Abstract

Equine luteinizing hormone (eLH) and chorionic gonadotropin (eCG) are composed of identical α and β polypeptide chains, but eCG subunits are much more heavily glycosylated and sialylated. Consequently, eCG exhibits a much longer half-life than eLH in blood. Recombinant eLH/CG, expressed in Sf9 and Mimic insect cells, were compared with one another and to the natural hormones eCG and eLH. Mimic cells are stably-transformed Sf9 cells, expressing five mammalian genes encoding glycosyltransferases involved in the synthesis of complex N-carbohydrate chains. Recombinant eLH/CG expressed in Mimic cells exhibited a higher apparent molecular weight (MW) than that expressed in Sf9 cells, suggesting that its N-glycosylation was, as expected, more complete. Nevertheless, the two recombinant eLH/CG exhibited lower MW than natural eCG from pregnant mare plasma. The two eLH/CG produced in Sf9 and Mimic cells were found to be active in in vitro LH and FSH bioassays, with potencies similar to those of eCG. By contrast, they exhibited no significant in vivo bioactivity, neither in the specific follicle-stimulating hormone (FSH) assay nor in the specific eCG assay. Although recombinant eLH/CG produced in Mimic cells bears more elaborate carbohydrate chains than recombinant eLH/CG from Sf9 cells, it exhibits no significant in vivo bioactivity, probably because of insufficient terminal sialylation of its carbohydrate chains, leading to its rapid removal from blood.

Introduction

Equine chorionic gonadotropin (eCG) formerly named pregnant mare serum gonadotropin (PMSG) belongs to the family of glycoprotein hormones. It is produced by trophoblast cells of endometrial cups in pregnant mares and plays a major role in the maintenance of early gestation (Allen & Moor 1972). Like the other members of the family, including luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH), eCG is composed of two dissimilar and noncovalently associated α- and β-subunits. Within the same species, the α-subunit is encoded by a single gene and is common to all glycoprotein hormones, whereas different genes encode β-subunits that confer specificity to the glycoprotein hormone heterodimers (Goverman & Pierce 1981, Combarnous 1992). In equids, in contrast to primates, both LH and CG β-subunits are encoded by the same gene (Sherman et al. 1992) and consequently the recombinant hormone is called eLH/CG. Both placental eCG and pituitary eLH exhibit dual LH and FSH activity ratios (Stewart & Allen 1979, Combarnous et al. 1984). Although eCG and eLH exhibit identical α and β polypeptide chains, their carbohydrate contents are different. Indeed, eCG is the most heavily glycosylated of all glycoprotein hormones, with 45% carbohydrate by weight (Harbon-Chabbat et al. 1961, Gospodarowicz 1972) versus 30% for eLH (Roser et al. 1984, Butnev et al. 1996). The α-subunit, composed of 96 amino acids, bears two complex-type N-linked oligosaccharide chains located at asparagines (Asn) 56 and 82 whereas the β-subunit composed of 149 amino acids has only one at Asn 13. In addition to N-glycans, both eLH and eCG β-subunits possess a carboxy-terminal peptide (CTP) of 28 amino acids (B122–149), which is O-glycosylated at the same twelve serine or threonine residues (Bousfield & Butnev 2001). Placental and pituitary hormones also strongly differ by their N-glycan termination with sialic acids (Siaα2,3 Gal) on eCG and sulphated N-acetylgalactosamines (SO4–4-GalNAc) on eLH (Smith et al. 1993, Matsui et al. 1994). The remarkable difference in their molecular weight is essentially due to the presence of longer disialylated poly-N-acetyllactosaminyl O-glycans on eCG, with a greater percentage of O-glycans attached at serine and threonine sites
These structural differences explain why eCG has such an exceptional half-life compared with eLH. Owing to its biological properties, eCG has been used for a long time in fertilization programs. Nevertheless, commercial preparations of partially purified eCG from PMSG could contain contaminants with potential sanitary risks. It is of great interest to produce, in large quantities, a bioactive substitute for eCG and other gonadotropins used as therapeutic agents. Only recombinant gonadotropins produced in mammalian cells have been shown so far to exhibit in vivo biological activity. Such hormones, like hFSH expressed in Chinese hamster ovary (CHO) cells (Keene et al. 1989), have even been put on the market as Gonal-F (Serono, Geneva, Switzerland) and Puregon (NV Organon, Oss, The Netherlands). However, the great advantage of non-mammalian systems like yeasts, insect cell lines or plants would be to produce recombinant

