Biological Functions of the COOH-Terminal Amino Acids of the α-Subunit of Tethered Equine Chorionic Gonadotropin

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ABSTRACT

Glycoprotein hormones have a common α-subunit that is involved in the signaling pathway together with G protein, adenylcyclase and cAMP induction; however, it is an unclear how this common structure is related to hormonal action. To determine the biological functions of the COOH-terminal amino acids in the α-subunit of these glycoprotein hormones, a tethered-molecule was constructed by fusing the NH_2-terminus of the α-subunit to the COOH-terminus of the β-subunit of equine chorionic gonadotropin (eCG). The following deletion mutants were created by PCR; Ile was inserted at position 96 to form Δ96, Lys was substituted at position 95 to form Δ95, His was inserted at position 93 to form Δ93 and Tyr was substituted at position 87 to form Δ87. Each mutant was transfected into CHO-K1 cells. Tethered-wt eCG, and Δ96, Δ95, and Δ93 mutants were efficiently secreted into the medium but the Δ87 mutant was not secreted. Interestingly, the RT-PCR, real-time PCR, and northern blot analyses confirmed that the RNA was transcribed in the Δ87 mutant. However, the Δ87 mutant protein was not detected in the medium or the intracellular fraction of the cell lysates. The LH- and FSH-like activities of the recombinant proteins were assayed in terms of cAMP production using rat LH/CG and rat FSH receptors. The metabolic clearance rate (MCR) was determined by injecting rec-eCG (2 IU) into the tail vein. The Δ95 and Δ93 mutants were completely inactive in both the LH- and FSH-like activity assays. The Δ96 mutant showed slight activity in the LH-like activity assay. In comparison to the wild type, the activity of the Δ96 mutant in the FSH-like activity assay was the highest among all the mutants. The MCR assay in which rec-eCG was injected showed a peak at 10 min in all the treatment groups, which disappeared 4 h after injection. These results imply a direct interaction between the receptor and the COOH-terminal region of the α-subunit. The data also reveal a significant difference in the mechanism by which the eCG hormone interacts with the rLH and rFSH receptors. The COOH-terminal region of the α-subunit is very important for the secretion and functioning of this hormone.

(Key words : eCG, Activity, COOH-terminal amino acids of the α-subunit)

INTRODUCTION

The glycoprotein hormone family consists of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH), which are secreted by the pituitary gland in all mammalian species, and chorionic gonadotropin (CG), which is secreted by placental trophoblast cells in primates and equids. These hormones consist of non-covalently associated α and β subunits. Within a species, the amino acid sequence of the α-subunit is identical across all glycoprotein hormones and is encoded by a single gene. The αβ dimer is the active form of the hormone, and biological specificity is conferred by the β subunit (Pierce and Parson, 1981, Combarnous *et al.*, 1981). Placental CG has been well documented in primates, equids, rodents, sheep, rabbits, and guinea pigs. Equine chorionic gonadotropin (eCG) is a placental glycoprotein hormone that is involved in the maintenance of pregnancy (Min *et al.*, 2004). eCG shows both LH- and FSH-like activities in many species but not in the horse (Aggarwal and Papkoff, 1981; Moyle *et al.*, 1990).

Previously, we prepared recombinant WT- and tethered-eCGs in CHO-K1 cells, and found that these recombinant derivatives had biological LH- and FSH-like activities comparable to those of the native hormone (Min *et al.*, 1996, 2004; Park *et al.*, 2009; Saneyoshi *et al.*, 2001). When the COOH-terminal region (Tyr-Tyr-His-Lys-Ser) of the α subunit was removed by treatment with carboxypeptidases, the recombinant mutants had only 0.4% activity relative to native lutropin in the receptor assay. Furthermore, no significant conformational changes were detected in circular dichroism measurements. These results suggested that the COOH-terminal region of the α subunit of the hormone is im-

