Function of the Tethered rec-eCG in Rat and Equine Receptors

Jong-Ju Park, Naidansuren JarGal, Jong-Taek Yoon and Kwan-Sik Min†

Animal Biotechnology, Graduate School of Bio. & Information Technology, Institute of Genetic Engineering, Hankyong National University, Ansung 456-749, Korea

ABSTRACT

The glycoprotein hormone family represents a class of heterodimers, that includes the placental hormone equine chorionic gonadotropin (eCG) and the anterior pituitary hormones- follitropin (FSH), lutropin (LH), and thyrotropin (TSH). The 4 hormones are heterodimers, with a common α-subunit and unique β-subunits. eCG is the most heavily glycosylated of the known pituitary and placental glycoprotein hormones. Recent observations using single chain glycoprotein hormone analogs in which, the β-and α-subunits are linked, implied that heterodimeric-like quaternary configuration is not a prerequisite for receptor/signal transduction. To study the function and signal transduction of tethered rec-eCG, a single chain eCG molecule was constructed and rec-eCG protein was produced. Molecular mass of the single chain is about 45 kDa. All mice were ovulated by tethered rec-eCG treatment. The dual activity of tethered rec-eCG was determined in receptor cell lines of nonequid species; in fact, this dual activity was proven in species other than horse. Tethered rec-eCG in equids does not bind to FSH receptors, suggesting that eCG is primarily an LH-like hormone in the horse. Taken together, these data suggest that tethered rec-eCG has dual activity in nonequid species *in vitro*. However, it has only LH-like activity in equid species *in vitro*.

(Key words : Tethered rec-eCG, Dual activity, LH/CG receptor, FSH receptor)

INTRODUCTION

Glycoprotein hormones control reproduction and development with their ability to bind receptors on gonadal tissues. Gonadotropins are widely used to induce ovulation and to stimulate oocyte production before performning assisted reproduction therapies such as *in vitro* fertilization (Xing *et al*., 2004). Equine chorionic gonadotropin (eCG) is a member of the glycoprotein hormone family, which includes luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH) (Min *et al*., 2004). The β-subunits of eCG and eLH have an identical primary structure (Min *et al*., 1994). eCG shows both LH- and FSH-like activities in many species but not in the horse (Aggarwal and Papkoff, 1981; Moore and Ward, 1980; Moyle *et al*., 1990; Papkoff *et al*., 1978). We previously prepared recombinant WT- and tethered-eCGs in CHO-k1 cells, and found that these recombinant derivatives have biological LH- and FSH-like activities that are comparable to native hormone (Min *et al*., 1996, 2004). We reported that the oligosaccharide at Asn^{56} of the eCG α-subunit plays an indispensable role in expressing LH- but not FSH-like activities. Interestingly, the deglycosylated eFSH mutant at Asn^{56} of the α-subunit did not show any FSH-like activity (Saneyoshi *et al*., 2001).

eCG does not bind to FSH receptors in equine follicles (Guillou and Combarnous, 1983) or testis (Moor and Ward, 1980), suggesting that eCG is primarily an LH-like hormone. It is of interest that eCG binds to donkey FSH receptors with similar affinity to its attachment to donkey LH receptors (Guillou and Combarnous, 1983), a finding that has been interpreted to indicate that eCG may have FSH-like activity in the donkey. Chopineau *et al.* (2001) reported that the hCG β-subunit mutants obtained by the co-expressed with either the human or the equine α-subunit did not display any FSH activity. They also insisted that the 102~104 sequence in eCG β-subunit appears to be of utmost importance for their binding to FSH receptors.

In an attempt to determine the role of the dual (LH- and FSH-like) activities of rec-eCGs in the equine receptor, we analyzed the responsiveness activity of the equine LH/CG receptor and equine FSH receptors. These results demonstrate that rec-eCG elicits a great response from the eLH/CG receptor, but that it does not elicit any signal from the eFSH receptor in the stimulation of cAMP synthesis.

† Corresponding author : Phone: +82-31-670-5421, E-mail: ksmin@hknu.ac.kr

MATERIALS AND METHODS

Materials

The expression vector pcDNA3 was purchased from Invitrogen (California, USA). CHO-K1 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Endonucleases were from Boehringer Mannheim (MA, USA) and Takara (Osaka, Japan). Polymerase chain reaction (PCR) reagents were from Takara (Japan). Ham's F-12, CHO-S-SFM II, Geneticin, Lipofectamine 2000 and fetal bovine serum (FBS) were from Gibco BRL (MD, USA). The QIAprep-Spin plasmid kit was purchased from QIAGEN Inc. (Hilden, Germany). PMSG enzyme-linked immunosorbent assay (ELISA) kit was from DRG (USA). The pCORON 1000 SP VSV-G tag expression vector and the cAMP kit were from Amersham Biosciences (UK). The oligonucleotides were synthesized by Green Gene Bio (Korea). Fetal bovine serum was from Hyclone laboratories (Utah, USA). Centriplus Centrifugal Filter Devices were purchased from Amicon Bio separations (MA, USA). ICR mice were purchased from Koatech (Seoul, Korea). All other reagents used were from Sigma-Aldrich (USA) and Wako Pure Chemicals (Osaka, Japan).