**Key to Symbols:**

- GlcNAc □
- Man ○
- Fucose ▼
- Gal ●
- GalNAc ■
- Sialic acid ◆

**Figure 1** Schematic representation of N- and O-glycans attached on placental eCG, pituitary eLH and recombinant eLH/CG expressed in Sf9 and Mimic cells. Monosaccharides are represented with indicated specific symbols. The location of these oligosaccharide chains on α and β subunit with or without CTP is represented.
proteins in larger amounts, at a lower cost, and in the absence of fetal calf serum. Some gonadotropins of zootechnical interest have already been expressed in baculovirus systems (Huang et al. 1991, Inaba et al. 1998, Kato et al. 1998, van de Wiel et al. 1998), in the methylotropic yeast Pichia pastoris (Fidler et al. 1998, 2003, Richard et al. 1998), and in plants (Dirnberger et al. 2001). However, only in vitro biological activities have been reported in these studies, with no information concerning the in vivo potencies of the recombinant hormones produced. Recently, single-chain eLH/CG (Galet et al. 1998), and in plants (Dirnberger et al. 2001). However, only in vitro biological activities have been reported in these studies, with no information concerning the in vivo potencies of the recombinant hormones produced. Recently, single-chain eLH/CG (Galet et al. 2001) and bFSH (Coulibaly et al. 2002) have been produced in the milk of transgenic rabbits. In vivo biological activity was determined only for eLH/CG, and it was found inactive because its half-life was too short, most likely because the carbohydrate moieties of recombinant glycoproteins in milk (Koles et al. 2004) are unusual compared with those found in serum glycoproteins. The present study deals with the analysis of the biological activities of recombinant eLH/CG produced in two insect cell lines, Sf9 and Mimic cells, the latter derived from Sf9 cells and expressing several mammalian glycosyltransferase genes (Hollister et al. 2002). Mimic cells have been claimed to produce glycoproteins with complex biantennary N-glycans as usually found in insect cells (Fig. 1). The present work was undertaken to determine whether recombinant eLH/CG expressed in Mimic cells is more completely glycosylated than that expressed in Sf9 cells and whether, consequently, it exhibits in vivo bioactivities.

Materials and methods

Cloning of complementary DNAs

Alignments of conserved amino acid sequences of equine (a_e), donkey (a_dk), human (a_h) and chimeric human/equine (a_hoe) gonadotropin α-subunits are shown in Figure 2, with corresponding nucleotide sequences. The few human nucleotides of chimeric a_hoe cDNA (Chopineau et al. 1997) were replaced with those encoding the equine α-subunit (a_e) (Ward et al. 1982, Chopineau & Stewart 1996) using common Bsm I restriction sites. This construct was used as a template to introduce XbaI restriction sites at each end and to remove the 3' untranslated region by PCR using primers A and B (Table 1). Then, the amplified fragment (430 bp) was digested and inserted downstream of the polyhedrin promoter (PH) at a unique XbaI site in a modified pGmAc116T baculovirus transfer vector (Poul et al. 1995) (Fig. 3A).

A full-length cDNA encoding the equine LH/CG β-subunit (β_e), including its signal peptide sequence and previously cloned into pCDM8 (Chopineau et al. 1995), was used. Its 3' untranslated region was removed and Bgl II and Hind III restriction sites were introduced at its ends by PCR using primers C and D (Table 1). The digested PCR product (535 bp) was inserted downstream of the protein p10 promoter (P10) into baculovirus transfer vector p119 (Chaabibi et al. 1993) using the Bgl II and Hind III cloning sites (Fig. 3B).

Truncated cDNA encoding equine LH/CG β-subunit missing its 122–149 sequence corresponding to the CTP (β_eACTP) was obtained from the full-length cDNA as a template by PCR using primers E and F, introducing a TGA stop codon at position 122 (Table 1). Then, the amplified product (446 bp) was subcloned into pGEM-T and reinserted into baculovirus transfer vector p119 using the indicated restriction sites and sequenced (Fig. 3B).

The α_e cDNA construct was also cloned in the mammalian expression vector pCDM8 and co-transfected with pCDM8-β_e (Chopineau et al. 1997) into COS-7 cells to produce the α_eβ_e eLH/CG heterodimer in order to compare its in vitro bioactivity with that of the previously studied chimeric α_hoeβ_e heterodimer.

PCR was carried out using Taq polymerase (Promega, Charbonnières, France) and amplified PCR products
were subcloned into pGEM-T (Promega) and digested using the restriction enzymes indicated (Fig. 3). Resulting fragments were low-melting gel-purified using NuSieve GTG agarose (BMA, Tébu, Le Perray-en-Yvelines, France) and directly ligated into specific baculovirus transfer vectors as described above. 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 (Meylan, France) and oligonucleotides from Eurogentec (Seraing, Belgium).

**Transient transfections of COS-7 cells**

COS-7 monkey kidney cells (ATCC-CRL 1651) were maintained at 37°C in Dulbecco’s modified Eagle’s medium, 5% CO₂, 3% horse serum, and 92% Dulbecco’s modified Eagle’s medium. 1 × 10⁶ cells per well were seeded in 6-well plates. 24 hours later, cells were transfected with the recombinant baculovirus vectors. Cells were harvested 2 or 4 days after transfection. To determine the activity of eLH/CG expressed in insect cells, we used the method described by Legardinier et al. (2005).

