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COOH-Terminal Animo Acids of Tethered-Human Glycoprotein Hormone α-Subunit Play an Important Role for Secretion

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ABSTRACT

Human chorionic gonadotropin (hCG) is a member of the glycoprotein hormone family which includes FSH, hCG, TSH. These hormone family is characterized by a heterodimeric structure composed a common α-subunit noncovalently linked to a hormone specific β-subunit. To determine α and β -subunits can be synthesized as a single polypeptide chain (tethered-hCG) and also display biological activity, the tethered-hCG and -FSH molecule by fusing the carboxyl terminus of the hCG β-subunit to the amino terminus of the α-subunit was constructed. To determine the importance of α COOH -terminal amino acid, we also deleted the α COOH-terminal amino acids. The expressing vectors were transfected into CHO-K1 cells. The tethered-wthCG and -wtFSH was efficiently secreted. The $\alpha\Delta$ 83hCG and $\alpha\Delta$83FSH mutants had no secretion. These results are the first conclusive evidence that COOH-terminal amino acids are very important for secretion in human glycoprotein hormone α-subunit. These results demonstrated that the $\alpha\Delta$83hCG and $\alpha\Delta$83FSH mutants could be play a pivotal role in the secretion of tethered-molecule.

(Key words : Tethered-hCG/FSH, C-terminal deletion mutant, Recombinant, CHO cells)

Ⅰ. INTRODUCTION

The glycoprotein hormones lutropin (LH), follitropin (FSH), thyrotropin and choriogonadotropin (CG) are heterodimers, which consists of a common α subunit and a unique β subunit that confer the receptor specificity of the ligand. The subunits combine non-covalently early in the secretory pathway, and formation of the heterodimer is crucial for binding to the gonadal and thyroid receptors(Min et al., 1996; Ben-Menahem et al., 2001). Site-directed mutagenesis has become an important tool for studying the structure and function of glycoprotein hormones. However, mutations in either α- or β-subunits can alter the folding and ultimately inhibit subunit assembly and secretion of the hormones (Matzuk & Boime, 1988; Min et al., 1997). To overcome these limitations, the genes encoding the common α-subunit and either the hCGβ, FSHβ, eCGβ, bovine LHβ and bovine FSHβ subunits were genetically secreted and were biologically active. The resulting polypeptide chains were efficiently secreted and were biologically active (Sugahara et al., 1995, 1996; Min., 2000, 2001; Min et al., 2003).

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Because gonadotropins share the α-subunit and the same signal pathway involving G protein, adenylylcyclase, and camp induction, it is an enigma how common structure is related to hormonal action. The human α-subunit is comprised of 92 amino acids (Fiddes & Goodman, 1979). Enzymatic truncation of the two a COOH-terminal residues, Lys91-Ser92, significantly reduced the bioactivity of lutropin, thyrotropin, and hCG (Yoo et al., 1991). Proteolytic removal of the five a COOH-terminal residues, Tyr88-Tyr89-His90-Lys91-Ser92, resulted in the partial or complete loss of receptor binding and bioactivity of hCG (Yoo et al., 1993). Mutagenic deletion of the hCG a COOH-ternimal 5 amino acids, but not the 4 amino acids, resulted in the complete loss of receptor binding and bioactivity (Chen et al., 1992). A αHis90-Lys91 play an essential role in cAMP induction of both hormones (hCG and FSH). In contrast to this common role, they are necessary for FSH binding to the FSH receptor but not for hCG binding to the LH/CG receptor. The hCG α COOH-terminal region makes 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).

Therefore, these COOH-terminal residues are important for dimer hCG and FSH, but it is unknown whether they are important for secretion and bioactivity in the tethered-molecules. In an attempt to determine the role of C-terminal mutants, we constructed C-terminal deletion mutants of tethered -hCG and -FSH from amino acids 92 to 83. Our results demonstrated that the $\alpha\Delta$83hCG and $\alpha\Delta$83 FSH mutants had no secretion into medium.