Construction of the *myc-tag* Tethered-eCG Transfer Vector

To obtain tethered eCG, cDNA encoding the full-length eCG β-subunit (20 amino acid residue signal sequence and 149 amino acid residues of the mature protein) was fused with the mature protein part of the *α*-subunit using the overlapping PCR mutagenesis method (Min *et al*., 2004).

The primers used are summarized in Table 1. The first PCR was performed using different sets of primers 1 and 2, and 3 and 4 (Table 1). Fragments were annealed and subjected to the second PCR using primers 1 and 4 to generate tethered eCGβα as shown in Fig. 1.

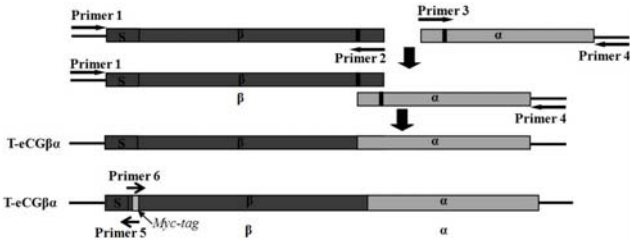

Fig. 1. Construction of tethered rec-eCGs by overlapping PCR mutagenesis. The *myc-tag* contains of 10 amino acids. S: signal region of eCG β-subunit; *Myc-tag*: *myc-tag* (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu).

The same method was used to add *myc-tag* (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) between the first amino acid and the second amino acid of the mature protein in eCG β-subunit (Fig. 1). These fragments were digested by the *Eco*RI and *Sal*I enzymes. Next, the fragments were ligated into the eukaryotic expression vector pcDNA3. All mutants were sequenced completely to confirm the Kozak site, *myc-tag* and PCR errors.

Transient and Stably Transfection into CHO Cell Lines

Cultured CHO-K1 cells were transfected with expression vectors using liposome transfection method as previously described (Min *et al*., 2004). The transfected cells were cultured for 48 h in serum-free medium (CHO-S-SFM-II), the media were harvested and centrifuged at 15,000 rpm for 10 min. Then the supernatants were collected and stored at −20℃ until assa-

Table 1. Oligonucleotide sequences of the primers used for the construction of each mutants

	Primer name	Primer Sequence
1	T-eCG*β a β*-subnuit 5′	5'- T**GAATTC**ACCATGGAGACGGTCCAG -3'
2	T-eCG*β a* reverse	5′-TCCATCAGGAAAAGAAGTCTTTATTGG-3′
3	T-eCG*β a* forward	5′-ATAAAGACTTCTTTTCCTTGATGGAGAG-3′
4	T-eCG*β a α*-subunit3′end	5'- CC**GTCGAC**TTTAAATCTTGTGGTGATAGCA -3'
5	T-*myc* eCG*β a* reverse	5'-TTCTTCAGAAATAAGCTTTTGTTCGGATGCCCAGACCCCGCC-3'
6	T-*myc* eCG*β a* forward	5'-AAGCTTATTTCTGAAGAAGACTTGAGGGGGCCACTGCGGCCA-3'
7	eLH/CGR RT-forward	5′-CTGTCAATTCTTGTGCCAAT-3′
8	eLH/CGR RT-reverse	5′-AGAGTCTGTATAGCAAGTCT-3′
9	eFSHR RT-forward	5′-CCATTCCCTGCCTAACTAT-3′
10	eFSHR RT-reverse	5′-GCAAGTTGTCTGAAGCTCAGAGG-3′

yed. Six to 8 pools of stably transfected cells were selected by incubation in growth medium [Ham's F12 media containing 10% FCS, penicillin (100 IU/ml), streptomycin (100 μg/ml), and glutamine (2 mM) supplemented with G418 (800 ug/ml) for 2~3 weeks post-transfection according to the method reported previously (Min *et al*., 1996). The culture media were collected and centrifuged at 15,000 rpm at 4℃ for 10 min to remove cell debris. Supernatants were collected and concentrated in an Amicon Stir Cell Concentrator and stored at −20℃ until assayed.