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portant (Parsons and Pierce, 1979). Yoo *et al.* (1993) reported that the among the truncated mutants (△92, △91, and △90), the △92 mutation had no effect on receptor-binding or cAMP induction by FSH and hCG. However, the △91 and △90 mutations abolished the ability of both hormones to induce cAMP synthesis. These authors also demonstrated that αLys^{91} is important for receptor modulation in the stimulation of c-AMP synthesis (Yoo *et al.*, 1991). A peptide containing 10 amino acids of the COOH-terminal of the α subunit, denoted peptide $\alpha^{83\sim92}$, was synthesized by Multiple Peptide Systems. Peptide $\alpha^{83\sim92}$ was functional not only in receptor binding but also in cAMP induction by the LH/CG receptor. However, peptide $\alpha^{83\sim92}$ did not bind to the FSH receptor or stimulate cAMP synthesis by the FSH receptor. These results indicated significant differences in the interactions of hCG with the LH/CG receptor and those of FSH with the FSH receptor (Yoo *et al.*, 1993).

The aim of this study was to determine the biological functions of the COOH-terminal residues of the α-subunit of eCG. Therefore, we constructed the deletion mutants Δ96, Δ95, Δ93, and Δ87 and analyzed their secretion and, LH- and FSH-like activities on the basis of the cAMP assay, and metabolic clearance rate.

MATERIALS AND METHODS

Materials

The cloning vector pCR2.1 and the expression vector pcDNA3 were purchased from Invitrogen (Groningen, Netherlands). Polymerase chain reaction (PCR) reagents were purchased from TaKaRa (Japan). The QIAprep-spin DNA purification kit and plasmid kit were purchased from QIAGEN Inc. (Hilden, Germany). Endonuclease was purchased from TaTaTa and Toyobo (Seoul, Korea). Ham's F-12, DMEM, fetal bovine serum (FBS), lipofectamine, Opti-MEM, CHO-S-SFMII, Geneticin, Gentamycin and HEPES were from Gibco BRL (MD, USA). CHO-K1 cells were obtained from the Japanese Cancer Research Resources Bank (JCRB; Tokyo, Japan). Centriplus-concentration tubes were purchased from Amicon Inc (MA, USA). The DRG PMSG ELISA kit was purchased from DRG (USA). The cAMP assay was purchased from cAMP Biotrak EIA system (Amersham, USA). Mice were purchased from Korea Animal Tech (Koatech, Korea). Oligonucleotides were synthesized by Green Gene Bio (Seoul, Korea). All other reagents used were 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-

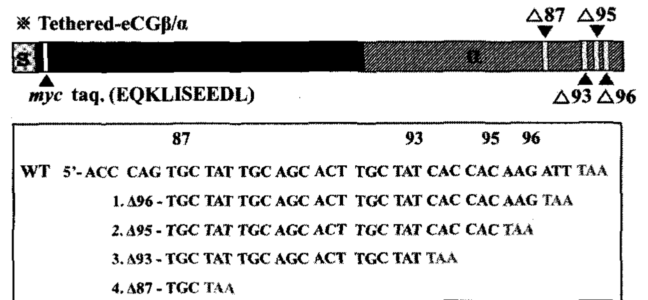


Fig. 1. Preparation of the COOH-terminal deletion mutants of α-subunit. The *myc-tag* contain of 10 amino acids. S: signal region of eCG β-subunit. *Myc-tag*: *myc-tag* (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu).

length eCG β-subunit (signal sequence of 20 amino acid residues and the mature protein of 149 amino acid residues) was fused with the mature protein of the α-subunit by overlapping PCR mutagenesis (Min *et al.*, 2004). Point mutations were introduced by PCR, and the sequence of the entire region of mutated cDNA was verified by automated DNA sequencing. The deletion mutants were created by PCR as follows: the Ile was substituted at position 96 to form 96, Lys was inserted at position 95 to form 95, His was substituted at position 93 to form 93 and Tyr was inserted at position 87 to form 87. Subsequently, each DNA fragment was inserted into the pCR2.1 cloning vector. These fragments were digested with the *EcoRI* and *SalI* enzymes and ligated into the eukaryotic expression vector pcDNA3 that had been digested with *EcoR* I /*XhoI*. All mutants were completely sequenced to confirm the presence of the Kozak site and, *myc-tag* and to rule out the possibility of any PCR errors (Fig. 1).

