Human Chorionic Gonadotropin \(\beta\)-Subunit Affects the Folding and Glycosylation of \(\alpha\)-Cys Mutants

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Abstract. Human chorionic gonadotropin (hCG) is a member of a family of heterodimeric glycoprotein hormones that contain a common \(\alpha\)-subunit but differ in their hormone-specific \(\beta\)-subunits. Both subunits have five and six disulfide bonds, respectively, which consist of cystine knot structure. It is evident from numerous studies that the structure of \(\beta\)-subunits is rigid, whereas that of \(\alpha\)-subunit is flexible and can be molded by a \(\beta\)-subunit. Previously, we reported that secreted forms of \(\alpha\) mutants where either cysteine residue in the disulfide bond 7–31 or 59–87 was converted to alanine contained a disulfide-linked homodimer in addition to a monomer. To study whether the hCG\(\beta\)-subunit affects the conformations of \(\alpha\) mutants, \(\alpha\)-subunits lacking either the 7–31 or 59–87 disulfide bond were expressed with wild-type (WT) hCG\(\beta\) in Chinese hamster ovary cells, and homodimer formation and glycosylation of dimerized \(\alpha\)-subunit were assessed by continuous labeling with \(\[^{35}\text{S}\]\)methionine/cysteine, immunoprecipitation with anti-\(\alpha\) or -hCG\(\beta\) serum, digestion with endoglycosidase-H or -F, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a non-reducing condition. Our data showed that a homodimer was not observed in the half-Cys mutants except one, where cysteine at position 7 was converted to alanine, in the presence of \(\beta\)-subunit. This finding indicated that hCG\(\beta\)-subunit rescued the \(\alpha\) half-Cys mutants from the formation of intermolecular disulfide-linked homodimer by preferentially combining with the \(\alpha\) mutants. In both free WT and all mutants treated with endoglycosidase-H, no or faint bands were recognized as the same migration as seen in endoglycosidase-F treatment. Even in the endoglycosidase-H sensitive cases, the amount of sensitive \(\alpha\)-subunits was less than 5% of total \(\alpha\)-subunits. In contrast to free \(\alpha\)-subunits, distinct endoglycosidase-H sensitive bands were seen in both WT and mutants, although the ratio was various. We concluded that hCG\(\beta\)-subunit affects the folding and glycosylation of the \(\alpha\)-subunit mutants.

Key words: Gonadotropin, \(\alpha\)-Subunit, Homodimer, Glycosylation

PLACENTAL hCG is a member of the glycoprotein family, which also includes pituitary TSH, LH, and FSH. These hormones are noncovalently associated heterodimers consisting of \(\alpha\)- and \(\beta\)-subunits. The unique \(\beta\)-subunit determines biological specificity, while \(\alpha\)-subunit has an identical amino acid sequence in all four members of the hormone family and shows considerable homology among different species [1, 2].

The \(\alpha\)- and \(\beta\)-subunits have five and six disulfide bonds, respectively. Recently, the crystal structure of hCG was demonstrated to have disulfide pairings in both subunits [3]. The structural feature of each subunit is a cystine knot formed by these disulfide bonds. We previously constructed \(\alpha\) mutants where either or both cysteine residues in each disulfide bond were converted to alanine. These mutants were expressed alone or with the wild-type (WT) hCG\(\beta\) gene in Chinese hamster ovary (CHO) cells [4]. The secreted forms of the \(\alpha\) half-Cys mutants contained a disulfide-linked homodimer in addition to a monomer. The disulfide bonds 7–31 and 59–87,
which lie outside the cystine knot, were eliminated
without significantly affecting assembly with the
hCGβ-subunit.

Although the α-subunit is common to the four-
glycoprotein hormones, it is posttranslationally
modified in the context of four different β-subunits
[5]. This modification results in the conformational
difference among α-subunits in these four hetero-
dimer forms. On the other hand, there are differences
in glycosylation between assembled (dimer) and unassembled (free, monomer) α-subunits [6, 7].
These studies suggest that the structure of the α-
subunit is not rigid but flexible.