Ⅱ. MATERIANLS AND METHODS

1. Materials

The expression vector pcDNA3 was purchased from Invitrogen (Groningen, Netherlands). CHO-K1 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Endonucleases were purchased from Takara Shuzo (Kyoto, Japan). Polymerase reagents were from Stratagene (La Jolla, CA). Ham's F-12, lipofectamine, DMEM, Wa/BSA, newborn calf serum (FBS) and Hepes were from Gibco BRL (MD, USA). The QIAprep -spin plasmid kit was from QIAGEN Inc. (Hilden, Germany). All the other reagents used were from Wako Pure Chemicals (Osaka, Japan) unless otherwise noted.

2. Mutagenesis

The tethered-hCG and hFSH molecules were constructed by previously method(Min et al., 1996). Expression vectors for the wild type hCG and FSH were constructed according to the method previously described (Min, 2001, 2002) (Fig. 1). The following primers were used in the construction of the tethered-hCG and -FSH.

Primer-1, (M13 RV),
5'-CAGGAAACAGCTATGAC-3':
Primer-4, (M13 M4),
5'-GTTTTCCCAGTCACGAC-3':

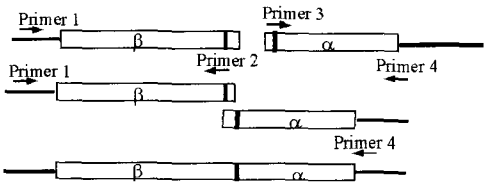

Fig. 1. Construction of tethered-hCG and -FSH by overlapping PCR mutagenesis. PCR was carried out the tethered-genes as described in Materials and Methods.

Primer 2 contains the first four codons of the mature(-subunit and the five codons of the hCG and FSH β-subunits carboxyl-terminal codons. Primer 3 also contains the sequence corresponding to the last

four carboxyl-terminal codons of the hCG and FSH β-subunits and the first five codons of the α-subunit form which signal sequence was removed.

We also constructed C-terminal deletion mutants ($\alpha\Delta 91$, $\alpha\Delta 89$, $\alpha\Delta 88$, $\alpha\Delta 87$, $\alpha\Delta 86$, $\alpha\Delta 84$, $\alpha\Delta 83$) of tethered-hCG and hFSH to determine the biological function (secretion, activity) of these mutants. PCR product was cloned into the pcDNA3. DNA sequencing was performed by the dideoxy chain-termination method using an AutoRead sequencing kit and an ALF DNA sequencer (Pharmacia LKB, Uppsala, Sweden). Sequencing analysis was performed with MacMolly Tetra computer software (Berlin, Germany) and compared with the database of GenBank/EMBL (MacMolly data).

3. Cell Culture and Expression

The expression vectors (pcDNA3-hCG β/α and pcDNA3-FSH β/α and mutants) were 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 μg/ ml) for 2 weeks post-transfection according to the method reported previously (Min, 2001). After incubation of selected stable cells (1×10^6) in 20 ml CHO-S -SFM -II at 37℃ for 48 h, the culture media were collected and centrifuged at 100,000×g, 4℃ for 60 min to remove the cell debris. The amount of recombinant wild type and tethered-hCG, -FSH and mutants was quantified.

4. Hormone Assay

The hCG and FSH proteins secreted by the stably transfected cells into serum-free media were collected and quantified by radioimmunoassay, as described in protocol (DPC; hCG IRMA and FSH IRMA). One hundred ul samples were added into the tubes prepared and added 100 μl of ^{125}I-hCG and -FSH Abs to every tube. After then, the tubes were shaken for 60 min. on a rack shaker and decanted thoroughly. Finally, the tubes were count for 1 min. in a gamma counter.

Ⅲ. RESULTS AND DISCUSSION

To systematically examine the roles of the α COOH-terminal region, we utilized the tethered -hCG and -FSH mutants deleted in the COOH -terminal region. A set of seven deletion mutants was generated in which the α-COOH terminal 10 amino acids were progressively truncated (Fig. 2).

