Cloning and functional expression of the equine luteinizing hormone/chorionic gonadotrophin receptor

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Abstract

Pituitary equine luteinizing hormone (eLH) and fetal chorionic gonadotrophin (eCG) have identical polypeptidic chains, but different linked carbohydrates. In equine tissues, eCG and eLH bind only to the LH/CG receptor (eLH/CG-R) and have no FSH activity. However, radio-receptor assays on equine luteal or testicular tissues have shown that eCG binds to the eLH/CG-R with only 2–4% of the binding activity of eLH. In order to study the structure–function relationship of eLH and eCG in a homologous system, we undertook the cloning and functional expression of the eLH/CG-R.

Based on sequence homologies among mammalian sequences for the LH/CG-R, overlapping partial fragments of LH/CG-R cDNAs were obtained from mare luteal RNA using reverse transcription-PCR and 5′-rapid amplification of cDNA ends. Ligations of the partial cDNA fragments encoded a part of the signal peptide followed by a putative 672 amino acid eLH/CG-R mature protein. The mature eLH/CG-R displayed 88.2–92.8% overall sequence homology with the other mam-

malian LH/CG-Rs and contained one unique seventh N-glycosylation site in its extracellular domain.

COS-7 cells were transiently transfected with a cDNA construct encoding an engineered complete signal peptide and the mature eLH/CG-R. Membrane preparations from transfected COS-7 cells bound ^{125}I -eLH with high affinity (K_d 3.8×10^{-10} M). On a molar basis, eCG competed with ^{125}I -eLH on membrane preparations with only 12.4% of the eLH binding activity. In transfected COS-7, both eLH and eCG increased the extracellular cAMP concentration in a dose-dependent manner, whereas eFSH did not. Furthermore, on a molar basis, eCG stimulated cAMP production with only 13.9% of the eLH stimulating activity.

We conclude that the cloned cDNA encodes a functional eLH/CG-R. The differences between eLH and eCG activities towards this receptor will be useful in studies of the influence of carbohydrates on gonadotrophin receptor binding and activation.

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Introduction

The luteinizing hormone/chorionic gonadotrophin receptor (LH/CG-R), together with those of follicle-stimulating hormone (FSH) and thyroid-stimulating hormone, belong to a subfamily of glycoprotein hormone receptors among the G-protein-associated seven-transmembrane-domain receptors (Dufau 1998, Ascoli *et al.* 2002). The LH/CG-R has an important role in reproductive physiology: it is primarily expressed in the gonads, and it targets the action of LH to Leydig cells in the testis and to thecal, granulosa and luteal cells in the ovary. In primates during pregnancy, the LH/CG-R also targets the action of chorionic gonadotrophin in luteal cells, male human fetal gonads and other potential target

organs that express LH/CG-R cDNA (Rao 1997, Themmen & Huhtaniemi 2000). The main intracellular signalling pathway activated by the LH/CG-R is the adenylyl cyclase pathway, leading to an increase in intracellular cAMP (Dufau 1998, Ascoli *et al.* 2002).

Radioreceptor assays performed on luteal membranes from cyclic and pregnant mares have revealed that the equine (e) LH/CG-R, like the primate one, binds two distinct hormones: pituitary eLH and fetal eCG (Stewart & Allen 1979, Ascoli *et al.* 2002). Furthermore, whereas eLH and eCG bind to both LH/CG and FSH receptors in all mammals studied, they have only an LH activity in equine tissues (Licht *et al.* 1979, Stewart & Allen 1979, 1981, Guillou & Combarnous 1983). Nevertheless, eCG binds to the eLH/CG-R with only 2–4% of the activity of eLH

Table 1 Nucleic acid sequences of primers used for the isolation and cloning of the eLH/CG-R cDNA. Location of nucleotides corresponds to the eLH/CG-R cDNA sequence on Fig. 1B. Primer P4 is a degenerate primer in which B=T/G/C, Y=C/T, K=T/G and R=A/G

