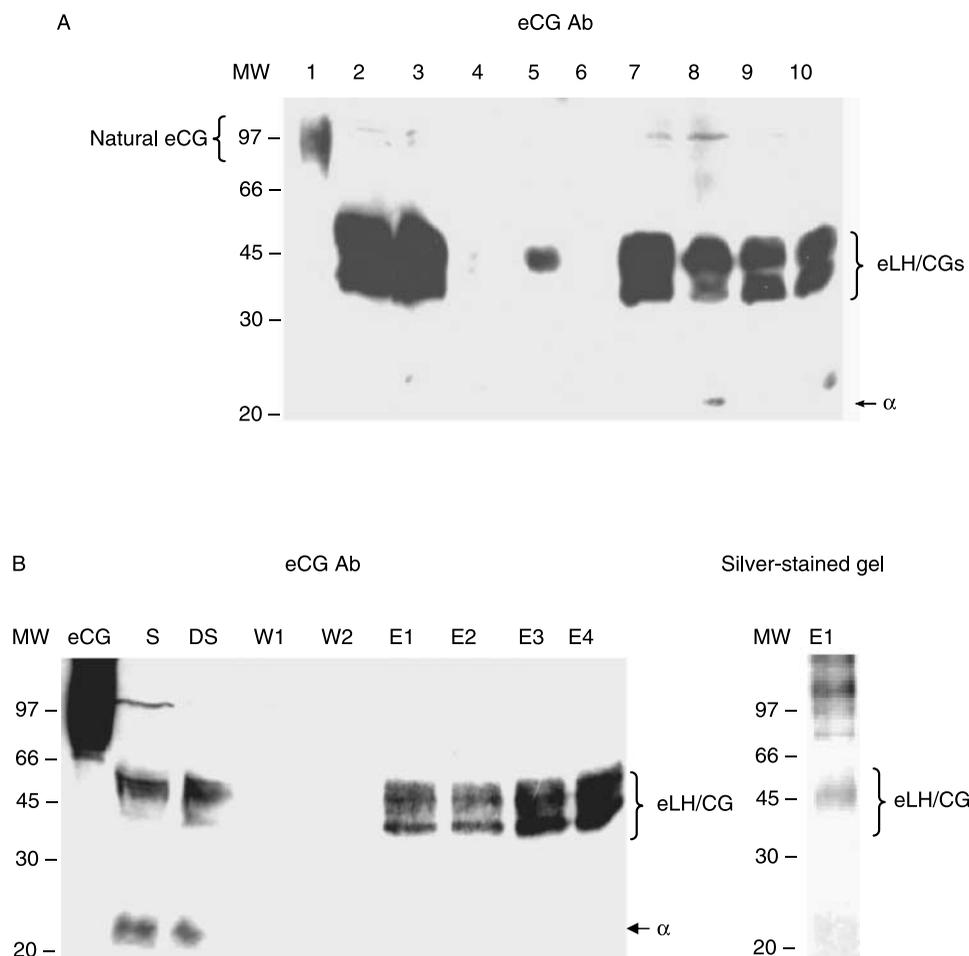


Figure 3 (A) Western blot analysis of recombinant untagged or polyhistidine-tagged heterodimeric variants of eLH/CG and β - α single-chain secreted by infected Sf9 cells. After 12% SDS-PAGE under non-reducing conditions, eLH/CG preparations were transferred to a nitrocellulose membrane. Recombinant hormones from co-infected Sf9 cells were revealed with a specific polyclonal anti-eCG antibody (A). Lane 1, natural eCG (50 ng); lanes 2 and 3, $\alpha \times \beta$; lane 5, β - α single-chain; lane 7, $\alpha \times \beta$ -His; lane 8, $\alpha \times \text{His}$ - β ; lane 9, His- $\alpha \times \beta$; lane 10, α -His $\times \beta$ (30 μ l conditioned media). (B) Western blot (left panel) and silver-stained gel electrophoresis (right panel) of $\alpha \times \beta$ -His purified from infected Sf9 insect cells. Secreted recombinant $\alpha \times \beta$ -His (S) was first desalted on a PD10 column (DS) before loading on a Ni-NTA column with a rate of 0.5 ml/min in a binding buffer. After two steps of washing with 10 and 25 mM imidazole (W1, W2), $\alpha \times \beta$ -His was eluted using elution buffer (E1–E4 1 ml fractions) containing 500 mM imidazole. Thirty microliters of these fractions were submitted to two 10% SDS-PAGE in non-reducing conditions; one gel was analyzed by western blotting using polyclonal anti-eCG antibody (eCG Ab) (left) and the other one was silver-stained (right).