**Table 1** Sequences of the primers used in PCR to amplify nucleotide sequences encoding the equivalent equine alpha subunit (A,B) and eLH/CG beta subunit with (C,D) or without CTP (betaACTP) (E,F). The initiation and stop codons are underlined and the restriction sites are in bold.

<table>
<thead>
<tr>
<th>Primers names</th>
<th>Primers sequences</th>
<th>Restriction sites</th>
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<tbody>
<tr>
<td>A=Alpha 5’</td>
<td>TCTAGACTGCAAAAAAATCCAGAGGAAGAAGA</td>
<td>Xba I</td>
</tr>
<tr>
<td>B=Alpha 3’</td>
<td>GCTCTAGACATTTAAAAACCTGCGATGAGA</td>
<td>Xba I</td>
</tr>
<tr>
<td>C=Beta 5’</td>
<td>GAAAGATCTAGAACCAAGAGGATGAGA</td>
<td>Bgl II</td>
</tr>
<tr>
<td>D=Beta 3’</td>
<td>CCCAAAGCTTGTAGTCTACGAAAGAAG</td>
<td>Hind III</td>
</tr>
<tr>
<td>E=BetaACTP 5’</td>
<td>CTAGAAGCTGAACCAAGAGGATGAGA</td>
<td>Bgl II</td>
</tr>
<tr>
<td>F=BetaACTP 3’</td>
<td>GTGAGAAGCTGGAAGTCAGGGCGTCC</td>
<td>Hind III</td>
</tr>
</tbody>
</table>
medium (DMEM) without sodium pyruvate; supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES buffer, 2 mM l-glutamine (Gln), 100 units/ml penicillin and 100 µg/ml streptomycin. COS-7 cells were transiently transfected using the calcium phosphate precipitation procedure. Briefly, cells were cotransfected at 75% of confluency in duplicate in 6 cm diameter Petri dishes with 1 µg of each construct in serum-free media. pCDM8-βe was co-transfected with pCDM8 containing αte, or αc cDNAs. After 48 h incubation at 37°C, media were recovered by centrifugation at 100 g for 5 min and stored at −20 °C until assayed. Unless otherwise stated, all other reagents used in cell culture were purchased from Invitrogen (Cergy Pontoise, France).

Cells and virus infection

The Sf9 subclone of Spodoptera frugiperda Sf21 cells (Vaughn et al. 1977) was maintained at 28 °C in supplemented TC-100 growth medium containing 5% heat-inactivated FBS, 50 units/ml penicillin G and 50 µg/ml streptomycin. Mimic cells (Invitrogen) were cultured in complete Grace’s Insect Medium, supplemented with 10% FBS and antibiotics as above. Both insect cell lines were maintained as adherent cells at a density of 2·0 × 10⁶ to 2·5 × 10⁶ cells per ml in 25 cm² flasks (Falcon) and passed over 80% confluency three times a week. To reach the same cell density as that obtained with Sf9 cells, more Mimic cells had to be passed because of their slower growth (but never more than 30 times, as recommended by the manufacturer). The viruses were propagated in both cell lines and recovered as described previously (Summers & Smith 1987). Cells were infected or co-infected with recombinant baculoviruses at a multiplicity of infection (MOI) ranging from 5 to 10 plaque forming units (PFU) per cell. After 45 min incubation with viral suspensions, fresh culture media were added and cells were incubated at 28 °C until day 5 post-infection. The viral titers were determined by plaque assay (Summers & Smith 1987).

Construction of the recombinant baculoviruses

Sf9 cells were cotransfected with 5 µg transfer vector and 500 ng purified viral DNA using a lipofection method (DOTAP, Roche, Meylan, France). Two distinct viral DNA preparations were used: (i) viral DNA extracted from the wild-type Autographa californica multiple nucleocapsid polyhedrosis virus (AcMNPV), when a polyhedrin-specific transfer vector such as pGmAc116T-αte was used or (ii) viral DNA extracted from the baculovirus AcSLP10 (Chaabibi et al. 1993) which possesses only one very late promoter, the P10 promoter, driving the expression of the polyhedrin gene when the P10 specific transfer vectors such as p119-βe and p119-βACTP were used. In each case, 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 recombinant baculoviruses NPV-αte (PH), SLP10-βe (P10) and SLP10-βeACTP (P10) were carried out as previously described (Summers & Smith 1987).