	Location	Sequence
Name and orientation		
P1 (sense)	203–224	5'-CTTTCAGAGGACTTAATGAGGT-3'
P2 (antisense)	829–849	5'-TCTAAAAGCACAGCAGTGGCT-3'
P3 (sense)	627–651	5'-GCTGGAGAAGATGCACAACGGAGCC-3'
P4 (antisense)	2076–2112	5'-GCTCTAGACAGTTAACABTCYKTRTAGCRAGTCTTG-3'
P5 (antisense)	501–525	5'-TTCGTTATTCATCCCTTGAAAAGCA-3'
P6 (sense)	[-8]–71	5'-GCTCTAGAATGAGAAGAAGGCTACTAGCACTACGACTATT ATTGGCACTACTTTTGTACCACCACTGCTGCCGCAAGC-3'

on testicular or luteal membranes from cyclic or pregnant mares (Stewart & Allen 1979, Guillou & Combarous 1983, Saint-Dizier *et al.* 2003). In contrast, eCG and eLH display similar affinity toward the porcine LH-R (Guillou & Combarous 1983). Thus it is of interest to determine whether this difference in binding affinities is attributable to differences in LH receptor sequences.

The protein structure of eCG is completely identical to that of eLH (Bousfield *et al.* 1987, Sugino *et al.* 1987), as a single gene encodes for their α -subunits (Stewart *et al.* 1987) and another gene encodes for their β -subunits (Sherman *et al.* 1992). Therefore, the differences in binding activity between eLH and eCG are likely to result from differences in size and structure of the N- and O-glycan chains linked to the polypeptidic α - and β -chains. Indeed, eCG contains much more and bulkier carbohydrates than eLH (Bousfield *et al.* 1996, 2001). Furthermore, glycan chains on eCG are mainly terminated by sialic acids, whereas more than 72% of eLH glycan chains are sulphated (Smith *et al.* 1993). It has been shown that the O-linked oligosaccharides of eCG have a negative influence on eCG binding activity tested on rat testicular membranes (Butnev *et al.* 1996, Bousfield *et al.* 2001). However, the significance of eLH and eCG carbohydrates in eLH/CG-R binding activity remains to be investigated. Furthermore, the possible influence of eLH and eCG carbohydrates on hormone-induced intracellular responses after eLH/CG-R activation has never been studied.

In order to study the structure–function relationship of eLH and eCG in a homologous system, we undertook the cloning of the complete nucleotide sequence and the functional expression of the eLH/CG-R cDNA, and studied the binding activity and hormone-stimulated cAMP production of the recombinant eLH/CG-R expressed in COS-7 cells.

Materials and Methods

Tissue collection and RNA isolation

Luteal tissues were obtained from one cyclic mare at day 8 post-ovulation and from three mares at days 15, 42 and 61

of pregnancy after ovariectomy as previously described (Saint-Dizier *et al.* 2003), and in accordance with the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction. Total RNA was extracted from fragments of frozen tissue using Trizol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. The resulting RNA was quantified spectrophotometrically and stored at – 80 °C until required for use.

Isolation and sequencing of full-length eLH/CG-R cDNA

Total pooled RNA (2 μ g) was reverse transcribed into first-strand cDNA using oligo(dT) primer and Rnase H–reverse transcriptase (Superscript II; Invitrogen Life Technologies) in the presence of deoxynucleotides according to the manufacturer’s recommendations. A 646 base pair (bp) cDNA fragment (cDNA fragment 1) encoding a part of the extracellular domain of the eLH/CG-R was first generated using primer pair P1 and P2 based on cDNA sequences similar among bovine (Lussier *et al.* 1996), porcine (Loosfelt *et al.* 1989), human (Minegishi *et al.* 1990) and murine (McFarland *et al.* 1989) LH/CG-Rs (Table 1). First-strand cDNA was subjected to 30 cycles of PCR amplification using Advantage 2 polymerase mix (BD Biosciences, Palo Alto, CA, USA) and primer pair P1 and P2. The reaction times were 1 min denaturation at 95 °C for the first cycle and 15 s per cycle thereafter, 40 s annealing at 60 °C and 1 min extension at 72 °C for the first 29 cycles, and 10 min extension on the final cycle at 72 °C. The 3′-end of the eLH/CG-R cDNA (cDNA fragment 2) was subsequently isolated using sense primer P3 designed from the cDNA fragment 1 previously obtained and a degenerate antisense primer (P4) based on bovine, porcine and human LH/CG-R cDNA sequences (Table 1). First-strand cDNA was amplified for 30 cycles using Advantage 2 polymerase mix (BD Biosciences) and primer pair P3 and P4 in the PCR conditions described above. Finally, the 5′-end of the eLH/CG-R cDNA (cDNA fragment 3) was isolated using the antisense primer P5 designed from the cDNA fragment 1 (Table 1) and the rapid amplification of cDNA ends technique, as described previously (Saint-Dizier *et al.* 2004).

