β-Subunit 94~96 Residues of Tethered Recombinant Equine Chorionic Gonadotropin are Important Sites for Luteinizing Hormone and Follicle Stimulating Hormone like Activities

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ABSTRACT

Equine chorionic gonadotropin (eCG) is a heavily glycosylated glycoprotein composed of non-covalently linked α- and β-subunits. To study the function and signal transduction of tethered recombinant-eCG (rec-eCG), a single chain eCG molecule was constructed, and the rec-eCG protein was prepared. In this study, we constructed 5 mutants (Δ1, Δ2, Δ3, Δ4, and Δ5) of rec-eCG using data about known glycoprotein hormones to analyze the role of specific follicle stimulating homone (FSH)-like activity. Three amino acids of certain specific sites were replaced with alanine. The expression vectors were transfected into CHO cells and subjected to G418 selection for 2~3 weeks. The media were collected and the quantity of secreted tethered rec-eCGs was quantified by ELISA. The LH- and FSH-like activities were assayed in terms of cAMP production by rat LH/CG and rat FSH receptors. Then, the metabolic clearance rate analyzed by the injection of rec-eCG (5 IU) into the tail vein was analyzed. The mutant eCGs (Δ1, Δ4, and Δ5) were transcripted, but not translated into proteins. Rec-eCG Δ2 was secreted in much lower amounts than the wild type. Only the rec-eCG Δ3 (β-subunit: Gln^{94}-Ile^{95}-Lys^{96}→Ala^{94}-Ala^{95}-Ala^{96}) was efficiently secreted. Although activity is low, its LH-like activity was similar to that of tethered eCGβα. However, the FSH-like activity of rec-eCGβαΔ3 was completely flat. The result of the analysis of the metabolic clearance rate shoed the persistence of the mutant in the blood until 4 hours after the injection. After then, it almost disappeared at 8 hours. Taken together, these data suggest that 94~96 amino acid sequences in eCG β-subunit appear to be of utmost importance for signal transduction of the FSH receptor.

(Key words : Tethered rec-eCG, LH- and FSH-like activities, β-subunit 94~96 residues)

INTRODUCTION

Equine choriogonadotropin (eCG) is a placental glycoprotein hormone that is involved in the maintenance of pregnancy. It is a member of the glycoprotein hormone family, which also includes the luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH) (Min *et al.*, 2004). Gonadotropins are used widely to induce ovulation and to stimulate the production of oocytes prior to assisted reproduction therapies such as *in vitro* fertilization (Xing *et al.*, 2004). The α/β dimers have the high biological activity, but dissociated subunits do not (Pierce and Parson, 1981). The β-subunits of eCG and eLH have identical primary structures (Min *et al.*, 1994). eCG shows both LH- and FSH-like activities in many animal species, but not in horses (Aggarwal and Papkoff *et al.*, 1981; Moyle *et al.*, 1990).

We have previously reported the preparation of recombinant wild type (WT) and tethered-eCGs in Chinese hamster ovary cells-k1 (CHO-K1) cells; we found that these recombinant derivatives have biological LH- and FSH-like activities that are comparable to the native hormones (Min *et al.*, 1996, 2004; Park *et al.*, 2009). Additionally, we reported that the oligosaccharide at Asn56 of the α-subunit of eCG plays an indispensable role in expressing LH- but not FSH-like activities. Interestingly, the eFSH mutant deglycosylated at Asn56 of the α-subunit did not show any FSH activity (Saneyoshi *et al.*, 2001). The hFSHβ mutant Q48A was 8 times less active than the WT hFSH when assayed for binding to hFSH receptors. hFSHβ mutations at V38A and Y39A affected receptor binding, however, neither of these mutations could individually cause more than a 2-fold decrease in receptor binding activity of hFSH (Roth and Dias., 1996). A peptide corresponding to FSH residues 33~53 blocked the binding of FSH to its receptor and stimulated steroidogenesis (Santa Coloma and Reichert., 1990; Santa Coloma *et al.*, 1990) and similar

