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Biosynthesis of a Biological Active Single Chain Equine Chorionic Gonadotropin

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Abstract The equine chorionic gonadotropin (eCG) subunits α and β are transcribed from different genes and associate noncovalently to form the bioactive eCG heterodimer. Dimerization is rate limiting for eCG secretion, and dissociation leads to hormone inactivation. The correct conformation of the heterodimer is also important for efficient secretion, hormone-specific post-translational modifications, receptor binding and signal transduction. To determine whether α and β subunits can be synthesized as a single polypeptide chain (tethered-eCG) and also display biological activity, the tethered-eCG molecule by fusing the carboxyl terminus of the eCGβ-subunit to the amino terminus of the α-subunit was constructed and transfected into chinese hamster ovary (CHO-K1) cells.

LH- and FSH-like activities were assayed in terms of testosterone production and aromatase activity in primary cultured rat Leydig cells and granulosa cells, respectively. The tethered-eCG was efficiently secreted and showed similar LH-like activity to the dimeric eCG α/β and native eCG. FSH-like activity of the tethered-eCG was also shown similarly in comparison with the native and wild type eCG α/β. Our data for the first time suggest that the tethered-eCG can be expressed efficiently and the produced product by the CHO-K1 cells is fully LH- and FSH-like activities in rat *in vitro* bioassay system. Our results also suggest that this molecular can imply particular models of FSH-like activity not LH-like activity in the eCG. Taken together, these data indicate that the constructs of tethered molecule will be useful in the study of mutants that affect subunit association and/or secretion.

Key words: equine chorionic gonadotropin; tethered-eCG; *in vitro* LH and FSH activity

Introduction

eCG belongs to the glycoprotein hormone family, which also includes the gonadotropins luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH). These hormones exist as heterodimers composed of a common α-subunit, which is noncovalently linked to a hormone-specific β-subunit [1]. The β-subunits of eCG and eLH has identical primary structures and they originate from one gene [2]. The difference between eCG and eLH lies in the structure of their carbohydrates, which are both sialylated and sulfated in LH and sialylated in CG [3,4].

eCG have been expressed for the first time in CHO-K1 cells, and these recombinant derivatives have biological LH- and FSH-like activities comparable to the native hormone by our report [5]. We also cloned eFSH β-subunit cDNA and expressed wild-type [6]. An interesting feature of eCG β-subunit is the most heavily glycosylated carboxyl -terminal peptide (CTP) region among all known hormones. There seem to be at least six, or even as many as eleven, O-glycosylation sites on the extended CTP of the eCG β-subunit [7-9].

Tethering a variety of multisubunit complexes into single chains has been performed by several studies to increase protein stability or activity [10-12]. In the case of the glycoprotein hormones, the tethered-hCG and FSH molecules not only were efficiently secreted but also displayed an increased biological activity *in vitro* and *in vivo* [13-16]. eCG is a unique member of the gonadotropin family since it appears to be a single molecule that possesses both LH- and FSH-like activities in many species, but not in horses [12,17-18]. This dual activity of eCG, LH- and FSH-like activities *in vitro*, in heterologous species is of fundamental interest to the study of the structure-function relationships of gonadotropins and their receptors. Thus, eCG is a distinct molecule in terms of its biological function and the structure of its carbohydrates. The function analysis of tethered-eCG is expected to yield valuable information on the dual activity of eCG, LH- and FSH-like activities *in vitro*.

Here, I constructed the single chain eCG molecule and transfected into CHO-K1 cells. This approach may be used to expand the spectrum of structure function studies of glycoprotein hormone analogs. Subunits gene fusion therefore appears to be a promising strategy, not only for the generation of long lasting eCG analogs, but also particularly

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for the development of recombinant mutants with desirable characteristics. These results indicate that the tethered-eCG is secreted efficiently, and that is similar to wild type eCG in the LH-and FSH-like activities.