Transient and Stable Transfection of the CHO Cell Lines

Cultured CHO-K1 cells were transfected with the expression vectors using the liposome transfection method described previously (Min *et al.*, 2004). The transfected cells were cultured for 48 h in serum-free medium (CHO-S-SFM-II) that was harvested and centrifuged at 15,000 rpm for 10 min. The supernatants were then collected and stored at −20℃ until further analysis. Six to eight pools of stably transfected cells were selected by incubating 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 posttranfection according to a previously described method (Min *et al.*, 1996). The culture media were collected and centrifuged at 15,000 rpm and 4℃ for 10 min to remove the cell debris. The supernatants were collected and concentrated in an Amicon stirred cell concentrator and stored at −20℃ until further analysis.

Hormone Quantitation and Western Blot Analysis of Tethered rec-eCGs

The recombinant hormones were quantified by PMSG ELISA using the anti-PMSG monoclonal antibody and enzyme conjugated with coupled to horseradish peroxidase, and TMB as the substrate. For western blot analysis, samples of the concentrated medium were subjected to reducing 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970). After SDS-PAGE, the proteins were transferred onto a polyvinyli-dene difluoride (PVDF) membrane (0.2 um) at 100 V for 2 h using a Bio-Rad Mini Trans-Blot electrophoresis cell. Subsequently, the membrane was incubated for 5 min with 2 ml Lumi-Light substrate solution and placed on a sheet of Saran Wrap with the protein side of the membrane facing upward. The membrane was covered with a second sheet of Saran Wrap and exposed on an X-ray film for 1~10 min.

RT-PCR, Real-time PCR and Northern Blotting Analysis

RT-PCR was performed with 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′-TCC-CATCACTGTGACCCTGATAAA-3′), incubated at 70℃ for 5 min and then placed on ice. The forward primer (5′-TTTCCTGATGGAGAGTTTACAACG-3′) was then added, and the reaction volume was made up with DEPC-DW. The cDNA synthesis reaction was performed using the following protocol: 42℃ for 60 min and, 94℃ for 5 min. PCR was carried out according to the following PCR protocol: 94℃ for 1 min followed by 30 cycles (94℃ for 1 min, 56℃ for 1.5 min, and 72℃ for 1 min). this was followed by a final extension at 72℃ for 8 min. Real-time PCR was carried out using the One-Step SYBR RT-PCR kit. The extracted RNA from the selected CHO cells was used as the template. The Real-time PCR amplification conditions were as follows: a total of 5 μg of RNA, 1× of 2× One-Step SYBR RT-PCR buffer, 5 pmol of each primer, 2.5 U of Takara Ex Taq HS (Takara, Japan), 50 U of M-MLV RTase (RNase H free), and 20 U of RNase inhibitor were added to the PCR tubes, and the reaction volume (25 μl) was made up with RNase free dH_2O. RNA electrophoresis was performed on an agarose gel containing 10× MOPS and 37% formaldehyde, and the total RNA concentration prepared to 10 μg/μl. After electrophoresis, the RNA was transferred onto a membrane with 20× SSC for at least 5 h or overnight. The probe was prepared by purifying the sample after PCR amplification. Probe labeling was performed with the DIG DNA labeling kit. EtOH precipitation was carried out on the following day and the membrane was hybridized. The probe concentration was 40 ng/ml and incubation was carried out for at least 6 h or overnight at 68℃ with gentle rocking. The bands were visualized the next day using an antibody conjugate and X-ray film for exposure.