Quantitation of expressed recombinant hormones in enzyme-linked immunosorbent assay (ELISA)

The concentrations of the recombinant eLH/CG dimeric hormones produced in COS-7 cells or insect cells culture media were determined in a sandwich ELISA as previously described (Galet et al. 2000). The microtitration plates from Nunc (Maxisorp, VWR, Strasbourg, France) were coated overnight at 4 °C with 100 µl per well 89A2 mouse monoclonal antibody (1 µg/ml) (Intervet, Boxmeer, Holland) recognizing the alpha chain as single subunit and as heterodimer in eCG (Chopineau et al. 1993). After five washes in 0·1% Tween 20–PBS (1·15 g/l Na₂HPO₄, 0·2 g/l KH₂PO₄, 8 g/l NaCl, 0·2 g/l KCl, Dulbecco) and saturation of non-specific sites with 0·2% bovine serum albumin (BSA) (Sigma, Saint-Quentin Fallavier, France) in 0·1% Tween 20–PBS, 100 µl per well of standard eCG NZY-01 (1500 IU/mg) (Lecompte et al. 1998) or media containing recombinant eLH/CG were incubated at different concentrations for 1 h at 4 °C. After five washes with 0·1% Tween20-PBS, a rabbit polyclonal antibody raised against highly-purified eCG (Cahoreau & Combarnous 1987) was added at a 1:100 000 dilution for 1 h at 4 °C. A peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, Interchim, Montluçon, France) added at 1:10 000 dilution for 1 h at 4 °C, and, after five washes, 100 µl/well SureBlue TMB peroxidase substrate was added (KPL, Eurobio, Les Ulis, France) and the mixture was incubated for 20 min at room temperature in the dark. The reaction was stopped with 50 µl 2N H₂SO₄ and 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, Downers Grove, IL, USA).

Western blot analysis of the recombinant dimeric hormones and free α- and β-subunits

Sf9 or transgenic Mimic insect cells were seeded in 25 cm² flasks and inoculated separately or simultaneously with recombinant NPV-αte (PH), SLP10-βe (P10) and SLP10-βeACTP (P10). Supernatants were recovered by centrifugation at 100 g for 5 min and aliquots (30 µl) were diluted in Laemmli’s 4 × buffer (Laemmli 1970) under non reducing or reducing
conditions (5% β-mercaptoethanol). Unheated or heated samples were electrophoresed in 10 or 14% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE). After separation, proteins were transferred overnight at 4°C onto nitrocellulose membranes (Schleicher and Shuell, Ecquevilly, France) within a mini-transblot apparatus at 20% v/v propanol-2/Tris–glycine (25 mM Tris, 0·192 M glycine) buffer from Amresco (Interchim, Montluçon, France). Membranes were saturated for 30 min at room temperature in Tris buffer saline (TBS; 25 mM Tris, 140 mM NaCl, 3 mM KCl pH 7·4) Amresco containing 0-05% Tween 20 and 5% non-fat milk and then incubated for 2 h with specific antibodies. Dimeric hormones were specifically revealed using rabbit polyclonal anti-eCG antibody (Cahoreau & Combarnous 1987) at 1: 50 000 dilution. Free α and β subunits with or without CTP were revealed using 10 µl mouse monoclonal antibody 89A2 (1 µg/ml) (Intervet) (Chopineau et al. 1993) and a rabbit polyclonal antibody (dilution 1:5000) raised against a synthetic LH/CG β1–9 peptide representing the nine N-terminal residues of LH and CG β-subunits from numerous species (M Chopineau & C Galet, unpublished). This peptide with highly-conserved sequence among all LH and CG β-subunits was conjugated to Keyhole Limpet hemocyanin using carbodiimide as the coupling agent. The membranes were washed three times for 10 min in TBS-0-05% Tween 20 and incubated for 45 min with peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch; Interchim, Montluçon, France) diluted 1:5000. After three washes, the membranes were analysed using SuperSignal, West Pico (Pierce).

In vitro LH and FSH bioassays

LH and FSH bioactivities of recombinant hormones were estimated by the in vitro stimulation of progesterone production respectively in mouse Leydig tumoral cells MLTC-1 (ATCC®CRL 2065) and in a mouse adrenal cortex tumor stably expressing the human FSH receptor, referred to as Y1 cells (kindly given by Ares Serono, Geneva, Switzerland) (Kelton et al. 1992). For the in vitro LH bioassay, about 1·5 × 10^5 MLTC-1 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 until 80% confluency. Just before stimulation, cells were incubated for 2 h in 0·5 ml serum-free RPMI media and then stimulated for 2 h with 0·5 ml serum-free RPMI media containing samples of recombinant hormones at different concentrations. The supernatants were recovered and stored at −20°C until assayed. To estimate the in vitro FSH bioactivity, 1·5 × 10^3 Y1 cells were seeded in 0·5 ml Ham’s F10 growth medium (supplemented with 15% horse serum (HS), 2·5% FBS, 2 mM l-Gln and 80 µg/ml Geneticin (G418) in 48-well plates and incubated at 37°C for 3 or 4 days. Twenty-four hours before the stimulation, cells were cultured in 0·5 ml F10 media that was richer in serum (5% HS, 0·8% FBS, 2 mM Gln and 80 µg/ml G418). Finally, Y1 cells were stimulated with 0·5 ml of the same samples as above in serum-free F10 medium containing 1 mg/ml BSA and 2 mM l-Gln. After an incubation of 4 h at 37°C, media were stored at −20°C until assayed.