Materials and Methods

Materials

The expression vector pABWN was generously provided by Dr. J. I. Miyazaki (Osaka University). CHO-K1 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Ham's F-12, CHO-S-SFM II and McCoy's 5a media, Medium 199, G418, and Lipofectamine were from Gibco BRL (MD, USA). Fetal bovine serum was from Hyclone Laboratories (Utah, USA). The standard eCG (1000 IU/mg, H119/H) for radioimmunoassay (RIA) and bioassay, the anti-eCG polyclonal (A540/R1H) and anti-rabbit precipitating (A223/SH) antibodies were purchased from UCB-Bioproducts (SA, USA). The eCG (3140 IU/mg) for radioiodination was provided by Teikoku Zoki Co. (Tokyo, Japan). $Na^{125}I$ was obtained from Du Pont NEN (DE, USA). Wild type recombinant eCG and expression transfer vector of wild type eCG (pABeCG α/β, 14.4 kb) were used as described previously [5]. All the other reagents used were from Wako Pure Chemicals (Osaka, Japan).

Construction of the Tethered-eCG Transfer Vector

The schematic diagram of wild type eCG α/β and tethered-eCG was shown in Fig. 1.

To obtain tethered-eCG, the 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 β-subunit using the method of overlapping PCR mutagenesis [19]. Expression vectors for the wild type eCG (pABeCG α/β, 14.4 kb) and tethered-eCG (pAB-Tethered-eCG, 11.8 kb) were prepared as shown in Fig. 2A and B. The following primers were used in the construction of the tethered-eCG:

Primer 1, M13 forward, (5'-GTTTTCCCAGTCACGAC-3')
Primer 2, (5'-TCCATCAGGAAAAGAAGTCTTTATTGG-3')
Primer 3, (5'-ATAAAGACTTCTTTTCCTTGATGGAGAG-3')
Primer 4, M13 reverse, (5'-CAGGAAACAGCTATGAC-3')

The first PCR was carried at using primers 1, 2, 3 and 4, respectively (step 1). The fragments were annealed and subjected to the second PCR using primers 1 and 4 to generate the tethered-eCG as shown in Fig. 2B. This fragment was digested by *Eco*RI/*Pst*I and ligated into the same sites of pUC119 (pUC Tethered-eCG) and was sequenced to ensure no errors were introduced during the PCR (step 2). Following *Xho*I/*Sal*I digestion, the fragment encoding tethered-eCG was ligated into the unique *Xho*I site of the pABWN expression vector (step 3). The direction of expression transfer vector (pAB-Tethered-eCG) was confirmed by restriction mapping using *Xho*I and *Sal*I.

Cell culture and functional expression

The expression vector (pAB-Tethered-eCG) was transfected into CHO-K1 cells by the liposome formulation (Lipofectamine) transfection method according to the supplier's instruction. Stable cell transfectants were selected by incubation in growth medium [Ham's F12 media containing penicillin (50 U/ml), streptomycin (50 mg/ml), glutamine (2 mM) and 10% FCS] supplemented with G418 (800 ug/ml) for 2 weeks posttransfection according to the method reported previously [5]. After incubation of selected stable cells (1×10^6) in 20 ml CHO-S-SFM-II at 37C for 48 h, the culture media were collected and centrifuged at 100,000×

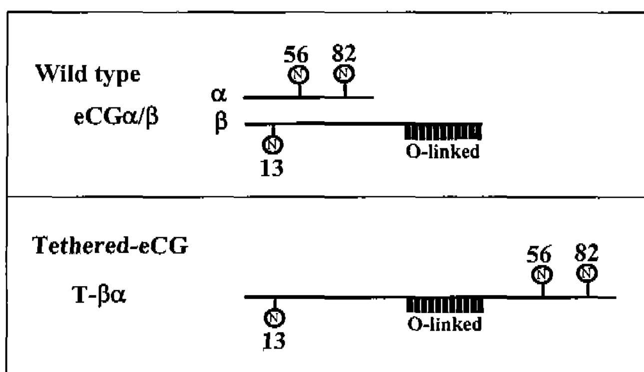

Fig. 1. Schematic diagrams of recombinant tethered-eCG. Recombinant eCG, wild type (eCG α/β), were shwon. A circled "N" denotes an N-linked oligosaccharde and "O-linked oligo saccharide denotes O-linked."