Collection of Intracellular Proteins by Cell Lysis

The selected wild type and △87 cell lines were thawed and divided into two groups each. The CHO-S-SFMⅡ medium was added to one of the two groups, and cell growing medium was added to the other. After 72 h, the medium was collected. For the cells cultured in the cell growing medium, the supernatant was removed, and the cells were collected. The cellular protein was extracted with the PRO-PREP protein extraction solution. The amount of secreted protein and intracellular protein in the cell lysates were estimated by ELISA.

***In Vitro* Bioassy for LH- and FSH-like Activities**

The LH- and FSH-like biological activities were determined by measuring total cyclic AMP production. Receptor cells were cultured in standard 96-well microplates with cell concentrations of 10^5~10^6 cells/ml and incubated overnight at 37℃. An aliquot (20 μl) of rec-eCG was added, and the reaction was incubated for 30 min. The lysis reagent (20 μl) was then added. Finally, a cyclic AMP kit was used according to the manufactures's protocol, and the optical density at 630 nm was recorded in a microplate reader. The concentration-response curves for the rec-eCG induced increases in c-AMP accumulation were constructed by measuring the total cAMP level in cells that had been incubated with at least five different concentrations of eCG in the presence of a phosphodiesterase inhibitor (Min *et al.*, 1998).

Metabolic Clearance Rate of Tethered rec-eCGβα and rec-eCGβαΔ3

The clearance of tethered eCGβα (wild type) and mutant rec-eCGβαs (Δ96, Δ95 and Δ93) was estimated in male ICR mice that were 7 weeks old. Each animal was injected with an infusion of the sample (tethered eCGβα or rec-eCGβαs, 5 IU). Blood samples were obtained from the eye vein and collected in heparinized microhematocrit tubes. Blood samples were collected every 10 min, 30 min, 2 h, and 4 h. The samples were centrifuged for 15 min at 5,000 rpm at 4℃, and the concentrations of tethered eCGβα and rec-eCGβα mutants in the plasma were estimated using the PMSG ELISA kit.

RESULTS

Production of Transient and Stably Tethered rec-eCG Mutants

PCR was carried out using primers that were designed on the basis of previously published sequence (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 β-subunit by site-directed mutagenesis. cDNA fragments of size 828 bp were predicted to be rec-eCGβα and mutant cDNAs, and these were amplified. Transient expression was collected from the supernatants 72 h after transfection. To establish stable cell lines, 6~8 stably transfected pools of cells expressing tethered rec-eCG were selected with G418. The rec-eCG protein secreted by the stably transfected cells into the serum free medium wase collected and quantified by ELISA (Fig. 2). The expression level of tethered eCGβα and rec-eCG mutants are shown in Fig. 2. The expression level of the △96 mutant was similar to that of the wild-type. In contrast, the expression level of the △95 and △93 mutants were approximately 3~4 times that of the wild-type. The △87 mutant protein was not secreted into the supernatant of the medium. After transfection, a stable cell line was established by G418 selection for a period of at least 2~3 weeks. The selected cell line was cultured and partly frozen in the LN_2 tank. Each cell line was selected six lines and samples from the serum free medium of each sample were subjected to ELISA. Some of the colonies showed very high expression (1,500 mIU/ml). However, no secretory protein was detected in the case of the △87 mutant.

RT-PC, Real-time PCR and Northern Blotting Analyses

To analyze mRNA expression in the tethered-eCG mutants, total RNA was extracted from transfected CHO-K1 cells. RT-PCR was used to verify the mRNA expression. The △96, △95, and △93 cell lines that showed the highest expression were subjected to RT-PCR, real-time PCR, and northern blotting. All samples were probed for mRNA expression (Fig. 3). An approximately 228 bp fragment was amplified. The data indicated that mRNA was expressed in all mutant cell lines, including the △87 mutant cells.