the highly specific monoclonal antibody directed against eCG α -subunit (α mAb 89A2) to catch recombinant dimeric hormones secreted in the serum-containing supernatant from infected Sf9 cells. For the sake of comparison, natural eCG was also diluted in the serum-containing culture medium. The supernatants from Sf9 cells infected with wild-type baculovirus AcMNPV and baculovirus AcSLP10- β were used as negative controls. The first control provides a measure of the background signal due to glycoproteins secreted by Sf9 cells in the absence of a recombinant

hormone. The second control demonstrates that the recombinant β -subunit is not retained by the anti-eCG α antibody (α mAb 89A2) used to catch the heterodimers. The location of *N*- and *O*-linked carbohydrate side chains of eLH/CG heterodimers produced in Sf9 cells are shown in Fig. 2.

Binding to mannose residues

As shown in Table 4, the recombinant heterodimeric hormones as well as the β - α single chain produced in

Table 4 Lectin ELISA for natural equine chorionic gonadotropin (eCG) and recombinant heterodimeric and single-chain variants of equine luteinizing hormone (eLH)/CG

	GNA (<i>Galanthus nivalis</i>) Man α -linked man (N-glycans) 0.20 μ g/ml	PNA (<i>Arachis hypogaea</i>) Gal β 1-3GalNAc (O-glycans) 0.40 μ g/ml
Lectin	Absorbance (mean \pm S.E.M.)	
Specificity concentration used		
Controls		
Wild-type AcMNPV	0.166 \pm 0.013	0.098 \pm 0.013
β^a	0.126 \pm 0.002	0.082 \pm 0.002
Dimeric eLH/CG		
$\alpha \times \beta$	0.352 \pm 0.017*	0.608 \pm 0.054*
His-tagged dimeric eLH/CG		
His- $\alpha \times \beta$	0.252 \pm 0.020*	0.728 \pm 0.071*
α -His $\times \beta$	0.399 \pm 0.016*	0.812 \pm 0.067*
$\alpha \times$ His- β	0.219 \pm 0.016*	0.598 \pm 0.039*
$\alpha \times \beta$ -His	0.355 \pm 0.023*	0.934 \pm 0.062*
Single-chain eLH/CG		
β - α	0.356 \pm 0.031*	0.572 \pm 0.049*
Natural hormone		
Natural eCG	0.073 \pm 0.005*	0.055 \pm 0.004*

Each material was tested at a concentration of 100 ng per well (100 μ l). Recombinant proteins used are shown in Table 3. *ANOVA test (Newman-Keuls); * $P < 0.001$ with wild-type AcMNPV as control.

^a β -subunit was used as a negative control; not captured by the specific anti-eCG α antibody (α mAb 89A2) coated on the microtiter plate.

Sf9 cells specifically interacted with GNA that is known to bind to terminal mannosyl residues of N-glycans (Shibuya *et al.* 1988). In contrast, the natural eCG did not bind to GNA, underscoring a complex-type N-glycosylation in which internal mannosyl residues are shielded from lectin.

Binding to O-glycans

PNA binds the core 1 Gal β 1-3GalNAc disaccharide of O-glycans (Goldstein & Hayes 1978) only in the absence of terminal sialic acids. Natural eCG did not bind to PNA contrary to all tested recombinant untagged or polyhistidine-tagged eLH/CG variants, which did bind to PNA. Even the β - α single chain, in which the potentially O-glycosylated-CTP was wedged between the α - and β -subunits, bound efficiently to PNA.

In vitro LH Biological activity of eLH/CG variants

In vitro LH bioactivities of recombinant eLH/CGs were determined by their steroidogenic activity in MLTC-1 cells expressing an endogenous LH receptor.

