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 mammalian 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 125I-eLH with high affinity (Kd 3.8×10−10 M). On a molar basis, eCG competed with 125I-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.


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 signaling pathway activated by the LH/CG-R is the adenyl cyclase pathway, leading to an increase in intracellular cAMP (Dufau 1998, Ascoli et al. 2002).

Radio-receptor 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.
on testicular or luteal membranes from cyclic or pregnant mares (Stewart & Allen 1979, Guillou & Combarnous 1983, Saint-Dizier et al. 2003). In contrast, eCG and eLH display similar affinity toward the porcine LH-R (Guillou & Combarnous 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 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/CGR binding activity remains to be investigated. Furthermore, the possible influence of eLH and eCG carbohydrates on hormone-induced intracellular responses after eLH/CGR 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/CGR cDNA, and studied the binding activity and hormone-stimulated cAMP production of the recombinant eLH/CGR 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 ovarectomy 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/CGR cDNA**

Total pooled RNA (2 µg) 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/CGR 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/CGRs (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/CGR 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/CGR 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/CGR 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).

### Table 1 Nucleic acid sequences of primers used for the isolation and cloning of the eLH/CGR cDNA. Location of nucleotides corresponds to the eLH/CGR 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

<table>
<thead>
<tr>
<th>Name and orientation</th>
<th>Location</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 (sense)</td>
<td>203–224</td>
<td>5′-CTTTCAGAGGA/CTTAATGAGGT-3′</td>
</tr>
<tr>
<td>P2 (antisense)</td>
<td>829–849</td>
<td>5′-TCTTAAAACGACAGCTGGCT-3′</td>
</tr>
<tr>
<td>P3 (sense)</td>
<td>627–651</td>
<td>5′-GCTGGAAGAGATGCACACGGAGAC-3′</td>
</tr>
<tr>
<td>P4 (antisense)</td>
<td>2076–2112</td>
<td>5′-CTCTC/AAACAGTTAAGCTG/TCGAGTC-3′</td>
</tr>
<tr>
<td>P5 (antisense)</td>
<td>501–525</td>
<td>5′-TTGC/ATATC/ACCCCTG/AAAGCA-3′</td>
</tr>
<tr>
<td>P6 (sense)</td>
<td>[-8]–71</td>
<td>5′-ATGGCCACTACTTTTGTTACCAACCACACTGCTGCCGCAAGC-3′</td>
</tr>
</tbody>
</table>
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), and sequenced. All 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 thermal 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. AFPS105A) 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/CGR N-terminal amino acid sequences

<table>
<thead>
<tr>
<th>pLH/CGR-R</th>
<th>eLH/CGR-R</th>
</tr>
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<tbody>
<tr>
<td>MRRRLULLLLLLLLLLLLPLPPQTLG</td>
<td>---------------LLPOQALRG</td>
</tr>
<tr>
<td>APCPEPCSPCPDQALRCPPF</td>
<td>APCPEPCSPCPDQALRCPPF</td>
</tr>
</tbody>
</table>

- Chimeric signal peptide

| MRRSLAILLLLLLPPPQALRG | APCPEP |

- Deduced nucleic acid sequence of the chimeric signal peptide

| MRRRSLLAILRLLLALLLPPLLPLQ |
| ATGAGAAGAGGGCTCAGGATGACATATTTTATTGACATTTCTTTGTTACCACCACCTGCTGCGGCAAGC |

- Nucleic acid sequence of the primer P6

| 5’ | CGCTCTAGAATGAGAAGAGGCTCAGGATGACATATTTTATTGACATTTCTTTGTTACCACCACCTGCTGCGGCAAGC |
| 3’ |

B

1. BglII

P1 P5 P3 P2

RT-PCR product (646 bp)

2. PstI

P3

RT-PCR product (1483 bp)

3. BglII

P5

RACE product (468 bp)

3’. XbaI

BglII

P5

Signal peptide + RACE product (533 bp)

1+2. BglII

P5

PstI

XbaI

1670 bp

1+2+3’. XbaI

BglII

P5

PstI

XbaI

eLH/CGR-R cDNA (2112 bp)

XbaI

pCDNA 3.1 (+)
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 125I-eLH binding by 50% (IC50), the equilibrium constant (Kd) and the maximal binding capacity (Bmax).

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 125I-cAMP in anti-cAMP antibody-coated tubes. The supernatants were then removed and the uncompeled radioactivity was counted in a gamma counter. The agonist concentrations inducing half-maximal stimulation of cAMP production (EC50) 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 92·2%, 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 (N23-L24-S25) 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 N23–L24–S25 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 125I-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 (Kd) of 3·8 × 10−10 M and a Bmax of 45·8 fmol/mg protein. The Kd measured for the recombinant eLH/CG-R was similar to those previously reported for eLH/CG binding sites in equine luteal or...
Mock-transfected cells and cells transfected with the expression vector without insert showed no binding to \( ^{125}\text{I}-\text{eLH} \).

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 \( ^{125}\text{I}-\text{eLH} \), 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 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.
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 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.
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 production with maximal doses of eLH and eCG respectively (Fig. 6). On a molar basis, eCG (EC50 3865 ng/ml) stimulated cAMP production with 13.9% of the stimulating activity of eLH (EC50 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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