Hormone Quantitation and Western Blot Analysis of Tethered rec-eCG

The recombinant hormones were quantified by PMSG enzyme-linked immunosorbent assay (ELISA) using anti-PMSG monoclonal antibody and enzyme conjugate with coupled to horseradish peroxidase and TMB as substrate. For Western blot analysis, samples of concentrated medium were electrophoresed on reducing 12% sodium dodecyl sulfate (SDS)-poly-acrylamide gels by the method of Laemmli (1970). After SDS-polyacrylamide gel electrophoresi (PAGE), the protein was transferred to polyvinyli-dene difluoride (PVDF) membrane (0.2 um) at 100 V for 2 h using a Bio-Rad Mini Trans-Blot electrophoresis cell. After blotting, the membrane was blocked with 1% blocking reagent for 1 h and bound by monoclonal anti-myc antibody (1:5,000) for 2 h. The blot was washed to remove unbound antibody, incubated with a secondary antibody linked to anti-mouse IgG-POD (37.5 ul/15 ml of blocking solution) for 30 min. and then washed. After then, the membrane was incubated for 5 min with 2 ml Lumi-Light substrate solution and placed it the protein side of membrane up on Saran Wrap. The membrane was covered with a second Saran Wrap and exposed on X-ray film for 1~10 min.

Enzymatic Release of N-linked Oligosaccharides

For western blot analysis, samples of concentrated medium were electrophoresed on a reducing or non-reducing 12.5% SDS-polyacrylamide gel by the method of Laemmli (1970). The tethered rec-eCG protein was analyzed to remove added glycans by *N*-glycosylation enzyme. For removal of all N-linked glycans, tethered rec-eCG was incubated 24 h at 37℃ with PNase F [2 ul enzyme (2.5 U/ml) /30 ul sample+8 ul of 5 x reaction buffers]. The reaction was stopped by boiling for 10 min, electrophoresed by SDS-PAGE, and analyzed by western blot. The protein was transferred to 0.2 um pore size nitrocellulose at 100 V for 2 h using a Bio-Rad Mini Trans-Blot electrophoresis.

***In Vivo* Biopotency of Tethered rec-eCG**

In vivo biopotency of tethered eCG was estimated by determining the ovulated oocyte number. hCG was injected into ICR mice at 48 h after one injection of tethered-eCG 10 IU. And then oocyte numbers were then investigated 18~20 h later by via counting the number of ova released into the oviducts.

Real-Time PCR and Cloning of Equine LH/CG and Equine FSH Receptors

Real-time PCR was used to confirm the expression of equine LH/CGR and equine FSHR in the testes and ovaries of adult horses. eLH/CGR primers (260 bp) [sense: 5'-CTGTCAATTCTTGTGCCAAT-3'; antisense: 5'-AGAGTCTGTATAGCAAGTCT-3'] and eFSHR primers (187 bp) [sense: 5'-CCATTCCCTGCCTAACTAT-3'; antisense: 5'-GCAAGTTGTCTGAAGCTCAGAGG-3'] were designed from the nucleotide sequence of the previously reported receptor (Robert *et al*., 1994; Saint-Dizier *et al*., 2004) and then expression was determined by real-time PCR.

To create full-length clones of eLH/CGR and eFSHR cDNAs, eLH/CGR primers [sense: 5'-CTGCTGCCGCAAGCGCTGCGCGGGGCG-3'; antisense: 5'-TTAACATGTATAGCAAGTCTT-3'] and eFSHR primers [sense: 5'-TGAATTCTTTAGTTCTGAGCTAAATGG-3'; antisense: 5'-AGGTACCACCATGGCCTTGCTCCTGGTCTCC-3'] were designed and then amplified by LA-PCR. The fragments were ligated into the pcDNA3 vector by *Kpn* 1 and *Xba* I enzymes cut and sequenced to ensure no errors were introduced during PCR. To remove the signal sequence, both receptors were re-amplified by the primers added to the *Xho* I site at the 5′-end and the *Eco* RI site at the 3′-end. Finally receptors were subcloned into the pCORON 1000 SP VSV-G tag expression vector by cut *Xho* I and *Eco* RI.

Selection of Cell Lines Expressing eLH/CG and e-FSH Receptors

Transfections of CHO cells were done using the liposome transfection method as previously described (Min *et al*., 2004). The receptor cDNAs were subcloned into the eukaryotic expression vector (pCORON 1000 SP VSV-G tag). Stably transfected cell lines were obtained after G418 selection and cloning as described elsewhere (Min *et al*., 1996, 2004). Establishment and properties of clonal cell lines expressing *vsvg*-eLH/CGR-wt [designated vsvg-eLH/CGR (wt-2-3)] and a clonal cell line expressing *vsvg*-eFSHR-wt [designated vsvg-eFSHR (wt-2-4)] were prepared by cAMP responsiveness for agonist cholera toxin.

Selection of Cell Lines Expressing Rat LH/CG and Rat FSH Receptors

Cloning of rat LH/CG and FSH receptors was previously described (Min *et al*., 1998; Min *et al*., 1999). The specific enzyme sites for rat receptors to remove

signal sequence were introduced by PCR, and the sequence of the entire cDNA region generated by PCR was verified by automated DNA sequencing. The receptor cDNAs were subcloned into the eukaryotic expression vector pCORON 1000 SP VSV-G tag for transfection. Transfections of CHO cells were done using a liposome transfection method as previously described (Min *et al*., 2004). Stably transfected cell lines were obtained after G418 selection and cloning as described elsewhere (Min *et al*., 1996, 2004). The establishment and properties of a clonal cell lines expressing *vsvg*-rLH/CGR-wt (designated *vsvg*-rLH/CGR (wt-2-6)) and a clonal cell line expressing *vsvg*-rFSH-wt (designated vsvg-rFSHR (wt-2-6)) were prepared by cAMP responsiveness for agonist cholera toxin.