The ability of $\alpha \times \beta$ heterodimer preparations to stimulate the production of progesterone in MLTC-1 cells was only slightly lower than eCG reference, whereas β - α single chain was half as active as the eCG reference (Table 5; Fig. 4). Among the polyhistidine-tagged heterodimers of eLH/CG, α -His $\times \beta$ and $\alpha \times$ His- β were 50 and 70% as active as the reference eCG

respectively, whereas His- $\alpha \times \beta$ and $\alpha \times \beta$ -His exhibited full *in vitro* LH bioactivity (Table 5, Fig. 5).

In Vitro LH receptor binding of recombinant eLH/CGs

For recombinant hormones that expressed a low biological activity, it was important to determine specifically their ability to bind to LH receptors using Leydig cell membrane preparations.

As shown in Table 5 and Fig. 6, the β - α single-chain and α -His $\times \beta$ were about 2.4-fold less efficient to bind to LH receptors than the $\alpha \times \beta$ counterpart and 2.8-fold less than that for natural eCG. To a lesser extent, $\alpha \times$ His- β was found 1.4-fold less potent than $\alpha \times \beta$. There is no significant difference in the LH receptor binding for $\alpha \times \beta$ and $\alpha \times \beta$ -His heterodimers. There is no significant difference in the S/B ratio for all natural and recombinant hormones indicating that there is no difference in their transduction efficiency after receptor binding.

Thermal denaturation of recombinant heterodimeric variants of eLH/CG

The thermal dissociation of recombinant eLH/CG variants as determined from two-site ELISA was found to begin at temperatures above 70 °C. Indeed, there was no difference for any hormone between the samples incubated for 5 min at 20 °C or at 70 °C. The $T_{1/2}$ values determined for the natural eLH and eCG and for the various recombinant eLH/CGs are shown in Table 6.

Table 5 *In vitro* luteinizing hormone (LH) binding and steroidogenic activity of recombinant heterodimeric and single-chain variants of equine LH (eLH)/chorionic gonadotropin (CG) relative to natural equine CG (eCG)

Hormone	Relative potencies ^a		
	Binding (B)	Steroidogenesis (S)	S/B ratio
Natural eCG	100 ^b	100 ^c	1.0
$\alpha \times \beta$	85 ± 5	93 ± 9	1.1 ± 0.2
His- $\alpha \times \beta$	ND	91 ± 6	ND
α -His × β	35 ± 7 [†]	51 ± 5 [‡]	1.5 ± 0.4
$\alpha \times$ His- β	60 ± 8 [*]	70 ± 7 [*]	1.2 ± 0.3
$\alpha \times \beta$ -His	85 ± 5	94 ± 6	1.1 ± 0.2
β - α single-chain	35 ± 8 [†]	51 ± 4 [‡]	1.5 ± 0.4

Student's *t*-test: significant difference with natural eCG (analyses from three to five experiments); **P* < 0.05, †*P* < 0.02, ‡*P* < 0.01. ND = not determined.

^aConcentrations of hormones were measured in the dimer-specific ELISA using a value of 10 000 IU/mg for pure eCG taken as reference.

^bIC50 = 140 ng/ml.

^cED50 = 55 ng/ml.

All recombinant dimeric eLH/CGs, with or without His tag at any position, displayed $T_{1/2}$ values identical to that of pituitary eLH (74.5 ± 1.5 °C) and only natural eCG displayed a significantly higher

$T_{1/2}$ value (77.4 ± 0.8 °C). As expected, single-chain eLH/CG exhibited a much higher $T_{1/2}$ value corresponding to its irreversible denaturation and precipitation.