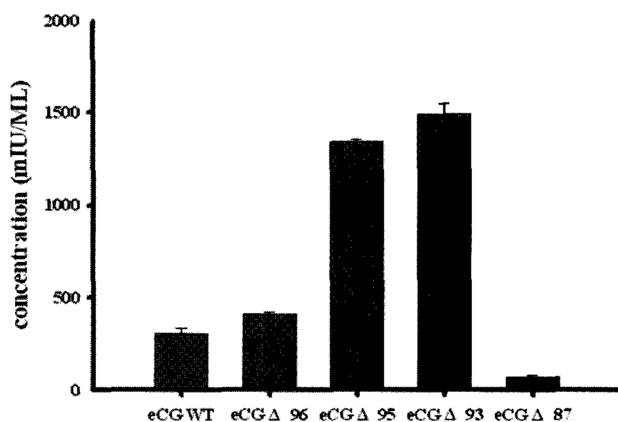


Fig. 2. Quantification of transient expression rec-eCG hormones by ELISA after transfection. After transfection at 72 hr, the medium was collected and centrifuged. And then transient expression quentity of tethered eCGs was analyzed by ELISA. Values are expressed as mean±SEM for at least three independent experiments.

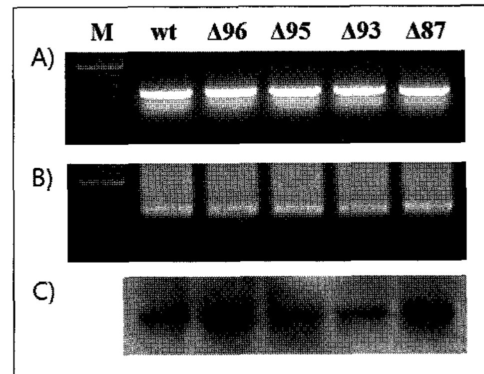


Fig. 3. RT-PCR, real-time PCR and Northern blotting. Each mutant transfected into CHO cells was washed two time with cold PBS and collected the cells. RNA was extracted and RT-PCR, real-time PCR and northern blot were determined to check mRNA expression. Each total RNA concentration was loaded 10 μg and then expo- ser time was 1 min. A) RT-PCR, B) Real-time PCR, C) Northern blot.

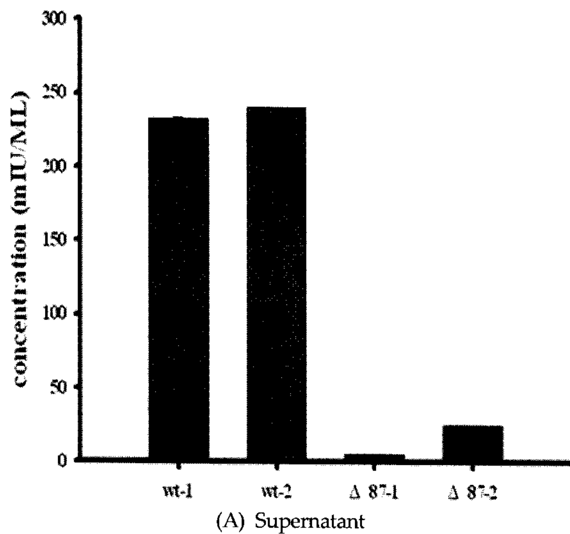
Collection and Western Blotting Analysis of the Intracellular Protein Fraction obtained after Cell Lysis

The mRNA of WT and mutants was transcribed in the CHO K1 cell lines. However, the rec-eCG protein was not detected in the medium containing the △87 cells (Fig. 4A). No recombinant-protein was detected in the intracellular protein fraction (Fig. 4B). The tethered rec-eCG hád an approximate size of 43~45 kDa. There was no difference between WT and mutants in terms of the molecular weight of the recombinant protein.

Biological Activity of Tethered rec-eCG

The effects of tethered rec-eCG on cAMP secretion in CHO cell lines expressing the rat LH/CG receptor and FSH receptor genes were determined to evaluate the LH- and FSH-like activities of the recombinant protein. Receptor cells were incubated with different concentrations of tethered rec-eCG. The cAMP concentration increased with an increase in the concentration of tethered rec-eCG (Fig. 6). WT showed a similar concentration-response curve in the LHR and FSHR assays over a concentration range of 10~100 ng/ml.