To elucidate whether hCGβ-subunit affects the
conformations of α mutants, α-subunits lacking
either the 7-31 or 59-87 disulfide bond were expressed
with hCGβWT in CHO cells, and the folding and
glycosylation of their dimerized α-subunits were as-
sessed.

Materials and Methods

Vector Construction

The construction of the vectors has been described
previously [4]. In brief, the cysteine codons at 7, 31,
7 and 31, 59, 87, or 59 and 87 were converted to
alanine codons by mutagenesis. DNA was trans-
formed into competent E. coli K12 JM109, and the
mutants were isolated using tetramethylammonium
cloride. After screening, the proper construction
was verified by DNA sequencing. The BamHI-BglII
fragment for each mutant was inserted into the
compatible BamHI site of the eukaryotic expression
vector, pM2 [8], downstream from the Harvey murine
sarcoma virus long terminal repeat [9]. Expression
vector for hCGβWT (pM2CGβ) was also described
previously [10].

Cells and Transfection

CHO cells were grown in F-12 medium supple-
mented with 100 U/ml penicillin, 100 μg/ml strepto-
mycin, 2 mM glutamine, and 5% fetal bovine serum
(FBS). The plasmids described above were trans-
fected alone or with pM2CGβ into the cells by the
calcium phosphate method [10]. The stable clones
were selected with 0.25 mg/ml of the neomycin
analog G418 (Life Technologies, Gaithersburg,
MD). Single colonies were isolated and maintained
in culture in the presence of 0.125 mg/ml G418.

α mutants were denoted as follows. α7, α31, and
α7-31 have amino acid substitution (cysteine → ala-
nine) at position 7, 31, and both, respectively. Thus,
they lack disulfide bond 7-31. α7-31 does not but
both α7 and α31 have a free thiol. Similarly, α59,
α87, and α59-87 are mutants with alterations at cyste-
eine 59, 87, and both, respectively (Fig. 1).

Metabolic Labeling and Immunoprecipitation

Stable clones were plated into 60 mm petri dishes
and grown to near confluency. CHO cells were
labeled for 8 h with 25 μCi/ml of Tran35S-label
(ICN, Irvine, CA; SA, > 1000 Ci/mmol) in labeling
medium (conditioned medium minus methionine,
cysteine, and G418, but supplemented with 7.5% 
dialyzed FBS). The media were divided into three
portions, immunoprecipitated, and treated as des-
cribed [11]. Polyclonal antisera against α- and
hCGβ-subunit were a gift from Dr. Irving Boime,
Washington University School of Medicine, St.
Louis, MO.

Enzymatic Digestion

Endoglycosidase treatment has been described
previously [12]. For endoglycosidase-H digestion,
immune complex was incubated in 18 μl H buffer
[50 mM sodium acetate, 0.5% Triton X-100, and
0.1% sodium dodecyl sulfate (SDS), pH 5.5] at 37°C
for 24 h with 2.5 mU endoglycosidase-H (Boehringer
Mannheim Biochemicals, Indianapolis, IN). Simi-
larly, the hCGβ-subunit was expressed alone or
with each α mutant in CHO cells, and the folding
and glycosylation of their dimerized α-subunits were
assessed.

Fig. 1. WT and mutant α-subunits. Changes in amino acid
are shown.
larly, endoglycosidase-F digestion was performed in 18 µl F buffer (50 mM sodium acetate, 50 mM EDTA, 10 mM sodium azide, 0.5% n-octylglucoside, and 0.1% SDS, pH 5.5) at 37°C for 24 h with 0.1 U endoglycosidase-F (Boehringer Mannheim Biochemicals, Indianapolis, IN). Samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a non-reducing condition as described [8]. The quantitation of α-subunit was performed by densitometry.