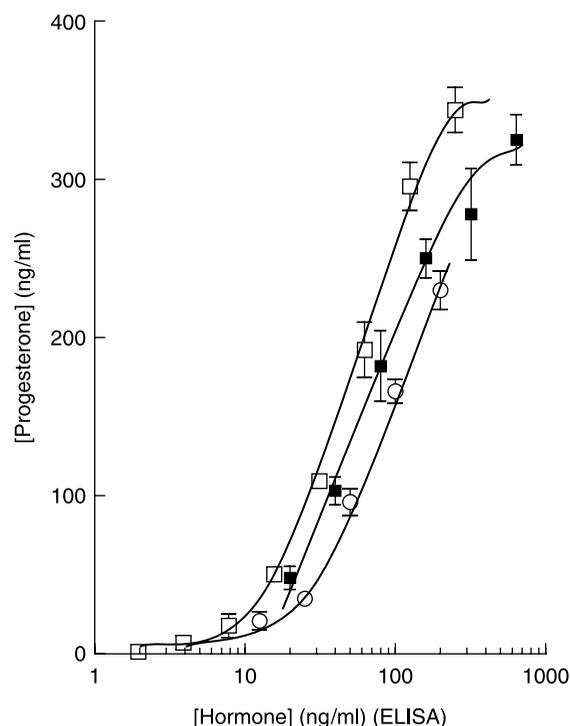


Figure 4 *In vitro* LH bioactivities of recombinant $\alpha \times \beta$ heterodimeric eLH/CG (■) and β - α single-chain (○), against natural eCG (□). Potencies are estimated by the production of progesterone in MLTC-1 cells expressing endogenous LH receptor. Hormone concentrations are indicated as ng of pure eCG (10 000 IU/mg) per ml as previously determined by dimer-specific sandwich ELISA. This figure is representative of three independent assays.

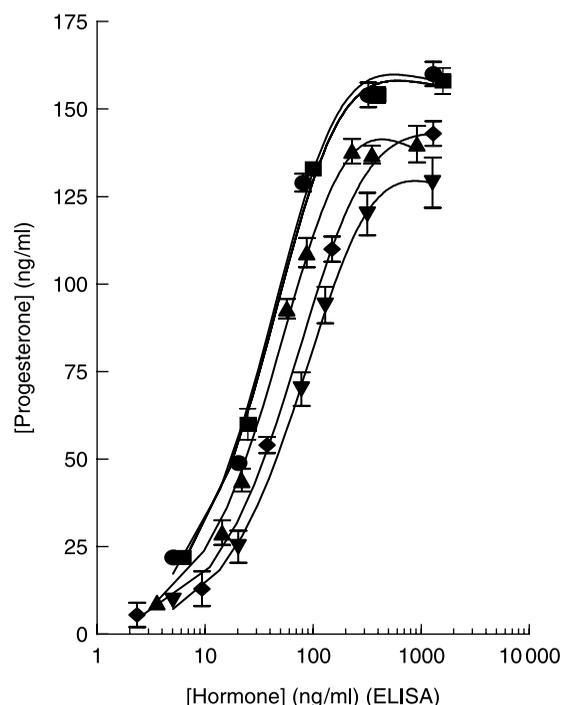


Figure 5 *In vitro* LH bioactivities of recombinant untagged $\alpha \times \beta$ (■) and polyhistidine-tagged heterodimeric variants of eLH/CG (▲, His- $\alpha \times \beta$; ▼, α -His × β ; ◆, $\alpha \times$ His- β ; ●, $\alpha \times \beta$ -His) represented by the production of progesterone in MLTC-1 cells expressing endogenous LH receptor. Hormones concentrations are indicated as nanograms of pure eCG (10 000 IU/mg) per ml as previously determined by specific sandwich ELISA. This figure is representative of three independent assays.