All amplified PCR products were subcloned into the TA (thymine adenine) cloning vector pCR II-TOPO (Invitrogen Life Technologies) and sequenced on both strands (Genome Express, Meylan, France). Sequence analyses were performed on the Infobiogen web site (<http://www.infobiogen.fr>).

Construction of the eLH/CG-R cDNA for expression studies

Comparison of the 468 bp cDNA fragment 3 with cDNA sequences of other mammalian LH/CG-Rs showed that this fragment encoded a partial signal peptide followed by the N-terminal extremity of the eLH/CG-R mature protein. Because of the high homology between the eLH/CG-R cDNA fragment 3 and the corresponding porcine LH/CG-R cDNA (93%; Loosfelt *et al.* 1989), a chimeric porcine/equine signal peptide was engineered to permit receptor expression at the membrane level (Fig. 1A). The nucleic acid sequence of this porcine/equine signal peptide was added to the 5'-end of the cDNA fragment 3 by PCR using a sense primer P6 (Table 1) and the antisense primer P5 under the following conditions: 1 min at 94 °C for one cycle; 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C for 25 cycles, and 10 min at 72 °C for one cycle. The 533 bp PCR product obtained (cDNA fragment 3') was purified, cloned into the TA cloning vector pCR II-TOPO (Invitrogen Life Technologies) and sequenced.

A full-length eLH/CG-R cDNA was engineered by fusing the overlapping fragments 1 and 2 at the unique site PstI, generating a 1483 bp fragment (1+2), and then fusing the overlapping fragments (1+2) and 3' at the unique site BglII (Fig. 1B). The resulting 2112 bp cDNA was cloned into the XbaI site of pCDNA 3.1 (+), a eukaryotic expression vector (Invitrogen Life Technologies), to yield pCDNA-eLH/CG-R. All the constructs were amplified in chemically competent *Escherichia coli* TOP 10 (Invitrogen Life Technologies), purified using the Nucleobond AX maxiprep plasmid kit and sequenced.

Transient transfection of COS-7 cells

COS-7 monkey kidney cells were maintained at 37 °C in a humidified 5% CO₂ incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10 mM Hepes. They were transfected at 65% confluency in 10 cm diameter Petri dishes with 12 µg pCDNA-eLH/CG-R using a calcium phosphate precipitation procedure previously described by Chopineau *et al.* (1997). For four dishes, 124 µl 2 M CaCl₂ was mixed with 48 µg pCDNA-eLH/CG-R and the volume adjusted to 1 ml with water. A DNA precipitate was formed by adding the mixture drop by drop to 1 ml Hepes-buffered saline (HBS) and 500 µl was

then added to each Petri dish, followed by incubation for 4 h. The medium containing DNA and calcium was then aspirated and 1.5 ml 15% glycerol in HBS was added for 90 s. The cells were then rinsed before being incubated with 6 ml supplemented DMEM for 48 h. Mock-transfected cells were treated in the same manner but without DNA, and control cells were transfected with the expression vector pCDNA without insert.

Homologous radioreceptor assays

Crude membrane preparations were made from transfected cells using a method previously described by Richard *et al.* (1997). Briefly, transiently transfected COS-7 cells in each Petri dish were washed with PBS (1.1 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 134 mM NaCl), then disrupted by thermic shock (5 min at 37 °C then 5 min at -80 °C) in 1 ml buffer A (10 mM Tris-HCl, pH 7.4, 0.1% BSA, 20% glycerol) supplemented with one tablet of protease inhibitors cocktail (Complete Mini, Roche Diagnostics), and collected by centrifugation for 30 min at 10 000 g. The pellets were resuspended in 1 ml buffer B (10 mM Tris-HCl, pH 7.4, 0.1% BSA), pooled, then aliquoted in smaller volumes and stored at -80 °C until required for use as binding fractions. The protein concentration of COS-7 binding fractions was determined by the method of Bradford (1976).