The basal cAMP levels were the same for the rat LH/CG receptor, and the maximal cAMP response was 1,650 fmol/10^6 cells, as reported earlier in our laboratory (Park *et al.*, 2009). The basal cAMP level for the rat FSH receptor was 100 fmol/10^6 cells, and the ma-



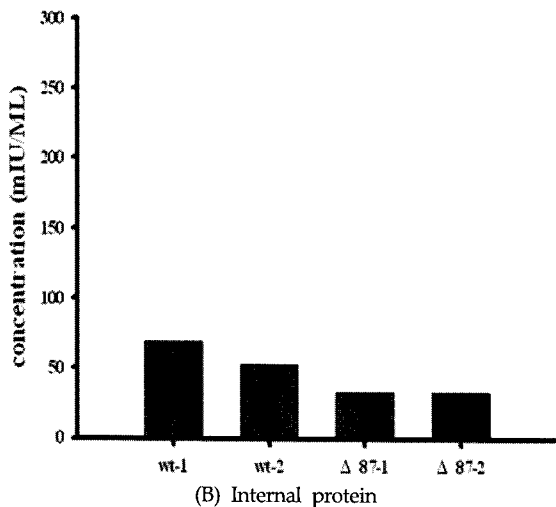


Fig. 4. Analysis of the supernatant and intracellular protein in the WT and △87. After transfection at 72 hr, the medium was collected and washed two time with cold PBS. And then cells were collected and centrifuged. After then supernatant was discard, the internal proteins by lysis buffer were collected and then quantity was analyzed by ELISA.

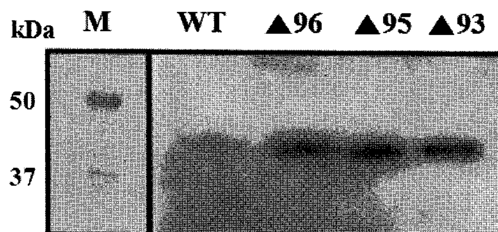
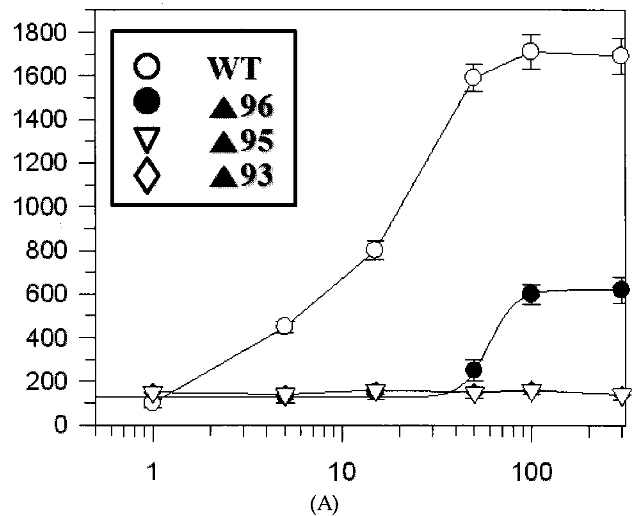


Fig. 5. Western blot analysis. Samples of each tethered-eCG were electrophoresed on a 12.5% SDS-polyacrylamide gel under reducing conditions. First antibody was used Anti-myc antibody (2 ul/10 ml). The second antibody used anti-mouse IgG-POD (37.5 ul/15 ml 1% blocking reagent). The bands of tethered-rec eCGs were detected. WT: wild type; ▲96: deleted No. of 96 aa; ▲95: deleted No. of 95 aa; ▲93: deleted No. of 93 aa in α-subunit of eCG.