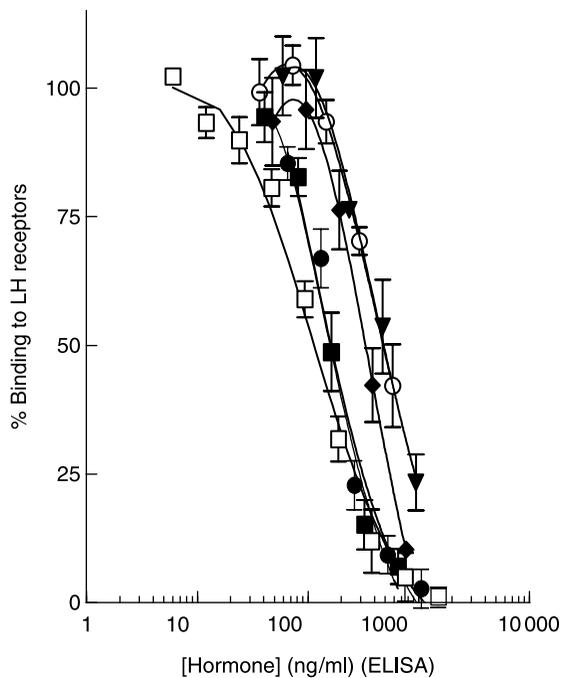


Figure 6 Competition curves of ^{125}I -oLH binding to membrane preparations from rat Leydig cells with recombinant β - α single-chain (\square), untagged $\alpha \times \beta$ (\blacksquare) or polyhistidine-tagged (\blacktriangledown , α -His \times β ; \blacklozenge , $\alpha \times$ His- β ; \bullet , $\alpha \times \beta$ -His) heterodimeric variants of eLH/CG and eCG reference preparation (\square). The cell membranes were incubated in triplicate with a constant amount of ^{125}I -oLH and increasing the concentrations of unlabelled eCG or recombinant eLH/CGs. Hormone concentrations are indicated as nanograms of pure eCG (10 000 IU/mg) per ml as determined by specific sandwich ELISA. This figure is representative of three independent assays.

Discussion

In previous papers, Boime & Ben-Menahem (1999) recommended single chain instead of heterodimeric gonadotropin expression because heterodimeric gonadotropins did not appear to be efficiently assembled or secreted (Corless *et al.* 1987, Matzuk & Boime 1988). In contrast, we previously reported a highly efficient assembly of the equine α - and β -subunits in a stable heterodimeric eLH/CG that was secreted in serum-containing culture medium of transfected COS-7 cells (Chopineau *et al.* 1997b) and infected Sf9 insect cells (Legardinier *et al.* 2005a).

In this study, we used the highly productive baculovirus–insect cell system to express single-chain and heterodimeric eLH/CG variants in order to compare their secretion rate, *in vitro* biological activity, and thermal stability with natural serum eCG and/or pituitary eLH as references. These biochemical and biological characteristics were also investigated for heterodimeric variants containing a polyhistidine tag fused either at the N- or the C-terminus of either the

Table 6 Thermal transition values of natural and recombinant equine gonadotropins

Transition temperature	
$T_{1/2}$ (°C) mean \pm S.D.	
Hormone	
Natural eCG	77.4 \pm 0.8 (n=3)
Pituitary eLH	74.5 \pm 1.5* (n=3)
$\alpha \times \beta$	73.7 \pm 1.0* (n=3)
His- $\alpha \times \beta$	73.2 (n=1)
α -His $\times \beta$	74.7 (n=1)
$\alpha \times$ His- β	72.7 (n=1)
$\alpha \times \beta$ -His	74.1 (n=1)
β - α	> 80 (n=1)
	} 73.7 \pm 0.8* (n=4)

Student's *t*-test: significant difference with natural eCG (analyses from three to four experiments); * $P < 0.05$.

α - or the β -subunit to determine if the location of the histidine extension could influence these properties.

The baculovirus–insect cell system has been used for a long time to express heterologous proteins (Miller 1988, Luckow & Summers 1989) and glycoproteins (Matsuura *et al.* 1987, Farrell *et al.* 1998, Altmann *et al.* 1999). Most of the time, recombinant AcMNPV-derived baculoviruses are constructed with heterologous genes placed under the control of the very late PH promoter or under the control of the very late P10 promoter. We showed in this study that heterodimeric eLH/CG variants and monomers were produced and secreted in high quantities, irrespective of the source of the baculovirus used. Nevertheless, these recombinant glycoproteins (monomers and heterodimers) were secreted in higher yield when expressed with AcSLP10-derived baculovirus (bearing only the P10 promoter) than when expressed with AcMNPV-derived baculovirus (bearing both P10 and PH promoters). Indeed, it was already shown that the production of recombinant proteins expressed under the control of the P10 promoter was increased when the expression of PH was impaired (Chaabihi *et al.* 1993). These differences of production are thus not due to differences in strength between very late PH and P10 promoters, which were shown to be equivalent.