In Vitro Bioassy for LH- and FSH-like Activities

LH- and FSH-like biological activities were estimated by measuring total cyclic AMP. Receptor cells were cultured in standard 96-well microplates with cell concentrations of $10^5 \sim 10^6$ cells/ml and incubated overnight at 37℃. A aliquots (20 μl) of rec-eCGs were added and incubated for 30 min. Lysis reagent (20 μl) was then added. Finally, a cyclic AMP kit was used according to the supplier's protocol and the OD was read at 630 nm with a microplate reader. Concentration-response curves for the rec-eCG induced increases in cAMP accumulation were obtained by measuring total cAMP level in cells that had been incubated with at least 5 different eCG concentrations in the presence of a phosphodiesterase inhibitor (Min *et al*., 1998).

RESULTS

Production of Transient and Stably Tethered rec-eCG

The tethered eCG and myc tag vectors were transfected into CHO-K1 cells. Transient expression was collected from the supernatants at 72h after transfection. To establish the stable cell lines, between 6 and 8 stably transfected pools of cells expressing tethered rec-eCG was selected for G418. The rec-eCG protein secreted by the stably transfected cells into serum free media were collected and quantified by ELISA (Fig. 2). The expression quantity was about 1,000～1,200 mIU/ml in transient transfection. There was no quantitative differences in the recognition of tethered eCG and tethered myc tag eCG detected by antibody. On the basis of these observations, myc tag rec-eCG was used to analyze the bio-activity.

Real-time PCR and Expression of eLH/CG Receptor and eFSH Receptor

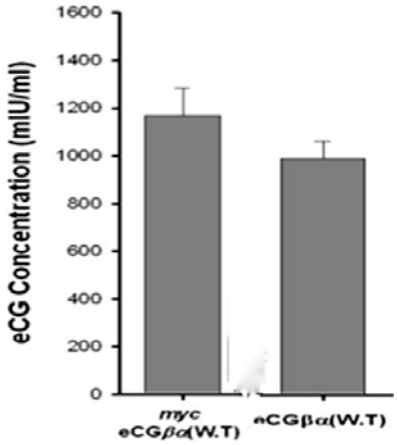

Fig. 2. Transient expression of tethered eCGs and *myc-tag* eCGs in CHO cells by ELISA. Left) Tethered rec-eCGs, Right) The added *myc-tag* in tethered rec-eCGs. Values are expressed as mean±SEM for at least three independent experiments.

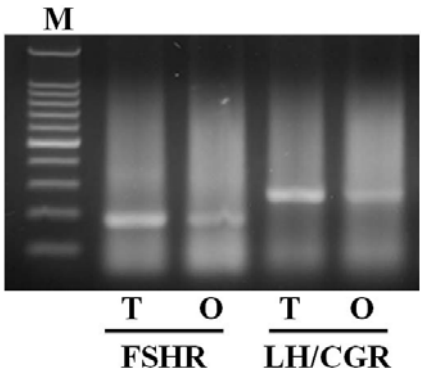

Fig. 3. Expression of eLH/CG receptor and eFSH receptor genes in equine ovary and testis. T: testis; O: ovary; M: marker.

Expression of eLH/CG and FSH receptors was detected by real-time PCR in equine testes and ovaries. The eLH/CG (260 bp) and eFSH receptors (187 bp) were amplified (Fig. 3) and full-length cDNAs of the LH/CG and FSH receptors were also amplified and sequenced. Both genes were transfected into CHO cell lines and we selected the clones expressing LH/CG and FSH receptor genes by G418. Finally, the clone was identified by cAMP responsiveness for agonist cholera toxin.

Western Blot and Treatment of N- and O-linked Oligosaccharide Digestion

Efficient translation of the tethered rec-eCG was detected about 43～45 kDa. By the N-linked digestion, molecular sizes of eCG were remarkably decreased to 30 kDa. It suggested that the oligosaccharides of about 10～15 kDa were added to tethered rec-eCG protein. However, O-linked digestion did not affect to the molecular weight of tethered rec-eCG (Fig. 4).

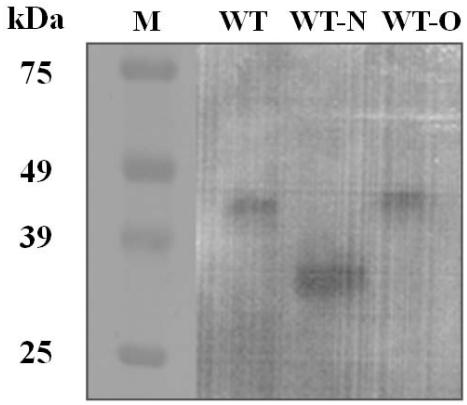

Fig. 4. Western blotting result for N-linked and O-linked glycosylation digestion. WT-N: digestion with N-linked glycosylation enzyme, WT-O: digestion with O-linked glycan enzyme.