Secreted progesterone recovered in media from MLTC-1 or Y1 cells was measured by a specific radioimmunoassay (Saumande & Batra 1985). Relative LH and FSH potencies of recombinant hormones in COS-7 cells and in insect cells were determined respectively with α_heβ_c and standard eCG NZY-01 as references. Relative biopotencies were calculated on the basis of ED_{50} corresponding to the dose necessary to obtain 50% of the maximal production.

Animals

Immature 25-day old female Wistar rats were obtained from the animal breeding facility of the laboratory. All the procedures used in this paper were in compliance with the European Community Council Directive of 24 November 1986 (86/609/EEC).

In vivo eCG and FSH bioassays

Supernatants containing recombinant eLH/CG from Si9 or Mimic cells were harvested 5 days postinfection and were centrifuged for 10 min at 1000 g. After dialysis, the samples were frozen at −80°C, lyophilised and diluted in 0·15 M saline on the basis of their immunoactivity in ELISA. The in vivo eCG bioactivity of recombinant hormones expressed in insect cells was determined in the eCG test of the Pharmacopoeia (Cole & Erway 1941). The in vivo FSH bioactivity was evaluated using a specific FSH bioassay of the Pharmacopoeia (Steelman & Pohley 1953). Five animals were injected s.c. for each dose of hormone and one to four doses were used per hormone to be tested.

Results

Production of recombinant eLH/CG expressed in COS-7 and insect cells

The quantities of recombinant hormones secreted by COS-7 and insect cells were estimated in a sandwich ELISA using standard eCG NZY-01 (1500 IU/mg) as reference and also compared with pituitary eLH (Fig. 4). Briefly, the pCDM8-β_c was cotransfected in COS-7 cells in serum free medium, either with pCDM8-α_he or with
the pCDM8-αc plasmid. After 48 h incubation, the media were recovered and assayed by ELISA. The productions of recombinant α2β2 and α2βc were 0·123 ± 0·003 µg/ml and 0·094 ± 0·006 µg/ml respectively. Mimic cells and Sf9 cells were both coinfected with NPV-αc(PH) and SLP10-βc(P10) recombinant baculoviruses. Supernatants from infected cells were recovered by centrifugation at 100 g for 5 min, 5 days post-infection and assayed by ELISA. Maximal productions of eLH/CG in Sf9 and Mimic cells were found to be 4·15 ± 0·49 µg/ml and 5·33 ± 1·32 µg/ml, respectively, 5 days post infection (n=5) and thus 30–50 fold more than in COS-7 cells (not shown).

Characterization of eLH/CG and its free subunits expressed in Sf9 and Mimic cells

To express free α and β subunits with or without CTP in the baculovirus insect cell system, Sf9 or Mimic cells were seeded in 25 cm² flasks and infected with recombinant NPV-αc(PH), SLP10-βc(P10) and SLP10-βcACTP(P10) respectively. Complete eLH/CG was
produced by coinfection of insect cells with NPV-α(e)(PH) and SLP10-β(e)(P10). After five days incubation at 28°C, supernatants were collected and analysed by Western blotting using specific antibodies.

Standard and recombinant dimeric hormones were detected using rabbit antisera directed against native eCG (Cahoreau & Combarnous 1987). Standard eCG exhibited a complex range of bands between 80 and 100 kDa while the recombinant dimeric hormones expressed in Sf9 cells and Mimic cells were more homogenous appearing as a doublet at about 45 and 50 kDa respectively (Fig. 5A). A strong specific signal was revealed at about 26 kDa for the α-subunit, with native heated eCG using 89A2 monoclonal antibody recognizing conformational α-subunit under non reducing conditions (Fig. 5B) (Chopineau et al. 1993). Recombinant α-subunit appeared as a major band at about 22 kDa in Sf9 cells versus 26 kDa in Mimic cells.

Using rabbit LH/CG beta (1–9) peptide antibody (C Galet & M Chopineau, unpublished observations), a specific immunoreactive band was detected under reducing conditions at about 50 kDa for the β-subunit of the native heated eCG. Equine LH/CG β-subunit expressed in Sf9 cells exhibited a doublet at about 26 kDa, whereas several immunoreactive bands were observed for that expressed in Mimic cells: a positive band appears at the same MW as that of the beta-chain expressed in Sf9 cells and another one at 28 kDa (Fig. 5C). A single band was detected for the βAEP at about 16 kDa in Sf9 cells and a doublet at the same MW in Mimic cells (Fig. 5D).