We previously reported the construction of a baculovirus transfer vector (p119-His) to overexpress the recombinant C-terminally polyhistidine-tagged proteins (Legardinier *et al.* 2005b) using equine-specific SP of α - and LH/CG β -subunits. Here, we described a new baculovirus transfer vector pGmAc115T-SP-His, designed for the secretion of any N-terminally polyhistidine-tagged proteins using an insect cell SP derived from the UDP-glucosyltransferase gene. Insect cells can recognize homologous and heterologous SPs and thus recombinant equine glycoproteins were secreted equivalently when joined either to the insect cell UDP-glucosyltransferase SP or equine-specific α - and eLH/CG β -subunit SPs.

The β - α single-chain derivative was secreted in two- to threefold lower quantities than the heterodimeric eLH/CG variants. It has been previously reported that single-chain hCG was also secreted at a lower rate than heterodimeric hCG in Sf9 cells infected by recombinant baculovirus (Narayan *et al.* 1995); however, the difference (1.8 vs 2.7 $\mu\text{g}/\text{ml}$) was less marked than that for eLH/CG (1.5 vs ~ 4 $\mu\text{g}/\text{ml}$). It has also been reported that single-chain human LH was secreted at a rate fourfold lower than its heterodimeric counterpart in CHO cells (Garcia-Campayo *et al.* 1997) but, in COS7 cells, single-chain and heterodimeric eLH/CGs were found to be secreted at similar, albeit low rates (0–13 $\mu\text{g}/\text{ml}$; Galet *et al.* 2000).

Untagged and polyhistidine-tagged heterodimeric eLH/CG variants were expressed efficiently and secreted as stable heterodimers in the medium of infected Sf9 insect cells with equivalent maximal productions suggesting that the histidine extension at various locations influenced neither subunit association and secretion of the different recombinant variants of eLH/CG nor their immunological recognition by conformation antibodies used in this study.

The β - α configuration was widely used to produce recombinant single-chain glycoprotein hormones of many species (Narayan *et al.* 1995, Boime & Ben-Menahem 1999, Dirnberger *et al.* 2001, Galet *et al.* 2001, Fidler *et al.* 2003, Min *et al.* 2004, Jablonka-Shariff *et al.* 2007), sometimes with heterologous CTP from hCG inserted between fused α - and β -subunits to enhance the secretion of these single-chain protein variants (Sugahara *et al.* 1996, Garcia-Campayo *et al.* 1997, Grossmann *et al.* 1997, Fares *et al.* 1998). These earlier studies showed that the CTP played the role of a flexible hydrophilic spacer allowing genetically fused α - and β -subunits to adopt a functional conformation and that secretion in the presence of CTP was enhanced due to its specific O-glycans. Indeed, our qualitative analysis of glycosylation using specific lectins showed that the β - α single-chain as well as all secreted heterodimeric variants of eLH/CG exhibited not only mannosylated N-glycans but also galactosylated core 1 O-glycans as previously shown for recombinant equine glycoproteins expressed in Sf9 insect cells (Legardinier *et al.* 2005b). Nevertheless, we can suppose that the three-dimensional conformation of single-chain eLH/CG could be altered when compared with heterodimeric eLH/CGs, as previously shown for hCG (Fralish *et al.* 2003). Unlike previous studies which reported that eLH/CG β - α single chain was fully bioactive *in vitro* (Galet *et al.* 2001, Min *et al.* 2004) and *in vivo* (Jablonka-Shariff *et al.* 2007), we report here that the β - α eLH/CG single-chain secretion by virus-infected insect cells was reduced when compared with heterodimeric eLH/CG and exhibited a slightly reduced *in vitro* LH bioactivity. Considering all these clues, it is of utmost interest to study the bioactivities of highly produced and fully

bioactive heterodimeric glycoforms rather than those of single-chain variants whose conformation is potentially less similar to that of natural eCG and eLH.