		82	83	84	85	86	87	88	89	90	91	92	
WT	NH_2-82 amino acids-	C	H	C	S	T	C	Y	Y	H	K	S	-C
αΔ91	NH_2-82 amino acids-	C	H	C	S	T	C	Y	Y	H	-C		
αΔ89	NH_2-82 amino acids-	C	H	C	S	T	C	Y	-C				
αΔ88	NH_2-82 amino acids-	C	H	C	S	T	C	-C					
αΔ87	NH_2-82 amino acids-	C	H	C	S	T	-C						
αΔ86	NH_2-82 amino acids-	C	H	C	S	-C							
αΔ84	NH_2-82 amino acids-	C	H	-C									
αΔ84	NH_2-82 amino acids-	C	-C										

Fig. 2. Mutant α-subunit. The carboxyl-terminal 7 deletion mutants of the α-subunit were progressively truncated by replacing amino codons with a stop codon in the α cDNA. -C: COOH.

The tethered-wthCG and -FSH were efficiently secreted to the dimer (Table 1). The deletion of hCG /hFSH α-subunits C-terminal ($\alpha\Delta 91$) was significantly reduced more than 2~6 times in the secretion of recombinant single-chain hormone. In hCG mutants, $\alpha\Delta 89$ mutant was a little decreased and then $\alpha\Delta 88$, $\alpha\Delta 87$, $\alpha\Delta 86$ and $\alpha\Delta 84$ mutants were detected as high concentration compared to wild type. The $\alpha\Delta 83$hCG and $\alpha\Delta 83$FSH mutants were not detected in this assay (Table 1). The correct conformation of the heterodimer is also important for efficient secretion, hormone-specific post-translational modifications, receptor binding

Table 1. Secretion of single chain hCG, hFSH and C-terminal deletion mutants

WT and mutants	hCG (mIU/ml)	FSH (mIU/ml)
WT	8,810	3.65
$\alpha\Delta$91	1,407	1.77
$\alpha\Delta$89	5,440	3.88
$\alpha\Delta$88	16,840	3.90
$\alpha\Delta$87	23,120	5.48
$\alpha\Delta$86	15,890	5.45
$\alpha\Delta$84	12,130	5.60
$\alpha\Delta$83	26	0.24

Values are the mean of triplicate observations.

and signal transduction.

We have been investigated the roles of oligosaccharides on glycoprotein hormones in the culture system of rat Leydig cells and granulose cells by using recombinant eCG (Min et al., 1996, 1997), eFSH (Saneyoshi et al., 2001), tethered-eCG (Min., 2001), and tethered-bovine LH and FSH (Min et al., 2003). Deletion of the αCOOH-terminal residues has the same type in the secretion. But, Yoo et al., (1991) demonstrated that the hCG α COOH-terminal region makes direct contact with the LH/CG receptor. The low affinity contact is necessary and sufficient for hCG's ability to activate the receptor and involves αLys^{90} and His^{91}. A α His90-Lys91 plays an essential role in cAMP induction of both hormones (hCG and FSH). In contrast to this common role, they are necessary for FSH binding to the FSH receptor but not for hCG binding to the LH/ CG receptor. The hCG α COOH-terminal region makes 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 demonstrate for the first time that the hCG/FSH α COOH-terminal region in $\alpha\Delta$83 is key role in the secretion of glycoprotein hormone. Thus, if the C-terminal deletion region is very important role in secretion, the deleted hormone in $\alpha\Delta$88-84 mutants could be produced the large scale of recombinant hormone in mammalian cells. It is suggest that α-subunit C-terminal region of human glycoprotein is very important for hCG and FSH secretion. Once important amino acids are identified, we can attempt to determine whether they are necessary for the hormone secretion and bioactivity. Then important amino acids were individually substitute with a series of other amino acids to determine effects of different side chains. Now, we are checking the LH-and FSH-like activities of these mutants. These results demonstrated that the $\alpha\Delta$83hCG and $\alpha\Delta$83FSH mutants could be play a pivotal role in the secretion of tethered-molecule. This approach posses a universal strategy to enhance both stability and bioactivity as well as to control specificity of noncovalently linked oliglomers, and may also be used to engineer molecules with novel activities or specificities.

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