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results have been reported for the corresponding regions of hCG and TSH (Morris *et al.*, 1990). hCG was, until recently, the only gonadotropin for which a crystallographic model had been resolved (Lapthorn *et al.*, 1994; Wu *et al.*, 1994). The 90~110 sequence of the β-subunit surrounds the α-subunit forming a 'seatbelt' which is fastened by the 26~110 disulfide bonds. Studies have shown that these 90~110 sequence influence gonadotropin specificity: the 93~110 region influences LH specificity (Hwang *et al.*, 1993; Han *et al.*, 1996), whereas the 100~110 region influences FSH specificity (Moyle *et al.*, 1994). Although the amino acid sequences of eCG, donkey CG (dCG), and zebra CG (zCG) are highly homologous, they have different FSH/LH activity ratios. The β-subunit of 3 CG genes are different in only 6 positions 51, 94, 95, 102, 103, and 106 (Chopineau *et al.*, 1999). The 102~104 sequences in the eCG β-subunit are crucial to the FSH activity of eCG, but they are not sufficient to elicit FSH activity in hCG (Chopineau *et al.*, 2001).

In an attempt to determine the role of these amino acids in determining the level of the dual activities (LH- and FSH-like activities) of eCGs, we substituted 5 specific sites (^{43}RVM^{45} to ^{43}AAA^{45}, ^{54}QPV^{56} to ^{54}AAA^{56}, ^{94}QIK^{96} to ^{94}AAA^{96} in the β-subunit and ^{33}KGC^{35} to ^{33}AAA^{35}, ^{42}PTP^{44} to ^{42}AAA^{44} in the α-subunit) with Ala by site-directed mutations. Our results demonstrate that the 94~96 amino acids of the β-subunit are important for the stimulation of cAMP synthesis.

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 from Boehringer Mannheim (MA, USA) and Takara (Osaka, Japan), polymerase chain reaction (PCR) reagents from Takara (Japan). Ham's F-12, CHO-S-SFM-Ⅱ, geneticin, lipofectamine 2000, fetal bovine serum (FBS) were purchased from Gibco BRL (MD, USA). The QIAprep-spin plasmid kit was purchased from QIAGEN Inc. (Hilden, Germany). Pregnant mare serum gonadotropin (PMSG) enzyme-linked immunosorbent assay (ELISA) kit was purchased from DRG (USA). cAMP kit was 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 obtained from Koatech (Seoul, Korea). All other reagents used were purchased from Sigma-Aldrich (USA) and Wako Pure Chemicals (Osaka, Japan).

Construction of the Tethered-eCG Mutant Transfer Vectors

To obtain tethered eCG, the cDNA encoding the full-length eCG β-subunit (20 amino acid residues of the signal sequence and 149 amino acid residues of the mature protein) was fused with the mature protein part of the α-subunit using the method of overlapping PCR mutagenesis (Min *et al.*, 2004). The point mutations were introduced by PCR-based strategies, and the sequence of the entire region of the point-mutated cDNA was verified by automated DNA sequencing.

The primers used are summarized in Table 1. The mutant eCGs were constructed using the method of overlapping PCR mutagenesis by using primers 1~10 of Table 1. The Arg^{43}-Val^{44}-Met^{45}→Ala^{43}-Ala^{44}-Ala^{45}, Gln^{54}-Pro^{55}-Val^{56}→Ala^{54}-Ala^{55}-Ala^{56}, and Gln^{94}-Ile^{95}-Lys^{96}→Ala^{94}-Ala^{95}-Ala^{96} mutations were introduced in the eCG β-