Table 2. The superovulatory potency of control eCG and tethered rec-eCG.

	Ovulated Oocyte number	
	Good	Bad
Control eCG	24	4
	32	2
	12	6
Tethered rec-eC	14	1
	13	0
	11	0

Immature mice were treated s.c. with a single injection (10 IU) of control eCG and tethered rec-eCG. And then hCG was treated before sacrifice 20 h later. Number of ovulated ova in the oviducts was counted.

In Vivo Biopotency of Tethered rec-eCG

In vivo biopotency of tethered rec-eCG was estimated by determining the ovulated oocyte number. hCG was injected into ICR mice at 48 h after one 10 IU injection of tethered rec-eCG. Oocyte numbers were investigated after 18~20 h. Native eCG was injected as a positive control, and it induced a large number of oocytes, however, many of them were of poor quality. In contrast, only o of the oocytes in the tethered rec-eCG treatment groups was of poor quality. Even though it resulted in lower numbers of oocytes, tethered rec-eCG was found to be a good reagent for inducing ovulation in experimental animals (Table 2).

Biological Activity of Tethered rec-eCG in Nonequid Species

The effects of the tethered rec-eCG on cAMP secretion in CHO cell lines expressing rat LH/CG receptor and FSH receptor genes were determined to evaluate their LH- and FSH-like activities. Receptor cells were incubated with various concentrations of tethered rec-eCG. The cAMP concentration increased in direct proportion to the concentration of tethered rec-eCG (Fig. 5). Over the range of 10~100 ng/ml, tethered rec-eCGs showed a similar concentration-response curve. Basal cAMP levels were 74.3 fmol/10^6 cells for the rat LH/CG receptor and maximal cAMP responses were 1,610 fmol/10^6 cells. Basal cAMP levels for the rat FSH receptor were 62.2 fmol/10^6 cells and maximal cAMP res-

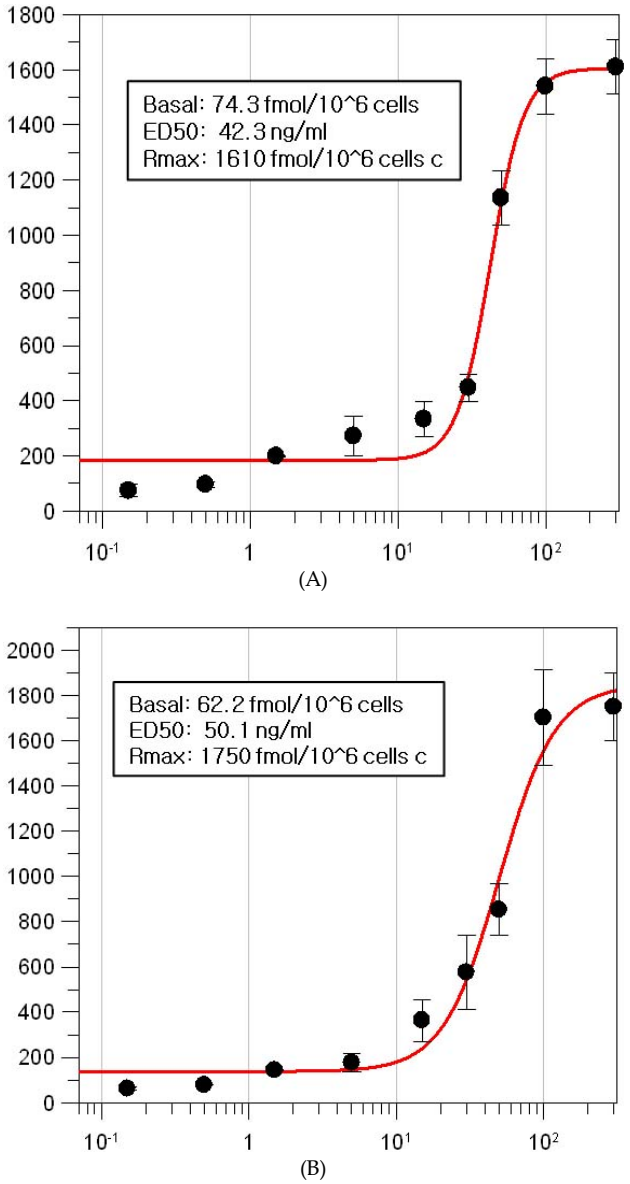

Fig. 5. Effects of increasing concentrations of rec-tethered eCG on accumulation in stably transfectants of CHO cells expressing rLHR-wt (A) and rFSHR-wt (B). Cells were incubated with the indicated concentrations of rec-eCG in medium containing 0.5 mM 3-isobutyl 1-methyl xanthine for 30 min. at 37℃ before total cAMP was assayed (see Material and Methods for details). Each point represents the average SEM of three independent experiments. Duplicate dishes were used for each experiment.