Homologous equine radioreceptor assays were performed on COS-7 binding fractions according to a method previously described for equine luteal membranes (Saint-Dizier *et al.* 2003). Equine LH (National Hormone and Peptide Program, lot no. AFP5130A) was radioactively labelled with ¹²⁵I-Na (Amersham Pharmacia Biotech) using Iodo-Gen (Pierce, Rockford, IL, USA). The specific activity of ¹²⁵I-eLH (2100 Ci/mmol; molecular weight (MW) 34 000) was calculated by column recovery as described previously (Bolton & Hunter 1973). For ¹²⁵I-eLH-binding displacement assays, crude COS-7 membrane preparations (15 µg proteins/tube) were incubated with a constant amount of ¹²⁵I-eLH (55 pM) and increasing amounts of unlabelled eLH, eCG or eFSH. Hormones used were all purified in our laboratory and their relative activities were all as follows: eLH 525, 0.8 × eLH-A activity; eCG 652, 5000 IU/mg; (Guillou & Combarnous 1983, Combarnous *et al.* 1984). eLH-A and eFSH-A are standard preparations described in these two references. For ¹²⁵I-eLH-binding saturation assays, COS-7 binding fractions were incubated with increasing amount of ¹²⁵I-eLH (seven points from 10 to 600 pM). Non-specific binding was determined in the presence of 100 IU human chorionic gonadotrophin (hCG; Chorulon, Intervet, Boxmeer, Netherlands). All reactions were incubated in triplicate in a final volume of 300 µl buffer B supplemented with 6 mM CaCl₂ at room temperature for 18 h. Reactions were stopped by the addition of 1 ml cold buffer B, and bound and free hormones were separated by

A

➤ Alignment of porcine and equine LH/CG-R N-terminal amino acid sequences

	10	20	↓	30	40
pLH/CG-R	MRRRSLALRLL	LALLLPPLPQTL	LG	APCPEPCSCR	PDGALRCPGP
eLH/CG-R	-----	LLPQALRG		APCPEPCRCF	PDGALSCPGP

➤ Chimeric signal peptide MRRSLALRLL LALLLPPLLPQALRG APCPEP

➤ Deduced nucleic acid sequence of the chimeric signal peptide

M R R R S L A L R L L L A L L L L P P L L P Q
ATGAGAAGAAGGTCACCTAGCACTACGACTATTATTGGCACTACTTTTGTACCACCACCTGCTGCCGCAAGC

➤ Nucleic acid sequence of the primer P6

5' GCTCTAGAAATGAGAAGAAGGTCACCTAGCACTACGACTATTATTGGCACTACTTTTGTACCACCACCTGCTGC
CGCAAGC 3'

B

centrifugation at 4000 *g* for 30 min at 4 °C. The resulting pellets were counted in a gamma counter.

The data on displacement and saturation plots were analysed with the GraphPad PRISM2.01 software package (San Diego, CA) in order to estimate the concentration of hormone required to reduce ^{125}I -eLH binding by 50% (IC_{50}), the equilibrium constant (K_d) and the maximal binding capacity (B_{max}).

cAMP assays

Transfected cells were rinsed and placed in DMEM supplemented with 0.1% BSA (Sigma) and 0.25 mM isobutylmethyl xanthine (Sigma) at 37 °C for 30 min, then stimulated in triplicate for 1 h with various concentrations of eLH, eCG and eFSH (eLH 525; eCG 652; eFSH 149) or human recombinant FSH (Gonal-F, Serono, Geneva, Switzerland). cAMP secreted into the extracellular medium was measured using a radioimmunoassay kit according to the manufacturer's instructions (Immunotech, Marseille, France). Briefly, cell supernatants were desiccated, resuspended in 100 μl PBS, and incubated overnight with 3×10^{-3} μCi ^{125}I -cAMP in anti-cAMP antibody-coated tubes. The supernatants were then removed and the uncompeted radioactivity was counted in a gamma counter. The agonist concentrations inducing half-maximal stimulation of cAMP production (EC_{50}) were calculated using the GraphPad PRISM201 software package.