**Results**

**Assembly of WT and mutant α-subunits with hCGβ-subunit**

When α7, α31, α59, and α87 mutants were subjected to nonreducing gels, disulfide-linked homodimers were seen in addition to monomers (Fig. 2, lanes 4, 7, 10, and 13) as reported previously [4]. To study the effect of β-subunit on α-subunit, α mutants were cotransfected with the hCGβ gene and stable cell lines producing both subunits were selected. For each mutant, clones were isolated synthesizing excess hCGβ-subunit to ensure that it would not limit dimer formation. α and β antisera detect, in addition to heterodimer, free α and free β, respectively. Thus, α-subunit immunoprecipitated with β antisera means only the dimerized α-subunit, although the immunoprecipitates with α antisera contain both free and dimerized α-subunits. The uncombined α-subunit is more heterogeneous and migrates slower on SDS-PAGE than the dimer form of α-subunit (lanes 1–3). This is due to alterations in the processing of the N-linked oligosaccharides [6, 7]. α homodimer was not observed in α31 (lane 8), α59 (lane 11), and α87 (lane 14), but seen in α7 (lane 5) in the presence of β-subunit. The α homodimer was not recognized in any of the α mutants when immunoprecipitated with β antisera (lanes 6, 9, 12, and 15), indicating that the α homodimer did not assem-

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**Fig. 2.** Assembly of α-WT and -mutants with hCGβ-subunit. Clones expressing α alone (lanes 1, 4, 7, 10, and 13) and both hCGβ (lanes 2, 3, 5, 6, 8, 9, 11, 12, 14, and 15) were continuously labeled with [35S]methionine/cysteine for 8 h. The medium from each clone were divided into two aliquots and immunoprecipitated with α (α) or hCGβ (β) antiserum, followed by 15% SDS-PAGE in a nonreducing condition. Arrow indicates the homodimer of α-subunit. M, markers (kilodaltons; Amersham) are shown.
Endoglycosidase treatment of WT and mutant α-subunits

The secretory forms of the α-subunit from CHO cells are a mixture of complex, high mannose, and hybrid types (>95% of the secreted material is resistant to endoglycosidase-H but sensitive to endoglycosidase-F) [8]. Endoglycosidase-H cleaves high mannose, noncomplex, and hybrid type oligosaccharides, whereas endoglycosidase-F cleaves all forms of N-linked oligosaccharides. Thus, endoglycosidase-H and -F treatment discriminates the oligosaccharide structure. Endoglycosidase-H sensitive forms should migrate to the same position as those digested with endoglycosidase-F. To characterize the oligosaccharide structure of the free α-subunit, we treated immunoprecipitated α-subunits with endoglycosidase-H or -F (Fig. 3). In both WT and all mutants treated with endoglycosidase-H, no or faint bands were recognized as the same migration as seen in endoglycosidase-F treatment (lanes 2, 3, 5, 6, 8, 9, 11, 12, 14, 15, 17, 18, 20, and 21). Even in the endoglycosidase-H sensitive cases, the amount of sensitive α-subunits was less than 5% of total α-subunits (Table 1). In α59 and α87, homodimeric bands after endoglycosidase-F treatment were heterogeneous, although monomeric bands were single (lanes 15 and 18).

Endoglycosidase-H sensitivity of α mutants dimerized with hCGβ-subunit

To characterize the oligosaccharide structure of dimerized α-subunit, α-subunits immunoprecipitated with hCGβ antisera were treated with endoglycosidase-H or -F. In contrast to free α-subunits, distinct endoglycosidase-H sensitive bands were seen in both WT and mutants (Fig. 4, lanes 2, 5, 8, 11, 14, 17, and 20), although the ratio was various (Table 1).