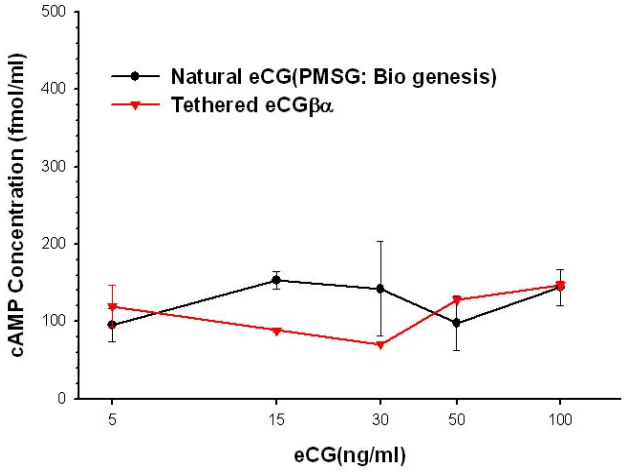

Fig. 6. Cyclic AMP response for agonist (natural eCG) and rec-tethered eCGβα in CHO cell expressing eFSHR genes. * Natural eCG: PMSG.

ponses were 1,750 fmol/10^6 cells. The concentration-response curve of tethered rec-eCG was almost similar between the rat LH/CG receptor and rat FSH receptor.

Biological Activity of Tethered rec-eCG in Equids

The effects of the tethered rec-eCG on cAMP secretion in CHO cell lines expressing equine LH/CG receptor and FSH receptor genes were determined to evaluate their LH- and FSH-like activities. Tethered rec-eCG bound to the LH/CG receptor in equine and had a very similar pattern to the rat LH/CG receptor (data not shown). However, the concentration-response curve was essentially flat in the eFSH receptor (Fig. 6), it showed no FSH-like activity, indicating that eCG does not bind to FSH receptors in horses.

DISCUSSION

Previously, we and others (Ben-Menahem *et al.*, 2001; Fares *et al.*, 1998; Garcia-Campayo *et al.*, 1997; Sugahara *et al.*, 1995) observed that single chain variants of the glycoprotein hormone family exhibit biological activity comparable to the corresponding heterodimers. The present study indicates that the eCG heterodimer can be expressed as a single chain encoding both subunits (α- and β-). Our results are consistent with those of earlier studies in demonstrating that eCG can be synthesized as biologically active single chains (Min *et al.*, 2004). Thus, the non-covalent heterodimeric structure is not critical for the glycoprotein hormone family function (Fares *et al.*, 1998).

The results presented here strongly suggest that tethered rec-eCG has intrinsic LH and FSH activities because it binds to both LH and FSH receptors and activates adenyl cyclase in other species. Moyle *et al.* (1978) reported that dual activity of eCG is probably not related to its carbohydrate content because removing sialic acid residues increases its potency equally well in systems known to possess LH and FSH receptors. Martinuk *et al.* (1991) reported that sialic acid was significant to the distribution and disappearance of eCG. The effects of carbohydrate removal on biological activity are primarily a function of clearance rate rather than a tissue-specific phenomenon. However, our group suggested that oligosaccharide at the α-subunit Asn^{56} is the essential factor in the LH-like activity of rec-eCG (Min *et al.*, 1996). These data suggest that tethered rec-eCG has dual activity in nonequids species *in vitro*. However, it has only LH-like activity in equids species *in vitro*. The chemical similarity of eLH and eCG was previously reported (Licht *et al.*, 1979).

In the present study, eCG bound to putative LH receptors in equine testis (Saint-dizier *et al.*, 2003). However, this occurred at about one-tenth the affinity of equine LH binding (Guillou and Combarnous, 1983). eCG binds to the eLH/CG receptor at only 2～4% of the activity of eLH on testicular or luteal membranes from cyclic or pregnant mares (Saint-Dizier *et al.*, 2003). In contrast, eCG and eLH display similar affinity toward the porcine LH receptor (Guillou and Combarnous, 1983). Unexpectedly, eCG bound to donkey FSH receptor with similar affinity to its attachment to donkey LH receptor (Guillou and Combarnous, 1983). However, donkey LH bound to equine testis preparations with about 10% of the activity of the horse LH (Roser *et al.*, 1984). eCG has LH-like activity in horse receptor and *in vitro* bioassays. In spite of the amino acid homology, it has lower LH activity than does horse LH (Murphy and Martinuk, 1991).