Results and Discussion

Comparison of the eLH/CG-R with other mammalian LH/CG-Rs

Figures 2 and 3 show the nucleotide and deduced amino acid sequences of the cloned eLH/CG-R cDNA, in addition to comparisons between equine, porcine, bovine, human and rat LH/CG-R amino acid sequences. The 2047 bp eLH/CG-R cDNA generated from cDNA fragments 1, 2 and 3 showed 92.3, 91.6, 88 and 85.7% homologies with reported cDNA sequences of porcine (Loosfelt *et al.* 1989), bovine (Lussier *et al.* 1996), human

(Minegishi *et al.* 1990) and rat (McFarland *et al.* 1989) LH/CG-Rs respectively, and showed 64.6% homology with equine FSH receptor (eFSH-R) cDNA (Robert *et al.* 1994). The predicted amino acid sequence of the full-length pCDNA-eLH/CG-R construct consisted of 27 residues of a putative signal peptide followed by 672 residues of a mature protein (Fig. 2). The eLH/CG-R mature protein was highly conserved among mammals (pig 92.8%, cow 92%, human 89.3% and rat 88.2%) and showed 57.9% homology with the eFSH-R. The putative eLH/CG-R transmembrane domain was composed of 266 amino acids residues, including seven hydrophobic segments potentially spanning the cytoplasmic membrane, followed by a 71 amino acid COOH-terminal intracellular domain.

An interesting finding was a seventh *N*-glycosylation site ($\text{N}^{23}\text{-L}^{24}\text{-S}^{25}$) in the N-terminal extremity of the 335 amino acid extracellular domain of the eLH/CG-R, i.e. one more than the six conserved *N*-glycosylation sites in the human, porcine, bovine and rat LH/CG-R sequences (Fig. 3) (Loosfelt *et al.* 1989, McFarland *et al.* 1989, Minegishi *et al.* 1990, Lussier *et al.* 1996). It would be of interest to investigate the possible functional role of this equine-specific *N*-glycosylation in eCG and eLH binding affinities through the use of recombinant eLH/CG-R with the mutated $\text{N}^{23}\text{-L}^{24}\text{-S}^{25}$ site.

Binding activity of recombinant eLH/CG-R expressed in COS-7 cells

To confirm that the cloned cDNA encodes a functional LH/CG-R, COS-7 cells were transiently transfected with the pCDNA-eLH/CG-R construct, and crude membrane preparations from the transfected cells were used in a homologous radioligand receptor assay. As shown in Fig. 4, increasing amounts of specific binding of ^{125}I -eLH were found in membrane preparations from transfected cells incubated with increasing doses of labelled eLH. Analysis of the saturation curve indicated a dissociation constant (K_d) of 3.8×10^{-10} M and a B_{max} of 45.8 fmol/mg protein. The K_d measured for the recombinant eLH/CG-R was similar to those previously reported for eLH/CG binding sites in equine luteal or

Figure 1 (A) Engineering of a chimeric porcine/equine signal peptide. The N-terminal amino acid sequence of the porcine LH/CG-R (pLH/CG-R, in grey, Loosfelt *et al.* 1989) and the translated amino acid sequence of the 5'-end of the eLH/CG-R cDNA fragment 3 were aligned. Potential cleavage sites of porcine and chimeric signal peptides were determined using the SignalP V1.1 program on the Expasy website (Expert Protein Analysis System; <http://www.expasy.org>) and are indicated with arrows. No potential cleavage site could be determined for the partial sequence of the eLH/CG-R using this program. The deduced nucleic acid sequence of the chimeric signal peptide was designed using the genetic code and limiting the number of G and C bases. The sense primer P6 was used in combination with the antisense primer P5 to add the nucleic acid sequence of the chimeric signal peptide to the 5'-end of the cDNA fragment 3. The primer P6 was also designed to allow ligation of the full-length eLH/CG-R cDNA into the pCDNA expression vector and thus contained an XbaI restriction site (underlined). (B) Construction of the pCDNA-eLH/CG-R. Horizontal lines indicate cDNA fragments and arrows indicate the different primers used. A full-length eLH/CG-R cDNA was engineered by fusing the overlapping fragments 1 and 2 at the unique site PstI, generating a 1483 bp fragment (1+2), and then fusing the overlapping fragments (1+2) and 3' at the unique site BglII. The resulting 2112 bp cDNA was cloned into the XbaI site of pCDNA 3.1 (+).