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

Primer name	Location	Primer sequence
T-eCGβα Δ1 reverse	β : 43~45	5′-CGGCAGGGCAGCTGGCATCACCCGCACCATGCT-3′
T-eCGβα Δ1 forward	β : 43~45	5′-AGCATGGTGCGGGTGATGCCAGCTGCCCTGCCG-3′
T-eCGβα Δ2 reverse	β : 54~56	5′-CTCACGGTAGGTGCACACTGGCTGGGGAATGGC-3′
T-eCGβα Δ2 forward	β : 54~56	5′-GCCATTCCCCAGCCAGTGTGCACCTACCGTGAG-3′
T-eCGβα Δ3 reverse	β : 94~96	5′-CCCGCAGTCAGTGGTCTTGATCTGGCAGGGCCC-3′
T-eCGβα Δ3 forward	β : 94~96	5′-GGGCCCTGCCAGATCAAGACCACTGACTGCGGG-3′
T-eCGβα Δ4 reverse	α : 33~35	5′-CGCTCTGGAGAAGCAGCAGCCCTTACACTGGTA-3′
T-eCGβα Δ4 forward	α : 33~35	5′-TACCAGTGTAAGGGCTGCTGCTTCTCCAGAGCG-3′
T-eCGβα Δ5 reverse	α : 42~44	5′-CTTCCTGGACCTTGCTGGAGTGGGGTACGCTCT-3′
T-eCGβα Δ5 forward	α : 42~44	5′-AGAGCGTACCCCACTCCAGCAAGGTCCAGGAAG-3′

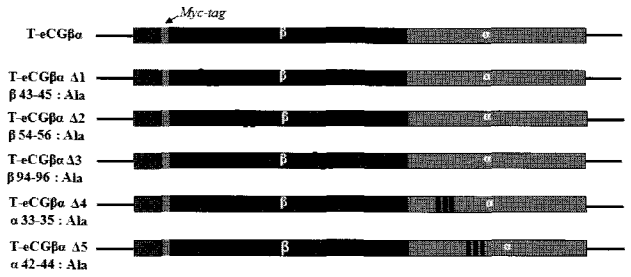

Fig. 1. Construction of tethered rec-eCGs by overlapping PCR mutagenesis. The *myc-tag* contain of 10 amino acids. S: signal region of eCGβ-subunit. *Myc-tag: myc-tag* (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu).

subunit by using the primer pairs 1~2, 3~4, and 5~6. The Lys^{33}-Gly^{34}-Cys^{35}→Ala^{33}-Ala^{34}-Ala^{35} and Pro^{42}-Thr^{43}-Pro^{44}→Ala^{42}-Ala^{43}-Ala^{44} mutation in the eCG α-subunit using the primer pairs 7~8 and 9~10 (Fig. 1). These fragments were digested by the enzymes *Eco*RI and *Sal*I and then ligated into the eukaryotic expression vector pcDNA3. All mutants were sequenced completely to confirm the Kozak site, *myc-tag,* and PCR errors.

Transient and Stable Transfection of CHO Cell Lines

Cultured CHO-K1 cells were transfected with expression vectors using the 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 assayed. Six to 8 pools of stably transfected cells were selected by incubating the cells in a 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 μg/ml) for 2~3 weeks after transfection, according to a previously reported method (Min *et al.,* 1996). The culture media were collected and centrifuged at 15,000 rpm, and incubated 4℃ for 10 min to remove cell debris. Supernatants were collected and concentrated in an Amicon Stirred cell concentrator and stored at −20℃ until assayed.