To consider a nickel-affinity purification of recombinant eLH/CG, we first assessed the influence of the presence and the position of a short polyhistidine tag on LH bioactivity. Our data are in agreement with previous studies arguing that the C-terminus of α -subunit and the N-terminus of the β -subunit must be intact because they contain determinants involved in receptor binding (Chen & Bahl 1992). Among the polyhistidine-tagged heterodimers that retained full *in vitro* bioactivity, we showed that α - β -His was caught on a nickel-affinity matrix without dissociation but was co-eluted with some contaminating proteins. Further purification steps are required to obtain a highly purified recombinant hormone. Nevertheless, this heterodimer is a good candidate for further purification of polyhistidine-tagged recombinant variants of eLH/CG, expressed in insect cells or other eukaryotic expression systems allowing a complex-type N-glycosylation or sialylated N/O-glycans.

Finally, short-term heat-dependent dissociation of eLH, eCG, and the recombinant heterodimeric and single-chain of eLH/CG described in the present work has been followed using two-site sandwich ELISA. All the natural and recombinant dimers exhibit transition temperatures ($T_{1/2}$) in the range of 73–77 °C. These values are very similar to that of 77 °C previously reported for pituitary porcine LH (Burova *et al.* 2001) and urinary human FSH obtained by high-sensitivity differential scanning calorimetry (HS-DSC) and short-term heat treatment followed by RRA and HPLC. HS-DSC is the only method that allows thermodynamic parameters to be obtained directly for protein unfolding. The heat-dependent dissociation of porcine LH as followed by RRA was found to be closely correlated to HS-DSC data on the same hormone (Burova *et al.* 2001). This indicates that the heat-promoted dissociation of pLH is due to the melting of its secondary and tertiary structures. Isolated subunits did not exhibit any transition indicating that they do not retain stable periodic structures (Burova *et al.* 2001). Therefore, the quaternary structure of the heterodimeric hormone is strictly dependent on the proper secondary and tertiary structures of its constitutive subunits and, symmetrically, these secondary and tertiary structures exist only in the heterodimer. Consequently, $T_{1/2}$ values of different gonadotropin heterodimers are good thermodynamic criteria for the evaluation of their respective global cooperative folding.

The observation that all natural heterodimeric gonadotropins exhibit identical transition temperatures around 73–77 °C indicates that the thermodynamic stabilities of their global three-dimensional structure are identical. This is not unexpected as they comprise one common α -subunit and different but closely related β -subunits.

The recombinant eLH/CGs with hexahistidine tags at the N- or C-terminus of either their α - or β -subunit exhibit $T_{1/2}$ values within the 95% confidence limit of that determined for wild-type eLH/CG. This indicates that the tag has no negative influence on the thermodynamic stability of the heterodimer irrespective of its position at any terminus of either subunit.

Recombinant heterodimeric eLH/CGs, with or without hexahistidine tags, exhibit $T_{1/2}$ values identical to that of pituitary eLH and their $T_{1/2}$ values are all significantly lower than that of the natural eCG (Table 6). Recombinant eLH/CGs, natural eLH, and natural eCG all essentially differ from one another by their carbohydrate moieties. The immature-type carbohydrate chains in recombinant eLH/CGs thus have the same impact as the mature carbohydrates of pituitary eLH on the conformation stability of the heterodimer. It can be concluded that the completion of carbohydrate side-chains up to their mature structure is not required for the proper cooperative folding of the α - and β -polypeptide chains in the glycoprotein hormone heterodimers.

Nevertheless, natural eCG has been found to exhibit a slightly (2.9–3.7 °C), but significantly, higher $T_{1/2}$ than pituitary eLH and recombinant eLH/CGs respectively (Table 6). These data are in agreement with our previous reports showing that carbohydrate side chains play a primary role in the stability of heterodimers at 37 °C (Galet *et al.* 2004) in which the interchange of disulfide bonds is involved (Belghazi *et al.* 2006). The carbohydrate moieties of eCG are much bulkier and more acidic than those in pituitary eLH and in recombinant eLH/CGs (Smith *et al.* 1993, Matsui *et al.* 1994, Bousfield & Butnev 2001). The present report reinforces the view that the carbohydrate chains in eCG play a stabilizing role on the heterodimeric structure of the hormone.

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