ATG AGA AGA AGG TCA CTA GCA CTA CGA CTA TTA TTG GCA CTA CTT TTG TTA CCA CCA CTG CTG CCG CAA GCG CTG CGC GGG	81
M R R R S L A L R L L L A L L L L P P L L P Q A L R G G	-1
GCG CCC TGC CCC GAG CCC TGC CGC TGC CCG CCC GAC GGC GCC CTG AGC TGC CCC GGC CCG CGG AGG AAC CTC TCC CGA CTA	162
A F C F E P C R C P P D G A L S C P G P R R <u>N L S</u> R L	27
TCA CTC ACC TAT CTT CCT ATC AAA GTA ATC CCA TCT CAA GCT TTC AGA GGA CTT GAT GAA GTT GTA AAA ATT GAA ATC TCT	243
S L T Y L P I K V I P S Q A F R G L D E V V K I E I S	54
CAG AGT GAT TCC CTG GAA AAG ATA GAA GCT AAT GCC TTT GAC AAC CTC CTC AAT TTG TCT GAA ATA CTG ATC CAG AAC ACC	324
Q S D S L E K I E A N A F D N L L <u>N L S</u> E I L I Q N T	81
AAA AAC CTA GTG TAC ATC GAG CCT GGA GCA TTT ACA AAT CTC CCC CGG TTA AAA TAC CTA AGC ATC TGT AAC ACA GGC ATC	405
K N L V Y I E P G A F T N L P R L A K Y L S I C N T G I	108
CGA AAG CTT CCA GAT GTT ACC AAG ATC TTC TCC TCT GAA ATT AAT TTC ATT CTG GAA ATT TGC GAT AAC TTA CAC ATA ACC	486
R K L P D V T T K I F S E I N F I L E I C D N L H I T	135
ACC ATA CCA GGA AAT GCT TTA CAA GGG ATG AAT AAC GAA TCC ATA ACA CTC AAA CTA TAT GGA AAT GGA CTT GAA GAA ATA	567
T I P G N A F Q G M N <u>N E S</u> I T L K L Y G N G L E E I	162
CAA AGT CAT GCA TTC <u>AAC GGG</u> ACG ACA CTG ATT TCC CTG GAG CTA AAG GAA AAT GCC CAG CTG GAG AAG ATG CAC AAC GGA	648
Q S H S F <u>N G T</u> T L I S L E K E N A Q L E K F M H E S	189
GCC TTC CAG GGG GCC ACA GGG CCC AGC ATC TTG GAT ATT TCT TCC ACC AAA CTG CAG GCC CTA CCT AGC TAT GGG CTG GAG	729
A F Q G A T G P S I L D I S S T K L Q A L P S Y G L E	216
TCC ATT CAG ACG CTA ATT GCC ACA TCA TCC TAT TCT CTA AAA AAA TTA CCG TCA AGA GAA AAA TTT ACC AAT CTC CTG GCT	810
S I Q T L I A T K I S L K L P S R E K F T N L H I S	243
GCC ACA CTG ACT TAC CCA AGC CAC TGC TGT GCT TTT AGA AAC TTG CCA ACA AAA GAA CAG AAT TTT TCA TTT TCC ATT TTT	891
A T L T Y P S H C C A F R N L P T K E Q <u>N F S</u> F S I F	270
GAA AAC TTT TCC CAA Q TGT GAA AGC ACA GCA AGG AAA CCA AAT AAT GGA ACA CTT TAT TCT GCC AGC TTT GCT GAG AGT	972
E <u>N F S</u> Q C A E S T L I R K P N <u>N G T</u> L Y S A S K M H E S	297
GAA CTG AGT GGC TGG GAT TAT GAC GAT GGT TTC TGC CCA CAC AAG ACA CTC CGA TGT GCT CCT GAA CCA GAT GCT TTT AAT	1053
E L S G W D Y D D G F C P H K T L R C A P E P D A F N	324