Hormone Quantitation and Western Blot Analysis of Tethered rec-eCGs

The recombinant hormones were quantified by performing PMSG ELISA using the conjugate of anti-PMSG monoclonal antibody and horseradish peroxidase and tetramethylbenzidine (TMB) as the substrate. For western blot analysis, samples of the concentrated medium were electrophoresed, under reducing conditions, on 12% sodium dodecyl sulfate (SDS)-poly-acrylamide gels following the method of proposed by Laemmli (1970). After SDS-polyacrylamide gel electrophoresis (PAGE), the protein was transferred to polyvinyli-dene difluoride (PVDF) membrane (0.2 μm) at 100 V for 2 h using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer cell. After blotting, the membrane was blocked with a 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 the unbound antibody, incubated with a secondary antibody (peroxidase-linked anti-mouse IgG 37.5 μl/15 ml of blocking solution) for 30 min, and then washed. The membrane was then incubated for 5 min with 2 ml Lumi-Light substrate solution and placed with its protein side up on Saran Wrap. The membrane was covered with a second Saran Wrap and exposed to an X-ray film for 1~10 min.

RT-PCR

RT-PCR was performed using the RT-PCR kit (RT/PCR PreMix). Total RNA (1.0 μg) extracted from the CHO-K1 cells was mixed with the reverse primer (5'-TCCCATCACTGTGACCCTGATAAA-3') and incubated at 70℃ for 5 min and then placed on ice. The forward primer (5'-TTTCCTGATGGAGAGTTTACAACG-3') was then added and diethylpyrocarbonate-treated double distilled water (DEPC-DW). cDNA synthesis reaction was performed as follows: 60 min at 42℃, 5 min at 94℃. Then, PCR cycles were performed according to the following conditions: initial denaturation for 1 min at 94℃, then 30 cycles (1 min at 94℃, 1 min 30 sec at 56℃, 1 min at 72℃) of annealing, and final extension for 8 min at 72℃.

In Vitro Bioassy for LH- and FSH-like Activities

LH- and FSH-like biological activities were estimated by measuring the amount of total cyclic AMP. Receptor cells were cultured in standard 96-well microplates, at cell concentrations of 10^5~10^6 cells/ml, and incubated overnight at 37℃. An aliquot (20 μl) of rec-eCG was 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 optical density was read at 630 nm with a microplate reader. Concentration-response curves for the rec-eCG induced increases in cAMP accumulation plotted for the level of total cAMP in cells that had been incubated with at least 5 different concentrations of eCG in the presence of a phosphodiesterase inhibitor (Min *et al.,* 1998).

Metabolic Clearance Rate of Tethered rec-eCGβα and -eCGβαΔ3

Theclerance rates of tethered eCGβα (WT) and mutant rec-eCGβαΔ3 (β: 94~96) were assessed using 8-week-old, male ICR mice and compared. The animal was injected with the sample (tethered eCGβα or rec-eCGβαΔ3, 5 IU) and blood samples were collected from the eye vein into heparinized microhematocrit tu-

bes. Blood samples were collected every 10 min, 30 min, 2 h, 4 h, 8 h and 24 h. The samples were centrifuged for 15 min at 5,000 rpm at 4℃, and the plasma concentration of tethered eCGβα and rec-eCGβαΔ3 were estimated using the PMSG ELISA kit.

RESULTS

Production of Transient and Stably Tethered rec-eCG Mutants

PCR amplification was performed using the primers designed from the sequences published previously (Park *et al.*, 2009). The *myc-tag* (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) was added between the first and second amino acids of the mature protein in the eCG β-subunit by site-directed mutagenesis. Subsequently, we amplified cDNA fragments (each with a predicted length of 828 bp) for the 5 mutants of rec-eCG (Δ1, Δ2, Δ3, Δ4, and Δ5). The tethered eCG mutant vectors were transfected into CHO-K1 cells. Transient expression was collected from the supernatant at 72 h 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 secreted by the stably transfected cells into serum-free media was isolated and quantified by ELISA (Fig. 2). The expression level of tethered eCGβα and mutant rec-eCGs are shown in Fig. 2. The Δ1, Δ4, and Δ5 mutants were not secreted, while mutant Δ2 was secreted only in a small amount. The mutant Δ3 (β: 94~96) was efficiently secreted. The secretion pattern of stably expressing cell lines was not different from that of transiently expressing cell lines, indicating that the *myc-tag* was not required for secretion. The amount of eCG expressed was 600~1,200 mIU/ml in transient expressing cells.