**Table 2 Summary of relative LH and FSH in vitro potencies of recombinant eLH/CG expressed in COS-7 cells and in insect cells as determined in Figures 6 and 7**

<table>
<thead>
<tr>
<th>Hormone</th>
<th>COS-7 cells</th>
<th>Insect cells</th>
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<tbody>
<tr>
<td></td>
<td>Relative LH biopotency</td>
<td>Relative FSH biopotency</td>
</tr>
<tr>
<td>αα;β1α;β1 eLH/CG</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>αα;β1α;β1 eLH/CG</td>
<td>66±1</td>
<td>54±26</td>
</tr>
<tr>
<td>Standard eCG</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>eLH/CG Sf9</td>
<td>27±5</td>
<td>54±1</td>
</tr>
<tr>
<td>eLH/CG Mimic</td>
<td>80±10</td>
<td>100</td>
</tr>
</tbody>
</table>

**Figure 6 In vitro LH and FSH bioactivities of recombinant dimeric hormones expressed in COS-7 cells.** These activities were determined by the production of progesterone in MLTC-1 cells (A) and Y1 cells (B), measured in specific progesterone radioimmunoassay (RIA). Concentrations of each dimeric hormone αα;β1α;β1 (□), αα;β1α;β1 (▲) were estimated by ELISA and each point corresponds to the mean±S.E.M. of three experiments.
In vitro bioactivities

In vitro LH and FSH bioactivities of recombinant hormones were determined by their ability to stimulate progesterone production by MLTC-1 and Y1 cells, respectively. The dimeric αβ5 hormone expressed in COS-7 cells exhibited LH and FSH potencies, respectively 22 and 27% lower than those of αβ5 (Figs 6A and B, respectively) leading to a slight non-significant decrease in its FSH/LH activity ratio (Table 2).

The ability of eLH/CG expressed in Sf9 cells to stimulate the production of progesterone in MLTC-1 and Y1 cells was lowered by 73 and 46% respectively compared with standard eCG (Fig. 7A). The in vitro LH biopotency of eLH/CG expressed in Mimic cells was only 20% lower than that of natural eCG and its in vitro FSH biopotency was unaffected (Fig. 7B). Equine LH/CG produced in Mimic cells possessed a FSH/LH ratio of 1.2, versus 2.0 when expressed in Sf9 cells (Table 2).

In vivo bioassays

The eCG in vivo bioassay (Cole & Erway 1941) was used to compare the biopotency of eLH/CG produced in Sf9 cells with that produced in Mimic cells. The unique injection of standard eCG leads to a dose-dependent increase in ovarian weight. Equine LH/CG expressed by Sf9 or Mimic cells had no effect on ovary growth (Fig. 8). Pituitary eLH was also compared with eCG in this in vivo bioassay, on the basis of their respective potencies in the sandwich ELISA. Equine LH isoforms were found to be 10–30 fold less active than natural eCG in the in vivo eCG assay (not shown). The FSH in vivo bioassay (Steelman & Pohley 1953) was carried out to specifically test FSH bioactivity of Mimic eLH/CG. The repeated injection of standard hFSH or eCG NZY-01 over 4 days induced a dose-dependent increase in ovarian weight under hCG stimulation. However, both recombinant eLH/CG had no effect on ovary growth (Fig. 9).

Discussion

Equine CG from pregnant mare serum possesses extensive N- and O-glycosylation with 45% of its mass
due to its carbohydrate moiety (Harbon-Chabbat et al. 1961, Gospodarowicz 1972), whereas equine LH exhibits the same sites of glycosylation but with only 30% of its mass as carbohydrate side chains (Roser et al. 1984, Butnev et al. 1996). In addition to complex sialylated biantennary N-glycans (Smith et al. 1993, Matsui et al. 1994), eCG possesses 12 uncommon disialylated poly-N-acetyllactosaminyl O-glycans located at the C-terminus of the β-subunit (Hokke et al. 1994, Bousfield & Butnev 2001). The N-glycans of eLH occupy the same three sites as those in eCG (Fig. 1) but exhibit sulfated terminal N-acetyl-galactosamine residues in addition to, or in place, of sialic acid residues (Smith et al. 1993, Matsui et al. 1994). The sites of O-glycosylation of eLH are also the same as is eCG but, on average, they represent a smaller proportion and the chains are shorter (Bousfield & Butnev 2001). Equine CG presents a much longer half-life (6 days) than eLH (1 h) in mares (Cole et al. 1967, Ginther et al. 1974) in relation to its carbohydrate content. Indeed, enzymatic removal of terminal sialic acid residues of eCG leads to a dramatic drop in its in vivo bioactivity in various species, including sheep (Martínuk et al. 1991) and rats (Rafelson et al. 1961). Consequently, the structure of carbohydrate side-chains in recombinant eLH/CG is expected to be an important issue for its clearance from blood, and consequently for its in vivo bioactivity. The present paper reports the production and biological characterization of eCG in vivo bioactivity of eLH/CG in insect cell media. These activities were determined by the Cole and Erway assay (Cole & Erway 1941). In brief, ~5 day-old rat females received a unique subcutaneous injection of standard eCG as a positive control (◆) or different doses of eLH/CG produced in SF9 (■) and Mimic (○) insect cells. Ovaries were collected from five animals per dose and their weight determined 48 h post injection. Each point corresponds to the mean±S.E.M. of five animals. The quantity of eLH/CG was determined by ELISA.