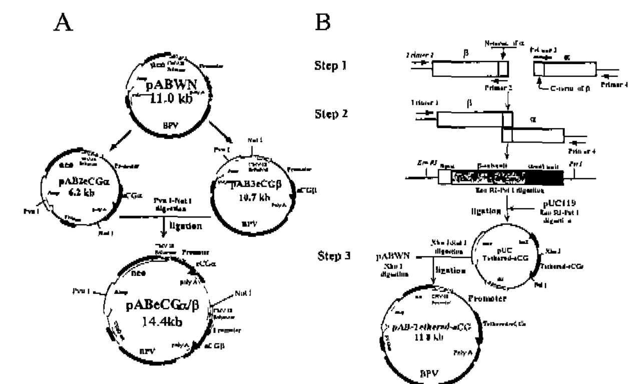

Fig. 2. Construction of tethered-eCG by overlapping PCR mutagensis and expression transfer vector. A: The construction of the wild type eCG expressin vector (pABeCG α/β: 14.4 kb) for transfection, as described previously [5]. B: The construction of the tethered-eCG expression vector. The expression vector pABWN (11kb) comprises a promoter based on the chicken b-actin promoter, containing cytomegalovirus-immediate early (CMV-IE) enhancer and a subregion of the bovine papilloma virus (BPV) (pAB-Tethered-eCG: 11.8 kb).

g, 4C for 60 min to remove the cell debris. The amount of recombinant wild type and tethered-eCG was quantified using RIA according to the supplier's protocol (UCB-Bioproducts). ^{125}I eCG trace was prepared by the Cloramin T-method [20]. The concentration, expressed as native eCG equivalents, of tethered recombinant eCG, was determined by RIA, in triplicate. The quantity of recombinant protein was estimated as equivalents of the native eCG standard as previously described [5].

Bioassay for LH- and FSH-like activities

The LH-like activities of the wild type and tethered recombinant eCGs were determined by testosterone production in the rat Leydig cell culture system as described [5]. The FSH-like activities were assayed based on the measurement of the amount of estradiol converted from 19-hydroxyandrostenedione in a culture of granulosa cells collected from immature rats as described [5]. The testosterone and estradiol concentrations of the conditioned media were measured by RIA, as described [21].

RESULTS

Cell lines of stably expressing tethered-eCG

The single chain eCG was constructed in which the carboxyl terminus (residue 149) of the β subunit was fused directly to the amino terminus of the α subunit. The tethered-eCG expression vector was transfected into CHO-K1 cells. Eight clones of stably transfected cells expressing tethered-eCG were selected for G-418 resistance. The clonal cell lines isolated with G418 were subjected to reverse transcription-PCR and Northern blotting analyses to select and establish those which stably expressed the tethered-eCG mRNA (Fig. 3). Recombinant eCGs secreted by the stably transfected cells into serum free media were collected and quantified by RIA. The concentration-response curves of the recombinant tethered-eCG were shown in Fig. 4. These competitive curves were directly proportional to each other, showing that there were no quantitative differences in the recognition of native eCG (Fig. 4A), wild type eCG and tethered-eCG (Fig. 4B) by the antibody. Based on these observations, the quantities of recombinant eCGs used in the bioassay are expressed as native eCG standard equivalent.

LH-like activity of recombinant wild type and tethered-eCGs

The effects of the recombinant eCGs on testosterone secretion in primary cultures of rat Leydig cells were determined to evaluate their LH-like activities. Leydig cells were incubated with various concentrations of native eCG, wild type or tethered-eCGs. The testosterone concentration increased in direct proportion to the concentration of native eCG (Fig. 5A). Over the range of 20-200 ng/ml, the activity of tethered-eCG was similar to that of the wild type eCG (Fig. 5B). The concentration-response curve of tethered-eCG was similar to that of the wild type eCG (Table 1). These results show that the tethered-eCG is capable of eliciting an *in vitro* LH-like biological response equivalent to that of heterodimeric eCG. These also suggest that the heterodimeric eCG is not essential for the LH-like activity of eCG.