RT-PCR of Cell Lines Transfected with Tethered rec-eCG Mutant Vector

To analyze the mRNA expression of tethered-eCG mutants, total RNA was extracted from transfected CHO-K1 cells. RT-PCR was then performed to verify the mRNA expression. About 228-bp fragments of the PCR products was amplified. The data indicated that mRNA was expressed in all the mutant cell lines. However, proteins of the Δ1, Δ4, and Δ5 mutants were not secreted into the medium, and the mRNA of the Δ1, Δ4 and Δ5 mutants was transcript into the CHO K1 cell lines. However, rec-eCG protein was not detected in the medium.

Western Blot Analysis

The size of the tethered rec-eCG protein was identified to be about 43~45 kDa. There is no difference between WT and Δ3 in their molecular weights. As previously reported, the molecular sizes of eCG mutants were remarkably decreased to 30 kDa by the N-

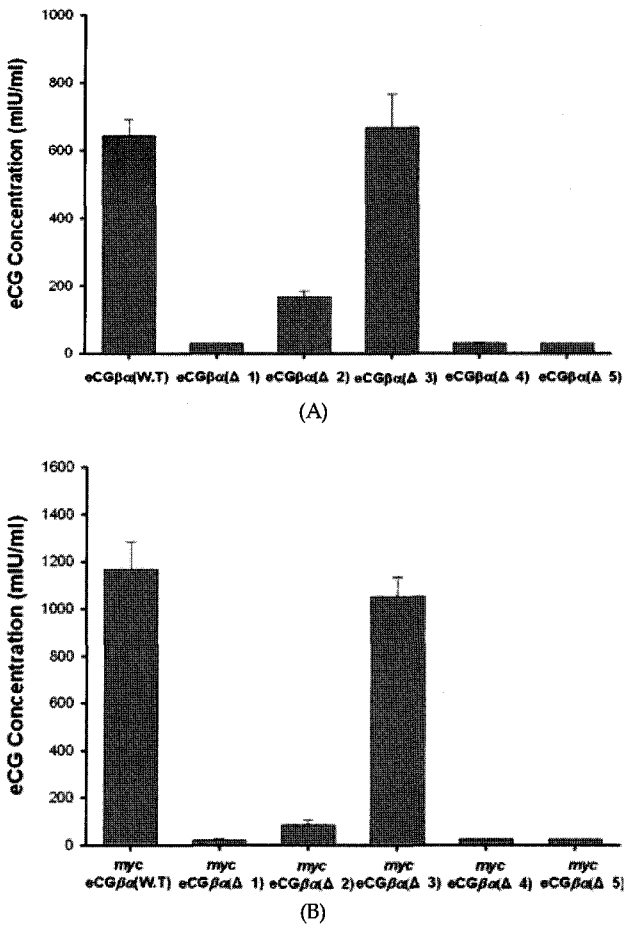

Fig. 2. Transient expression of tethered eCGs and *myc-tag* eCGs in CHO cells by ELISA. A) Tethered rec-eCGs, B) The added *myc-tag* in tethered rec-eCGs. Values are expressed as mean±SEM for at least three independent experiments.

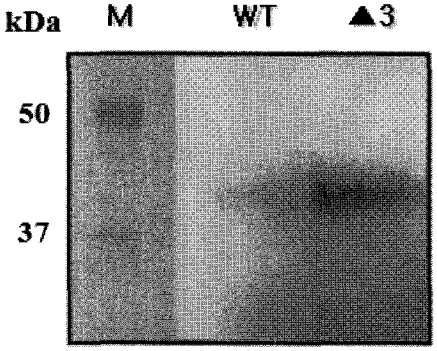

Fig. 3. Western blot analysis. WT: wild type; ▲3: β-subunit 94~96 mutant. Samples of each tethered-eCG were electrophoresed on a 12.5% SDS-polyacrylamide gel under reducing conditions. The bands of tethered-rec eCGs were detected.