Figure 9 FSH in vivo bioactivity of eLH/CG in insect cell media. These activities were determined by the Steelman and Pohley assay (Steelman & Pohley 1953). In brief, 25 day-old rat females were injected six times with a constant dose of hCG of 20 IU and different doses of eLH/CG produced in SF9 (■) and Mimic (○) insect cells or standard hormones (◆, eCG; ▲, hFSH) used as positive controls. Ovaries were collected from five animals per dose and their weight determined 24 h after the last injection. Medium containing 10% BFS was tested as control (■). Each point corresponds to the mean±S.E.M. of five animals. The quantity of eLH/CG was determined in a specific sandwich ELISA.
of recombinant eLH/CG produced in Sf9 and Mimic insect cell lines using the baculovirus expression system. The correction of the original chimeric αgly sequence into fully αgly sequence permitted the production of eLH/CG with its genuine sequence in COS-7 cells, as well as in Sf9 and Mimic cells. The replacement of the human N-terminal APDV sequence in αgly by the equine N-terminal FPDGEFTT sequence in αgly led to a slight decrease in both LH and FSH in vitro activities of the recombinant heterodimer formed with βe. Since the quantity of hormones tested in bioassays are determined by ELISA, this decrease actually relates to the bioactivity to immunoactivity ratio. Therefore, it cannot be ruled out that the observed decrease could in fact be due to a slightly better recognition of α,βe than αgly,βe in the ELISA. This would not be unexpected, as the α-specific 89A2 monoclonal antibody used in the sandwich ELISA was raised against authentic eCG, thus having an N-terminal FPDGEFTT sequence in its α-subunit. Moreover, the α-subunit N-termini of hCG and hFSH are known to be on the opposite side relative to their hormone binding sites to their cognate receptors (Lapthorn et al. 1994, Wu et al. 1994, Fox et al. 2001).

The in vivo biological activity of eCG strongly depends on the presence of sialylated complex saccharide side chains leading to its slow rate of elimination from blood (Klett et al. 2003). The baculovirus expression system allows high levels of production of recombinant proteins, but N-glycans processed in insect cells such as Sf9 cells are not complex and sialylated N-glycans such as those found in placental eCG. To overcome this problem, we expressed recombinant eLH/CG in Mimic cells that have been designed to produce biantennary, terminally sialylated N-glycans (Hollister et al. 2002). Recombinant eLH/CG with both subunits possessing the native amino acid equine sequences was successfully produced in two insect cell lines using the baculovirus expression system, with yields of 4–5 mg/l that are similar to those previously reported for the production of porcine FSH in insect cells (1 mg/l) (Kato et al. 1998) and of bovine FSH (1–5 mg/l) (van de Wiel et al. 1998).

Recombinant eLH/CG and its subunits expressed in Sf9 and Mimic cells exhibited lower apparent molecular weights compared with natural eCG and its subunits, highlighting the presence of shorter glycans in these cells. Nevertheless, the higher apparent molecular weights found for eLH/CG and its subunits expressed in Mimic cells compared with Sf9 cells strongly suggest that glycosylation was indeed improved in Mimic cells as expected (Hollister et al. 2002). The major differences in MW between natural and recombinant hormones seem to be essentially due to very short O-glycans in the CTP of eLH/CG produced in insect cells. Indeed, the α-subunit, which is only N-glycosylated, exhibited the same apparent MW when expressed in Mimic cells as that of α-subunit from natural eCG. On the contrary, the β-subunit and eLH/CG exhibited significantly lower MW than their natural counterparts, suggesting that the major effect on MW arises from weaker O-glycosylation of the CTP. Indeed, the CTP is O-glycosylated in insect cells since its elimination in βαCTP leads to a single band, while full-length β-subunit exhibits higher and heterogeneous MW. In Sf9 cells, O-glycans are constitutive by single N-acetylgalactosamine (GalNAc) residues or by GalNAc-Gal disaccharides (Thomsen et al. 1990) but for the time being no information on this matter is available for Mimic cells. Recombinant glycoproteins produced in Mimic cells referred to as SISWT-1 were claimed to bear sialylated biantennary N-glycans (Hollister et al. 2002, 2003). Sialylation in Mimic cells is unexpected since Sf9 cells do not synthesize the CMP-sialic acid nucleotidic donor (CMP-NeuAc) as a substrate for α2,6-sialyltransferase. Our observation that recombinant eLH/CG from Mimic cells do not exhibit any in vivo activity in spite of full in vitro activity strongly suggests that the hormone is probably not sialylated. Indeed, desialylation of natural eCG leads to the complete loss of its in vivo activity without affecting its in vitro activity (Morell et al. 1971).