CCC TGT GAA GAT ATT ATG GGC TAT GAC TTC CTT AGG GTC CTG ATT TGG CTG ATT AAT ATC CTA GCC ATC ATG GGA AAT GTG	1134
P C E D Y D F L R V L I W L I N I L A I M G N S	351
ACT GTC CTC TTT GTT CTC CTG ACC AGT CAT TAC AAA CTA ACA GTG CCC CGT TTT CTC ATG TGC AAT CTC TCT TTT GCA GAC	1215
<u>T V L F V L L T</u> S H Y K L T V P R <u>F L M C N L S F A D</u>	378
TTT TGC ATG GGG CTC TAT CTG CTG CTC ATT GCC TCA GTT GAT TCC CAA ACC AAA GGC CAG TAT TAT AAC CAT GCC ATA GAT	1296
F C M G L Y L L I A S V D S Q Y Y N H A I D	405
TGG CAG ACA GGG AGT GGG TGT AGT GCT GCT GGC TTT TTC ACT GTA TTT GCA AGT GAA CTT TCT GTC TAC ACC CTC ACA GTC	1377
W Q T G S G C <u>S A A G F F T V F A S E L S V Y T L T V</u>	432
ATC ACA CTA GAA AGA TGG CAC ACC ATC ACC TAT GCT ATT CAG CTG GAC CAA AAA CTA CGA TTA AGA CAT GCC ATT CCA ATT	1458
<u>I T L</u> E R W H T I T Y A I Q L D Q K L R L R H A I P I	459
ATG CTT GGA GGA TGG CTC TTT TCT ACT CTA ATT GCT ATG TTG CCC CTT GTG GGT GTC AGC AAT TAC AGG AAG GTC AGC ATT	1539
<u>M L G G W L F S T L I A M L P L V G V</u> S N Y R K V S I	486
TGC CTC CCC ATG GAT ATA GAA ACC ACT CTC TCA CAA GTC TAC ATA TTA ACC ATC CTG ATA CTC TTT	1620
C L P M D I E T T L S Q <u>V Y I L T I L I L N V V A F F</u>	513
ATC ATT TGT GCT TGC TAC ATT AAA ATT TAT TTT GCA GTT CAA AAT CCA CAA CTG ATA GCT ACC AAC AAA GAC ACA AAG ATT	1701
<u>I I C A C Y I</u> K I Y F A V Q N P Q L I A T N K D T K I	540
GCT AAG AAA ATG GCA GTC CTC ATC ACC GAT GTC ACC TGC ATG GCA CCT ATC TCT TTT GCT ATT TCA GCT GCT TTC	1782
A K K <u>M A V L I F T D F T C M A P I S F F A I S A A F</u>	567
AAA GTG CCC CTT ATC CTA GTA ACC AAT TCT AAA GTT TTA CTG GTT CTC TTT TAT CCT GTC AAT TCT TGT GCC AAT CCA TTT	1863
K V P L I T V T N S K <u>V L L V L F Y P V N S C A N P F</u>	594
CTG TAC GCG ATT TTC ACA AAG GCA TTC CGA AGA GAT TTC TTT CTG TTG CTG AGC AAA TTT GGC TGC TGT AAA CAT CGG GCT	1944
<u>L Y A I F T K</u> A F R R D F F L L L S K F G C C K H R A	621
GAA CTT TAT AGA AGG AAG GAT TTT TCA GCT TAT ACC TCC AAG TGG AAG AAT GGC TTC ACT GGA TCA AAT AAG TCT TCC CAG	2025
E L Y R R K D F S A Y T S K W K N G F T G S N H A I S S Q	648
CCC ACC TTG AAG CTG ACC ACA TTG CAC TGC CAA TAT ACA GCT GTC CCA GAC AAG ACT TGC TAT ACA GAG TGT TAA	2100
P T L K L T T L H C Q Y T A V P D K T C Y T E C *	672