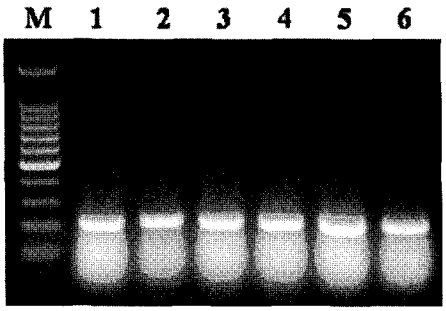

Fig. 4. RT-PCR of tethered rec-eCGs. Primer (228 bp): Forward; 5'-TTTCCTGATGGAGAGTTTACA-ACG-3' Reverse; 5'-TCCCATCA-CTGTGACCCTGATAAA-3', M: size marker, No.1: wild type, No.2: Δ1, No.3: Δ2, No.4: Δ3, No.5: Δ4, No.6: Δ5.

linked digestion. It is suggested that the oligosaccharides of about 10~15 kDa were added to the tethered rec-eCG protein. Thus, WT and Δ3 were efficiently secreted and translated into the CHO-K1 cell lines.

Biological Activity of Tethered rec-eCG in rLH/CGR and rFSHR

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 the LH- and FSH-like activities of rec-eCG mutants. 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, WT of the tethered rec-eCGs showed a similar concentration-response curve in the LHR and FSHR. For cells expressing the rat LH/CG receptor, the basal cAMP levels were 73.3 fmol/10^6 cells and maximal cAMP responses were 1,610 fmol/10^6 cells. Basal cAMP levels for the rat FSH receptor were 62.1 fmol/10^6 cells and maximal cAMP responses were 1,610 fmol/10^6 cells; for cells expressing the rat FSH receptor, these value 62.1 fmol/10^6 cells and 1,610 fmol/10^6 cells, respectively.

The concentration-response curve for the mutant Δ3 was essentially flat for the LHR and FSHR cell lines. With respect to the mutant Δ3, the basal cAMP levels were 58.0 fmol/10^6 cells and 63.2 fmol/10^6 cells and the maximal cAMP responses were 601.0 fmol/10^6 cells and 330 fmol/10^6 cells for cells expressing the for rat LH/CG receptor and those expressing the rat FSH receptor, respectively. Thus, mutant Δ3 did not show FSH-like activity, indicating that amino acids sites at the Gln^{94}-Ile^{95}-Lys^{96} position on the β-subunit of tethered-eCG were indispensable for such activity.

Clearance Rates of Tethered eCGβα and rec-eCGβαΔ3

The clearance rates of the WT and Δ3 (β: 94~96) were determined by injecting mice with the respective eCGs, and the plasma concentration was analyzed by ELISA at different time points after the injection. Fig. 6