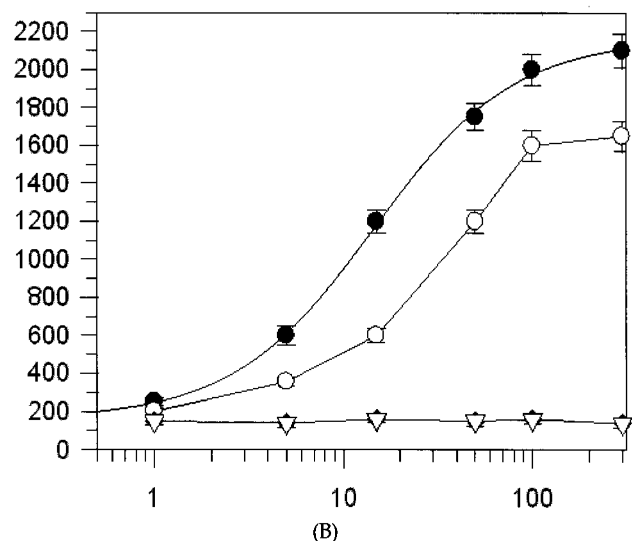


Fig. 6. Effects of increasing concentration of rec-tethered eCG on accumulation in stably transfectants of CHO cell expressing r-LHR-WT (A) and rFSHR-WT (B). Cells were incubated with the indicated concentrations of rec-eCG in medium containing 0.5 mM 3-isobutyl 1-methyl xanthine for 30 min at 37 before 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.

ximal cAMP response was 1,690 fmol/10^6 cells. With the respect to the LH-like activity, the △95 and △93 mutants were completely inactive, while the △96 mutant showed slight activity at a concentration of 50 ng/ml (Fig. 6A). With respect to the FSH-like activity, the △95 and △93 mutant showed essentially no activity, but the △96 mutant was highly active in comparison to wild-type eCG (Fig. 6B). Thus, the Δ93 and Δ95 mutants did not show any LH-like and FSH-like activities, indicating that the amino acid sites at position 93 and 95 on the α-subunit of tethered-eCG pl-

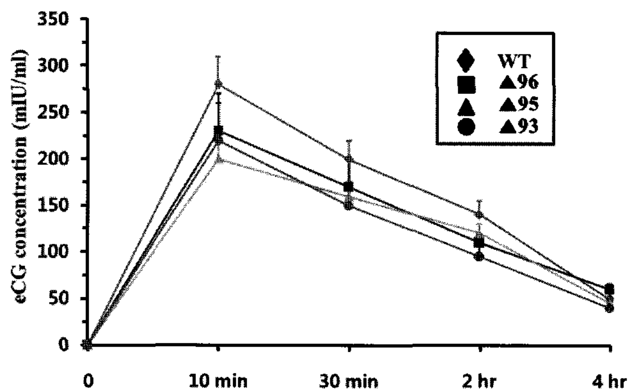


Fig. 7. Metabolic clearance rate of tethered eCG β/α and deleted mutants. Serum samples were analyzed for eCG content using ELISA. Values are expressed as mean±SEM for at least three independent experiments.

ay a pivotal role in the activity of cells expressing the rLH and rFSH receptors. These data also indicated that the Δ96 mutant of rec-eCG differed in terms of its interaction with the rLH and rFSH receptors.

Clearance Rates of Tethered eCGβα and rec-eCGβα Mutants

The clearance rates of WT and mutant proteins were determined by injecting these into mice and determining the plasma concentration at different times after injection using ELISA. The disappearance of WT eCG and the three mutant samples is shown in Fig. 7. The pattern in which the concentration of the mutant samples decreased was similar to that of WT. A peak was observed at 10 min in all the treatment groups, but it disappeared at 4 h after the injection. The clearance of rec-eCG was very fast since approximately 50% of the hormone disappeared after 2 h.

DISCUSSION

eCG is a glycoprotein hormone that is a unique member of the gonadotropin family. It has both LH and FSH-like activities in many animals. All gonadotropins have a common α subunit. The function and significance of this common α subunit in gonadotropins is unclear in the non-covalently associated complex of the α and β subunits.