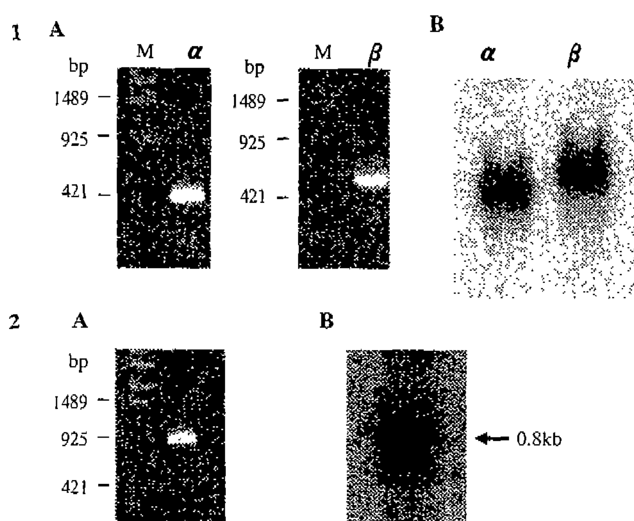

Fig. 3. RT-PCR and Northern blotting analyses of wild type eCG and tethered-eCG expressed by stable CHO-K1 cell clones. The RNA was extracted from each cell lines and PCR-amplified. The Total RNA (10 ug) were electrophoresed in 1% (W/V) agarose gel containing 0.66% M formaldehyde and transferred to a nylon membrane. 1; A: PCR; B: Northern blot (wild type eCG); 2; A: PCR; B: Northern blot (tethered-eCG).

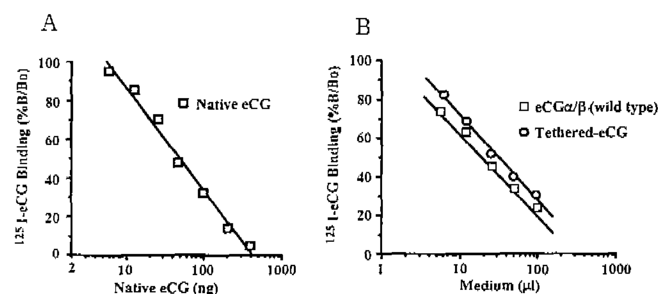

Fig. 4. Quantification of recombinant eCGs by RIA. The recombinant eCGs [eCG α/β (wild type) and Tethered-eCG] secreted by the stably expressed cells into serum free media were collected and subjected to RIA. Based on the result, the quantity of the recombinant proteins is expressed as an equivalent of the native eCG standard (1,000 IU/mg) as described in the text.

FSH-like activity of recombinant wild type and tethered-eCGs

The FSH-like activities of recombinant eCGs on aromatase activity were evaluated by measuring estradiol production by primary cultures of rat granulosa cells. The estradiol con-

Table 1. Hormone expression in CHO-K1 cells and bioactivity of recombinant tethered-eCG relative to wild type eCG

Recombinant eCGS	Hormone expression (ng/100ul)a	In vitro bioactivity (%) LH	FSH
ECG α/β (wild type)	208±29	100	100
Tethered-eCG	201±22	100	96

[a]Determined by RIA. Values are the means±S.E. of triplicate experiments. The EC_{50} values used to determine the relative potencies were determined from the concentration-response curves for the in vitro Leydig cell and granulosa cell bioassays (Fig. 5B and D).

centration increased in direct proportion to the concentration of native eCG added to the culture medium **(Fig. 5C).** Native eCG and wild type recombinant eCG showed very similar concentration-response curves. The FSH-like activity of tethered-eCG showed a little decreased concentration-response curves in comparison with eCG α/β **(Fig. 5D).** These results show that the tethered-eCG is biologically active *in.*

Discussion

The present study indicates that eCG heterodimer can be expressed as a single chain encoding both subunits: α and β. thus, the noncovalent heterodimeric structure is not critical for the function of the glycoprotein hormone family.