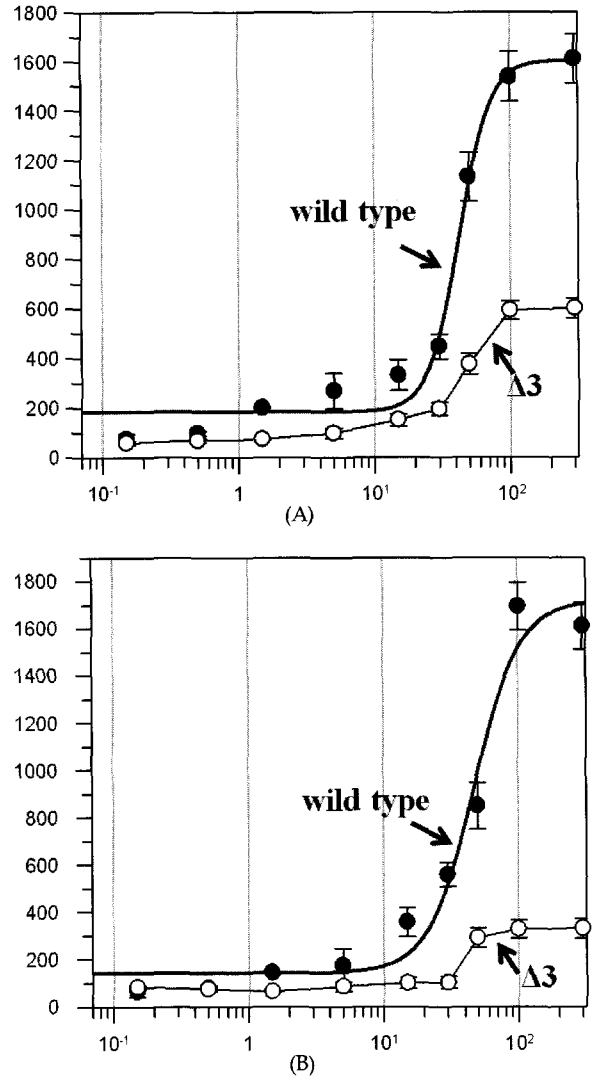

Fig. 5. Effects of increasing concentration of rec-tethered eCG on accumulation in stably transfectants of CHO cell expressing r-LHR-WT (A) and rFSHR-WT (B). Cells were incubated with the indicated concentrations of rec-eCG in medium containing 0.5 mM 3-isobutyl 1-methyl xanthine for 30 min. at 37 before 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.

showed that the presence of Δ3 was almost equally decreased in comparison to WT. eCGs was not detectable at 8 hrs after injection. The clearance of the rec-eCG was very rapid since about 50% of the hormone disappeared after 2 h. Hence, the estimated clearance of the rec-eCG was about 2~4 h.

DISCUSSION

In order to characterize the dual-activity in eCG, 5

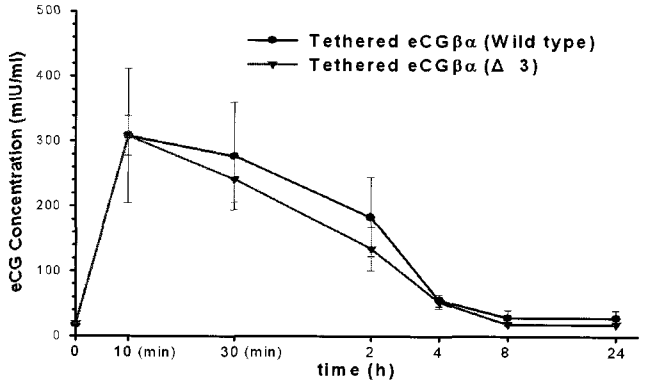

Fig. 6. Metabolic clearance rate of tethered rec-eCGβα (wild type) and mutant rec-eCGβα⊿3 (β:94~96). Rec-eCG was administrated as a 5 IU. Serum samples were analyzed for eCG content using ELISA. Each point represents mean eCG concentration (n=4). Values are expressed as mean±SEM for at least three independent experiments.

mutants were constructed by using site-directed mutagenesis method. Expression vectors for each of the mutants were transfected into CHO-K1 cell lines, which then prepared rec-eCGs. The quantity of rec-eCG was estimated by ELISA.Tethered rec-eCGs produced by the CHO-K1 cells showed LH- and FSH-like activity, which was confirmed by the results of cAMP analysis of cell lines expressing the rat LH/CG receptor and rat FSH receptor. The present study indicates that the specific FSH-activity site was found in tethered eCG. The 94~96 amino acids in the eCG β-subunit play a pivotal role in the FSH-like activity of rec-eCG.