Discussion

It is of interest that homodimers of α mutants, which were seen when α gene alone was expressed, were not created in the presence of hCGβ-subunit except α7. This finding suggests that intermolecular crosslinking of unpaired thiols in α mutants is fragile and that hCGβ-subunit controls the folding of α-subunit. The folding of the hCGβ-subunit has been well documented [13]. Formation and rearrangement of disulfide bonds occur during folding. Assembly with the α-subunit takes place before the final disulfide bond 26-110 is formed. This closing of the 26-110 bridge locks the seat belt and secures the αβ dimer preventing disassembly. The portion of α-
The degree of endoglycosidase-H sensitivity was calculated as the percentage of the amounts of endoglycosidase-H sensitive α to the total amounts of α. Values are the average of four independent experiments.
subunit effects and resultant conformational change may account for the difference in endoglycosidase-H sensitivity between free and heterodimerized \( \alpha \)-subunits. Likewise, the different endoglycosidase-H sensitivity among heterodimerized \( \alpha \)-subunits seems to be caused by the disparity of the protein backbone structures. We previously demonstrated the different receptor binding and signal transduction activity of heterodimers containing \( \alpha \)-Cys mutants [4]. The different glycosylation, in addition to structural change, may affect biological activities of mutant hCG.

It has been reported that there are significant differences in the intracellular behavior between \( \alpha \) and hCG\( \beta \) half-Cys mutants [4, 17]. All the half-Cys mutants of hCG\( \beta \)-subunit were secreted but heterodimer formation was inhibited or much less efficient than hCG\( \beta \)WT. In contrast, five of ten \( \alpha \) half-Cys mutants were not secreted but all secreted mutants assembled with hCG\( \beta \)-subunit. Recently, we reported that the secreted forms of some hCG\( \beta \) half-Cys mutants were partially sensitive to endoglycosidase-H [18]. Although free \( \alpha \) half-Cys mutants demonstrated sensitivity to endoglycosidase-H as shown here, the extent was much less than those seen in hCG\( \beta \) mutants. This finding also supports our previous conclusion that the roles for the disulfide bonds differ between the two subunits.

An unexpected observation was the different behavior of monomer and homodimer in \( \alpha \)59 and \( \alpha \)87. Although \( \alpha \)-subunit bears different types of N-linked oligosaccharide chains, the form after endoglycosidase-F treatment should be single because endoglycosidase-F removes all types of N-linked sugar. Monomeric forms were homogeneous, whereas homodimers were heterogeneous after digestion of \( \alpha \)59 and \( \alpha \)87 with endoglycosidase-F (Fig. 3). This inconsistency may be due to the following: 1) homodimeric forms consist of several variants constituted by mispaired disulfide links; 2) homodimeric forms contain O-linked oligosaccharides.

Regarding the first point, a free thiol created by half-Cys mutation may cause the scrambling and interchange of the resulting disulfide bonds. Unstable linkage in turn yields various conformations, resulting in different migration on nonreducing SDS-PAGE. With respect to O-linked glycosylation of \( \alpha \)-subunit, free bovine \( \alpha \)-subunit has been shown to have O-linked sugar at Thr\(^{39} \) in human \( \alpha \)-subunit) [19]. In human, \( \alpha \)-subunit expressed in the choriocarcinoma cell line, JAR, has O-linked sugar [20]. But no O-linked sugar has been demonstrated in \( \alpha \)-subunit synthesized in pituitary [21]. Recently we demonstrated that substitution of Pro\(^{40} \) and Arg\(^{42} \) by Ala resulted in the attachment of O-linked sugar in the human \( \alpha \)-subunit [12]. This finding indicates that O-glycosylation is associated with amino acid change or subsequent conformational change. Thus, it is likely that the different conformation around Thr\(^{39} \) in homodimer from that in monomer results in the addition of O-linked sugar. This may be particularly true of \( \alpha \)59 where two bands are apparent before and after endoglycosidase-F digestion.

A summary of results is illustrated in Fig. 5. hCG\( \beta \)-subunit rescued the \( \alpha \) half-Cys mutants from the formation of homodimer by assembling with the \( \alpha \) mutants. Endoglycosidase-H sensitivity of the \( \alpha \)-subunit mutants was different between free and heterodimerized forms. We concluded that hCG\( \beta \)-subunit affects the folding and glycosylation of the \( \alpha \)-subunit mutants.
References