We have been studying the functions of the COOH-terminal region of the α subunit of tethered-eCGβ/α and used PCR to construct four mutants. The tethered rec-eCGs produced in the CHO-K1 cells showed LH and FSH-like activities as determined by cAMP analysis in cell lines expressing the rat LH/CG receptor and rat FSH receptor. The △96, △95 and △93 mutants were efficiently secreted but the △87 mutant was not secreted. RT-PCR, real-time PCR, and northern blotting analyses confirmed that the mRNA of the △87 mutant was transcribed. However, it was why the mRNA could not be translated. One possibility is that amino acid substitution leads to changes in the conformation and folding of the protein. A cAMP analysis result of the COOH-terminal deletion mutant was different in the receptor. These data indicate that rec-eCG differs in its interaction with the LH and FSH receptors and also show for the first time that rec-eCG mutants with a deleted COOH-terminal region have a specific receptor action.

Enzymatic truncation of two COOH-terminal residues of the α subunit, i.e., Lys91 and Ser92, was shown to significantly reduce the bioactivity of lutropin, thyrotropin, and hCG (Yoo *et al.*, 1991). Proteolytic removal of five COOH-terminal residues of the α subunit, i.e., Tyr88, Tyr89, His90, Lys91, and Ser92, resulted in the partial or complete loss of the receptor binding ability and bioactivity of hCG (Yoo *et al.*, 1993). In hCG, mutagenic deletion of five, but not four, COOH-terminal residues of the α subunit resulted in the complete loss of receptor binding ability and bioactivity (Chen *et al.*, 1992). The His90 and Lys91 residues play an essential role in cAMP induction of both hormones (hCG and FSH). In contrast, although these residues are necessary for FSH binding to the FSH receptor, they are not required for hCG binding to the LH/CG receptor. The hCG COOH-terminal region is in direct contact with the LH/CG receptor, and this low affinity contact is necessary and sufficient to activate the receptor for signal generation (Yoo *et al.*, 1993). Our results are the first to show that the COOH-terminal region in α87 plays a key role in the secretion of glycoprotein hormone. It is suggested that the COOH-terminal region of the α-subunit of human glycoprotein is very important for hCG and FSH secretion (Min and Yoon, 2002). These results indicate that the COOH-terminal region differs in terms of activity toward the LH/CG receptor and FSH receptor. This approach offers a universal strategy for enhancing the stability and bioactivity as well as controlling the specificity of noncovalently linked oligomers, and it may also be used to engineer molecules with novel activities or specificities. Although, these COOH-terminal residues are important for dimeric eCG, it is unclear whether these are as important for the secretion and bioactivity of tethered-molecules.

Peptide $\alpha^{83\sim92}$ is capable of not only binding to the receptor but also including cAMP in the case of the LH/CG receptor. Moreover, the COOH-terminal region of the α-subunit has to adopt a specific structure in order to be recognized by the receptor, and this structure in not in the case of peptide $\alpha^{83\sim92}$ (Yoo *et al.*, 1993). The failure of the FSH receptor to interact with peptide $\alpha^{83\sim92}$ also indicates a specific requirement for not on-

ly the five COOH-terminal residues but also the surrounding contiguous structure. The difference in the receptor binding characteristics of FSH and hCG may be attributed to the distinct conformations of the COOH-terminal region in FSH and hCG (Yoo *et al.*, 1993). The results presented here show that the COOH-terminal residues of the *a* subunit of eCG are involved in the LH- and FSH-activities of eCG. It is unclear whether these amino acids directly influence the receptor activity by binding to the receptor or whether they indirectly influence the conformation of the molecule. Alternatively, the differences may be due to the distinct structures and mechanisms of the two receptors that recognize the COOH-terminal region of the α subunit of the respective hormones. A single-chain analog should also be constructed to produce additional hormone-specific bioactivity generating compounds that are potentially efficacious and have only FSH-like activity.

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