We have studied the functional role of dual-activity of eCG. We cloned cDNAs of α- and β-subunits of eCG derived from equine placenta (Min *et al.*, 1994) and observed that rec-eCG αAsn^{56}β produced into mammalian cells didn't exhibit LH-like activity *in vitro* Leydig cells. The oligosaccharide site at Asn^{56} in the α-subunit of eCG was found to be crucial the LH-like activity of eCG (Min *et al.*, 1996). It was previously reported that tethered eCGβ α was similar to dimeric eCG α/β in terms of the level of the LH-and FSH-like activities (Min *et al*, 2004). The amino acids of mutants (Δ1, Δ2, Δ3, Δ4 and Δ5) were substituted with alanine. The *myc-tag* was then added between the first amino acid and second amino acid in the mature protein of β-subunit of eCG. The secretion of the protein for *myc-tag* (*myc*-rec-eCGβ α) was not different from that of rec-eCGβ αWT. The rec-eCGβ α Δ3 (94~96 amino acids) mutant was efficiently secreted, while the rec-eCGβ α Δ2 (54~56 amino acids) mutant was weakly secreted. However, the proteins of Δ1, Δ4, and Δ5 mutants were not secreted into the medium. It was shown that the mRNAs of the Δ1, Δ4 and Δ5 mutants were transcript. However, it is not clear why the mRNA could not be translated. A possible explanation may be the amino acid substitution may have induced changes in the structure conformation and folding of the protein.

The LH- and FSH-like activities of mutant Δ3 were analyzed by the cAMP responsiveness in cell lines expressing rat LH/CG receptor and FSH receptor. FSH-activity remained almost unchanged in all treatments. The amino acid residues 94~96 of the β-subunit of hCG had little influence on hCGR binding. These observations support that a region of residues (94~96 amino acids) primarily influences LHR binding by its effect on hormone conformation rather than by directly acting an essential high-affinity receptor (Moyle *et al.*, 1995). In the present study, the 94~96 amino acids of eCG β-subunit appear to be of utmost importance for signal transduction of FSH-like activity. In this model, the tethered eCGβ α (wild type) and mutant eCGβ α Δ3 (β: 94~96) had long half-lives, of 4~8 h. Desialylation studies have shown the long half lives of eCG can be attributed to the presence of sialic acids (Martinuk *et al.*, 1991). In contrast, sulfated oligosaccharides have been implicated in the rapid clearance of the bovine LH (Baenziger *et al.*, 1992). The pharmacokinetics of a recombinant single β α chain of equine LH/CG produced in the milk of transgenic rabbit has been described previously. β αLH/CG shows an extremely rapid clearance (~10 min), which could be explained the absence of its *in vivo* biological activity (Galet *et al.*, 2000). Thus, rec-eCG produced in CHO cell lines can be thought to be attached to the sialic acids. Up to very recently, hCG was the only gonadotropin for which a crystallographic model had been constructed (Lapthorn *et al.*, 1994, Wu *et al.*, 1994), and all other gonadotropins were thought to have the same global conformation based on the conservation of the disulfide bonds. However, a recent communication described the crystal structure of hFSH and although its overall structure closely resembles hCG, several differences in the β-subunits were revealed, including those at residues 94~104, proving again that this region is of utmost importance for gonadotropin specificity.

The present results show that amino acids (94~96) of the eCG β-subunit play some role in the FSH-activity of eCG. Little is known on how these amino acids influence the FSH-activity; i.e., whether they act directly by binding to the receptor or indirectly by influencing the conformation of the molecule via the "seatbelt". However, the site of LH- and FSH-like activities in eCG was successfully identified. The identification of the specific bioactive site of rec-eCG by site-directed mutagenesis will be useful for analyzing the structure-function relationships of gonadotropins in the horse as well as other animals. A single-chain analog can also be constructed to include additional hormone-specific bioactive generating potentially efficacious compounds that have only FSH-like activity.

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(Received: 9 March 2010 / Accepted: 23 March 2010)