Figure 2 Nucleic acid and deduced amino acid sequences of the recombinant eLH/CG-R expressed in COS-7 cells. The putative cleavage site of the chimeric porcine (grey)/equine peptide signal is indicated by an arrow. The equine-specific *N*-glycosylation site is indicated in bold letters double underlined; other double underlines indicate the six *N*-linked glycosylation sites conserved among other mammalian LH/CG-Rs. Single underlines indicate the seven predicted transmembrane-spanning domains.

testicular membranes (Evans *et al.* 1982, Broadley *et al.* 1994, Motton & Roser 1997, Saint-Dizier *et al.* 2003). Mock-transfected cells and cells transfected with the expression vector without insert showed no binding to 125 I-eLH.

In order to compare the binding activity of eLH and eCG on recombinant eLH/CG-R, competitive binding

studies were performed on membranes from transfected COS-7 cells. As shown in Fig. 5, increasing doses of eLH and eCG competed with the membrane-bound 125 I-eLH, which indicates that both eCG and eLH bound to the recombinant eLH/CG-R expressed on COS-7 membranes. However, on a molar basis, eCG (IC_{50} 427 ng/ml; MW 44 000) bound to the recombinant eLH/CG-R with

Figure 3 Alignment of the amino acid sequences of equine, porcine, human, bovine and rat LH/CG receptors. Dots and dashes indicate identities and gaps respectively. The putative transmembrane domains are boxed. The potential *N*-glycosylation sites are highlighted in grey. The amino acids deduced from the degenerate P4 primer at the C-terminal extremity are in italics.

only 12.4% of the binding activity of eLH (IC₅₀ 41 ng/ml; MW 34 000).

The ratio of eCG binding activity to eLH activity for the recombinant eLH/CG-R (12.4%) was not very different from those previously found in testicular tissue (4%; Guillou & Combarnous 1983) and luteal tissue from cyclic (2–4%; Stewart & Allen 1979) or pregnant (2.5–3.9%;

Saint-Dizier *et al.* 2003) mares. Thus, as for the native receptor, eLH was found to exhibit much greater affinity than eCG for the recombinant eLH/CG-R expressed in COS-7 cells. As eLH and eCG exhibit similar affinities toward the porcine LH-R (Guillou & Combarnous 1983), this difference could be attributable to the specific seventh *N*-glycosylation site of the eLH/CG-R.

Figure 4 Saturation curve of ^{125}I -eLH binding to membrane preparations from COS-7 cells transiently transfected with the pCDNA-eLH/CG-R construct. Cell membranes were incubated in triplicate with ^{125}I -eLH in concentrations ranging from 10 to 600 pM with or without 100 IU cold hCG. The experiment was repeated three times and a representative assay is shown.

Figure 5 Competition curves of ^{125}I -eLH binding to membrane preparations from COS-7 cells transiently transfected with the pCDNA-eLH/CG-R construct. Cell membranes were incubated in triplicate with a constant amount of ^{125}I -eLH (55 pM) and increasing concentrations of unlabelled eLH and eCG. The experiment was repeated once and a representative assay is shown.

Gonadotrophin stimulation of cAMP production by transfected COS-7 cells

The ability of the recombinant eLH/CG-R to stimulate the cAMP pathway was tested by challenging the transfected cells with increasing concentrations of eLH and eCG. Stimulation with both hormones resulted in dose-dependent increases in cAMP production, which were about 20-fold and 15-fold increased over the basal rate of

Figure 6 Effect of various gonadotrophins on media cAMP concentrations in COS-7 cells transiently transfected with the pCDNA-eLH/CG-R construct. COS-7 cells were cultured for 1 h in the presence or absence of increasing doses of eLH, eCG, eFSH or human recombinant FSH (hrFSH). The data are expressed as the mean $\pm$ S.E.M. of triplicate observations from a single experiment. The experiment was repeated once and a representative assay is shown.

production with maximal doses of eLH and eCG respectively (Fig. 6). On a molar basis, eCG (EC_{50} 3865 ng/ml) stimulated cAMP production with 13.9% of the stimulating activity of eLH (EC_{50} 418 ng/ml). Neither pituitary eFSH nor recombinant hFSH stimulated cAMP production in transfected cells (Fig. 6). Mock-transfected cells and cells transfected with the expression vector without insert showed no increase in cAMP after hormone stimulation (data not shown).

Our results indicate that the binding of both eLH and eCG to the recombinant eLH/CG-R leads to activation of the adenylate cyclase/cAMP pathway, which is the main intracellular signalling pathway activated by mammalian LH/CG-Rs (Dufau 1998, Ascoli *et al.* 2002). As eLH and eCG have identical polypeptide chains (Bousfield *et al.* 1987, Sugino *et al.* 1987), the hormone carbohydrate compositions of eLH and eCG seem to be implicated in the binding affinity of the hormone for the recombinant eLH/CG-R, leading to different degrees of coupled intracellular responses.

In conclusion, the recombinant eLH/CG-R expressed in COS-7 cells provides us with a valuable model with which to study the influence of linked glycan chains on gonadotrophin receptor binding and activation.

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