Yoon *et al.* (2007) suggested that recombinant eCG can induce ovulation, hormone profiles, and inter-ovulatory intervals in cycling mare. They showed that increasing doses of rec-eCG (0.3, 0.6, 0.75, and 0.9 mg) showed increasing effectiveness at inducing ovulation within 48 h of treatment. Treatments with the 0.7 mg and 0.9 mg doses of rec-eCG resulted in 90% and 80% ovulation rates, similar to hCG treatment (85.7%). Thus, rec-eCG is a reliable and effective ovulatory agent that does not significantly alter endogenous hormone profiles or affect inter-ovulatory intervals.

The loss of biological activity of eCG after desialylation was previously reported (Aggarwal and Papkoff, 1981; Min *et al.*, 1996). The importance of sialic acid to the bioactivity of eCG *in vivo* was apparent in the 53% and 80% desialylated preparations, which were much less effective in stimulating folliclulogenesis or superovulation than were low doses of control eCG, even at doses of up to 500 IU (Martinuk *et al.*, 1991). Three desialylated eCG preparations (20%, 53%, and

80% sialic acid removed) determined the pharmacokinetic parameter for the disappearance, superovulatory, and ovarian weight responses. The 20% desialylated eCG induced superovulatory and ovarian weight responses, but 100～500 IU were required to achieve the same result as that produced by control eCG. The present data suggest that tethered rec-eCG has the same ovulation potency oocytes, however, only one oocyte in the tethered rec-eCG treatment group was of poor quality. Thus, rec-eCG has more potent FSH-like activity than control eCG.

Chopineau *et al.* (2001) reported that the 102～104 sequence in eLH/CG β-subunits appears to be of utmost importance for binding to FSH receptors. However, these results obtained with equid β-subunits are not transposable to other gonadotropins since similar mutations in hCG β did not lead to any increase in FSH activity. Galet *et al.* (2000) suggested that transgenic animal of single chain eCG was produced in a rabbit and presented similar *in vitro* LH and FSH bioactivities. However, eCG protein had an extremely rapid clearance (10 min), which could explain the absence of *in vivo* biological activity. Thus, the rabbit mammary gland is not appropriate for production of recombinant active eLH/CG. Multiple transcripts of eLH/CGR (7, 5.7, 4.9, 3.9, 2.8, 1.8 and 0.6 kb) were identified in the primary CL at all examined stages. Three of them (5.7, 2.8 and 0.6 kb) were coded for truncated eLH/CGR lacking the transmembrane domain. The affinity of eLH/CG binding sites did not change during pregnancy whereas the number of eLH/CG binding sites decreased significantly after the onset of eCG secretion (Saint-Dizier *et al*., 2004). On a molar basis, eCG competed with ^{125}I-eLH on membrane preparations with only 12.4% of the eLH binding activity. eCG stimulated cAMP production with only 13.9% of the eLH stimulating activity. The differences between eLH and eCG activities toward this receptor will be useful in studies focusing on the influence of carbohydrates on gonadotropin receptor binding and activation (Saint-Dizier *et al*., 2004).

The present study demonstrates the potential for engineering proteins of multiple functional domains by fusing 2 separate gene products into a single chimeric protein. In addition to the increased practical utility for such constructs, single-chain eCG provides a potentially valuable model to investigate a variety of structure-function problems independent of the assembly step.

REFERENCES

1. Aggarwal BB, Papkoff H (1981): Relationship of sialic acid residues to *in vitro* biological and immunological activities of equine gonadotropins. Biol Reprod 24:1082-1087.
2. Ben-Menahem D, Jablonka-Shariff A, Hyde RK, Pixley MR, Srivastava S, Berger P, Boime I (2001): The position of the α and β subunits in a single chain variant of human chorionic gonadotropin affects the heterodimeric interaction of the subunits and receptor-binding epitopes. J Bio Chem 276:29871-29879.
3. Chopineau M, Martinat N, Galet C, Guillou F, Combarnous Y (2001): β-subunit 102～104 residues are crucial to confer FSH activity to equine LH/CG but are not sufficient to confer FSH activity to human CG. Journal of Endocrinology 169:55-63.
4. Fares FA, Yamabe S, Ben-Menahem D, Pixley M, Hsueh AJ, Boime I (1998): Conversion of thyrotropin heterodimer to a biologically active single-chain. Endocrinology 139:2459-2464.
5. Galet C, Menck Le Bourhis C, Chopineau M, Le Griec G, Perrin A, Magallon T, Attal J, Viglietta C, Houdebine LM, Guillou F (2000): Expression of a single β α chain protein of equine LH/CG in milk of transgenic rabbits and its biological activity. Mol Cell Endocrinol 174:31-40.
6. Garcia-Campayo V, Sato A, Hirsch B, Sugahara T, Muyan M, Hsueh AJ, Boime I (1997): Design of stable biologically active recombinant lutropin analogs. Nature Biotechnology 15:663-667.
7. Guillou F, Combarnous Y (1983): Purification of equine gonadotropins and comparative study of their acid-dissociation and receptor-binding specificity. Biochim Biophys Acta 755:229-236.
8. Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.
9. Licht P, Gallo AB, Aggarwal BB, Farmer SW, Castelino JB, Papkoff H (1979): Biological and binding activities of equine pituitary gonadotropins and pregnant mare serum gonadotrophin. J Endocrinol 83: 311-322.
10. Martinuk SD, Manning AW, Black WD, Murphy BD (1991): Effects of carbohydrates on the pharmacokinetics and biological activity of equine chorionic gonadotropin *in vivo*. Biol Reprod 45:598-604.
11. Min KS (1999): Functional expression of lutropin/choriogonadotropin receptor cDNAs in 293 cells. Korean J Animal Reprod 23:347-352.
12. Min KS, Hiyama T, Seong HH, Hattori N, Tanaka S, Shiota K (2004): Biological activities of tethered equine chorionic gonadotropin (eCG) and its deglycosylated mutants. J Reprod Dev 50:297-304.
13. Min KS, Liu X, Fabritz J, Jaquette J, Abell AN, Ascoli M (1998): Mutations that induce constitutive activation and mutations that impair signal transduction modulate the basal and/or agonist-stimu-