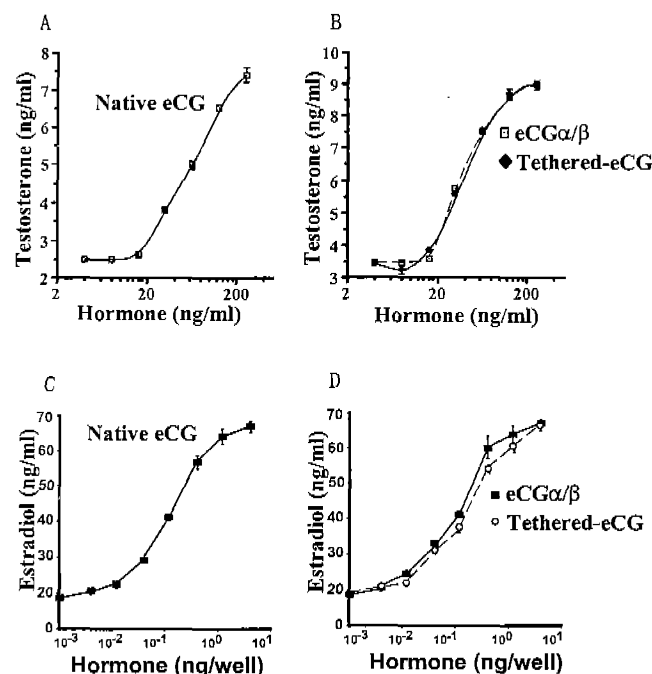

Fig. 5. Biological activity of eCG α/β (wild type) and tethered-eCG. LH-(A and B) and FSH (C and D)-like activities of recombinant eCGs were evaluated. Values are expressed as the mean±SEM for at least three separate experiments.

This data for the first time indicates that the recombinant tethered-eCG can be expressed efficiently and the produced product by the CHO-K1 cells is fully LH- and FSH-like activities in rat *in vitro* bioassay system. Recently, the tethered molecule has been proven by several experiments on the hCG and hFSH. Sugahara et al. [13-14] suggested that the tethered hCG and FSH not only was efficiently secreted but also displayed an increased biological activity in vitro and in vivo and retain a biologically active conformation similar to that seen in the heterodimer of CHO-K1 cells. Narayan et al. [16] reported a similar result that the receptor binding and signal transduction of the two single chain hCGs (single chain hCG or single chain hCG devoid of CTP) were as active as wild type heterodimeric hCG that was expressed in baculovirus-infected insect cells. It is generally accepted that O-linked oligosaccharides on the CTP of the hCG β-subunit are not important for in vitro LH-like activity [5,22-25]. Matzuk et al. [22] suggested that the O-linked oligosaccharides on the hCG b-subunit participated in prolonging the circulating half-life of LH in vivo, and resulted in increased biological activity.

Equine LH has high affinity for both rat LH and FSH receptors but in horses binds only to the LH receptor [12, 26]. In addition, eCG does not seem to bind to the horse FSH receptor [27-28], but it binds to the donkey FSH receptor [28-29], so that the molecular mechanism of eCG function is obscure. Binding specificity was flexible and varied independently of signal transduction activity during vertebrate evolution [12]. As shown in the present study, eCG has been used as a potent agent for induction of folliculogenesis in domestic and laboratory animals [21,26]. These observations demonstrate that the specificity can be manipulated independently in a rational model to create analogues with novel ligand binding properties.

Finally, these new findings are the result of the producing combination of the information gained from both genetic and biochemical approaches. Tethered-eCG can be permit development of potent new analogues that stimulate ovarian development in rodents. The constructs of tethered molecule will also be useful in the study of mutants that affect subunit association and/or secretion. A single-chain analog can also be constructed to include additional hormone-specific bioactive generating potentially efficacious compounds that have only FSH-like activity. Recombinant eCGs including the mutants, which lack oligosaccharides, will be useful tools for analyzing the structure-function relationships of gonadotropins in the horse as well as other species.

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