Both eLH/CGs expressed in Sf9 and Mimic cells exhibited full in vitro LH and FSH bioactivities, confirming that recombinant hormones possess a conformation similar to that of natural eCG. Equine LH/CG expressed in Mimic cells and Sf9 cells exhibit in vitro FSH/LH ratios that are not significantly different. On the basis of their concentrations measured by ELISA, their in vivo bioactivity was not detectable at the doses used and they are less than 1% of that of natural eCG. Recombinant eLH/CG produced in Mimic cells was also found to be inactive in the FSH in vivo bioassay (Steelman & Pohley 1953) that does not require a hormone with a very long half-life, in contrast to the eCG in vivo bioassay (Cole & Erway 1941). The absence of in vivo bioactivity of recombinant eLH/CG may be the result of a rapid clearance of the hormone in the plasma, as is the case for single-chain eLH/CG expressed in the milk of transgenic rabbits (Galet et al. 2001). Sialic acids are mainly responsible for prolonged half-life in plasma, suggesting they are partially or totally lacking on eLH/CG produced in Sf9 cells and in Mimic cells. Native eLH is known to bear sulphated N-glycans and sialylated O-glycans. Although displaying a similar in vitro FSH/LH ratio (Guillou & Combarnous 1983), native eLH that bears fewer sialic acids compared with eCG exhibits a much shorter half life. The presence of sialylated O-glycans is also involved in the half-life, since the elimination of the O-glycosylated CTP from hCG led to a drastic decrease of its half-life (Matzuk et al. 1990). The absence of in vivo bioactivity of eLH/CG expressed in Sf9 cells can be explained by the presence of trimannose side-chains that can be recognized by the macrophage mannose receptor (Childs et al. 1990),
leading to removal of the hormone from blood. Although eLH/CGs expressed in Mimic cells present more elaborate carbohydrate side chains, they might still be recognized by the MMR, since its carbohydrate recognition domains bind fucose and N-acetylglucosamine in addition to mannose, and its cystine-rich domain binds sulfated GalNAc and various sulfated glucosamine in addition to mannose, and its cystine-rich domain binds sulfated GalNAc and various sulfated carbohydrates (Leteux et al. 2000). The rapid elimination of porcine LH from blood in rats has been shown to occur by rapid trapping by kidneys (Klett et al. 2003) but it does not seem to involve MMR, since the half-life of pLH is not increased in MMR-/- mice compared with wild-type mice (Lee et al. 2002). Nevertheless, we have no clue at the moment whether recombinant hormones expressed in insect cells are eliminated from the circulation in the same way as pLH. We have shown in the present paper that eLH exhibits low in vivo bioactivity (3–10% that of eCG) but this activity is easily detectable, in contrast to that of recombinant eLH/CG. This indicates that recombinant hormones are, indeed probably eliminated from blood more rapidly than pLH and eLH. Therefore, the involvement of MMR present at the surface of macrophages cannot be ruled out in the elimination of recombinant eLH/CG.

In brief, we succeeded in producing two recombinant eLH/CG in the baculovirus expression system using Sf9 cells and their transgenic progeny Mimic cells. Although having full in vitro LH and FSH bioactivities, eLH/CG expressed in both cell lines did not exhibit significant in vivo bioactivity. Nevertheless, our results clearly show that the glycosyltransferase activities in Mimic cells lead to larger carbohydrate moieties on baculovirus-expressed proteins. However, other modifications such as the addition of O-glycans and sialic acid residues are still very incomplete and will need further attention. At the least, Mimic cells are expected to synthesize glycoproteins with N-glycans up to terminal galactose residues and so are good substrates for a more complete in vitro sialylation in contrast to those of eLH/CG from Sf9 cells. Two strategies can be proposed to engineer N- and O-glycosylation: one by modifying the cell genome of insect cells and the other by introducing all necessary glycosyltransferase genes in the baculovirus genome. A recent study showed the successful production of sialylated human α1-antitrypsin in Estigmene acra Ea4 cells transfected by a single recombinant baculovirus co-expressing N-acetylgalactosaminyl transferase II, β1,4-galactosyl transferase and α2,6-sialyltransferase in addition to human α1-antitrypsin (Chang et al. 2003).

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