lated internalization of the lutropin/choriogonadotropin receptor. J Bio Chem 273:34911-34919.
14. Min KS, Shinozaki M, Miyazawa K, Nishimura R, Sasaki N, Shiota K, Ogawa T (1994): Nucleotide sequence of eCG α-subunit cDNA and its expression in the equine palcenta. J Reprod Dev 40:301-305.
15. Moore WT Jr, Ward DN (1980): Pregnant mare serum gonadotropin. An *in vitro* biological characterization of the lutropin-follitropin dual activity. J Bio Chem 255:6930-6936.
16. Moyle WR, Erickson G, Bahl OP, Cristakos S, Gustowski J (1978): Action of PMSF and asialo-PMSG on rat Leydig and granulosa cells. Am J Physio 235:E218-E226.
17. Moyle WR, Matzuk MM, Campbell RK, Cogliani E, Dean-Eming DM, Krichevsky A, Barnett RW, Boime I (1990): Localization of residues that confer antibody binding specificity using human chorionic gonadotropin/luteinizing hormone β subunit chimeras and mutants. J Bio Chem 265:8511-8518.
18. Murphy BD, Martinuk SD (1991): Equine chorionic gonadotropin. Endocr Rev 12:27-44.
19. Papkoff H, Bewley TA, Ramachandran J (1978): Physiochemical and biological characterizations of pregnant mare serum gonadotropin and its subunits. Biochim Biophys Acta 532:185-194.
20. Robert P, Amsellem S, Christophe S, Benifla JL, Bellet D, Koman A, Bidart JM (1994): Cloning and sequencing of the equine testicular follitropin receptor. Biochem Biophys Res Commun 30:201-207.
21. Roser JF, Papkoff H, Murthy HM, Chang YS, Chloupek RC, Potes JA (1984): Chemical, biological and immunological properties of pituitary gonadotropins from the donkey (*Equus asinus*): comparison with the horse (*Equus caballus*). Biol Reprod 30: 1253-1262.
22. Saint-Dizier M, Chopineau M, Dupont J, Daels PF, Combarnous Y (2003): Expression and binding activity of luteinizing hormone/chorionic gonadotropin receptors in the primary corpus luteum during early pregnancy in the mare. Biol Reprod 69:1743-1749.
23. Saint-Dizier M, Foulon-Gauze F, Lecompte F, Combarnous Y, Chopineau M (2004): Cloning and functional expression of the equine luteinizing hormone/chorionic gonadotrophin receptor. J Endocrinol 183:551-559.
24. Saneyoshi T, Min KS, Jing Ma X, Nambo Y, Hiyama T, Tanaka S, Shiota K (2001): Equine follicle-stimulating hormoe: molecular cloning of β subunit and biological role of the asparagine-linked oligosaccharide at asparagines (56) of α-subunit. Biol Reprod 65:1686-1690.
25. Sugahara T, Pixley MR, Minami S, Perlas E, Ben-Menahem D, Hsueh AJ, Boime I (1995): Biosynthesis of a biologically active single peptide chain containing the human common α and chorionic gonadotropin β subunits in tandem. Proc Natl Acad Sci USA 92:2041-2045.
26. Xing Y, Lin W, Jiang M, Cao D, Myers RV, Bernard MP, Moyle WR (2004): Use of protein knobs to characterize the position of conserved α-subunit regions in lutropin receptor complexes. J Bio Chem 279:44427-44437.
27. Yoon MJ, Boime I, Colgin M, Niswender KD, King SS, Alvarenga M, Jablonka-Shariff A, Pearl CA, Roser JF (2007): The efficacy of a single chain recombinant equine luteinizing hormone (reLH) in mares: induction of ovulation, hormone profiles, and inter-ovulatory intervals. Domest Anim Endocrinol 33:470-479.

(Received: 25 November 2009 / Accepted: 14 December 2009)