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Human Chorionic Gonadotropin β-Subunit Affects the Folding and Glycosylation of α-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 α-subunit but differ in their hormone-specific β-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 β-subunits is rigid, whereas that of α-subunit is flexible and can be molded by a β-subunit. Previously, we reported that secreted forms of α 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 hCGβ-subunit affects the conformations of α mutants, α-subunits lacking either the 7–31 or 59–87 disulfide bond were expressed with wild-type (WT) hCGβ in Chinese hamster ovary cells, and homodimer formation and glycosylation of dimerized α-subunit were assessed by continuous labeling with [^{35}S]methionine/cysteine, immunoprecipitation with anti-α or -hCGβ serum, digestion with endoglycosidase-H or -F, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a non-reducing condition. Our data showed that α homodimer was not observed in the half-Cys mutants except one, where cysteine at position 7 was converted to alanine, in the presence of β-subunit. This finding indicated that hCGβ-subunit rescued the α half-Cys mutants from the formation of intermolecular disulfide-linked homodimer by preferentially combining with the α 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 α-subunits was less than 5% of total α-subunits. In contrast to free α-subunits, distinct endoglycosidase-H sensitive bands were seen in both WT and mutants, although the ratio was various. We concluded that hCGβ-subunit affects the folding and glycosylation of the α-subunit mutants.

Key words: Gonadotropin, α-Subunit, Homodimer, Glycosylation

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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 α- and β-subunits. The unique β-subunit determines biological specificity, while α-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 α- and β-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 α 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) hCGβ gene in Chinese hamster ovary (CHO) cells [4]. The secreted forms of the α half-Cys mutants contained a disulfide-linked homodimer in addition to a monomer. The disulfide bonds 7–31 and 59–87,

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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 heterodimer 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 assessed.

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 transformed into competent *E. coli* K12 JM109, and the mutants were isolated using tetramethylammonium chloride. After screening, the proper construction was verified by DNA sequencing. The *Bam*HI-*Bgl*II fragment for each mutant was inserted into the compatible *Bam*HI site of the eukaryotic expression vector, pM^2 [8], downstream from the Harvey murine sarcoma virus long terminal repeat [9]. Expression vector for hCGβWT (pM^{2}CGβ) was also described previously [10].

Cells and Transfection

CHO cells were grown in F-12 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 5% fetal bovine serum (FBS). The plasmids described above were transfected alone or with pM^{2}CGβ 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 → alanine) 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 cysteine 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 Tran^{35}S-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 described [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-

	7	31	59	87
α WT	Cys -	Cys	Cys -	Cys
α 7	**Ala**	Cys	Cys -	Cys
α 31	Cys	**Ala**	Cys -	Cys
α 7-31	**Ala**	**Ala**	Cys -	Cys
α 59	Cys -	Cys	**Ala**	Cys
α 87	Cys -	Cys	Cys	**Ala**
α 59-87	Cys -	Cys	**Ala**	**Ala**

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-

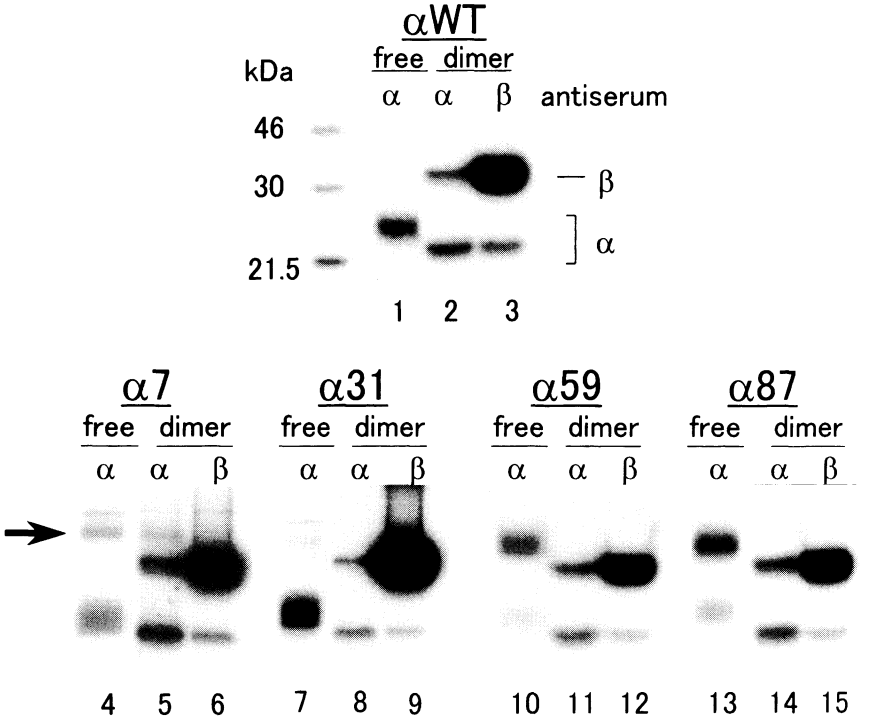

Fig. 2. Assembly of α-WT and -mutants with hCGβ-subunit. Clones expressing α alone (lanes 1, 4, 7, 10, and 13) and both hCGβ (lanes 2, 3, 5, 6, 8, 9, 11, 12, 14, and 15) were continuously labeled with [^{35}S]methionine/cysteine for 8 h. The medium from each clone were divided into two aliquots and immunoprecipitated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

ble with β-subunit to form a trimer.

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 antiserum 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).

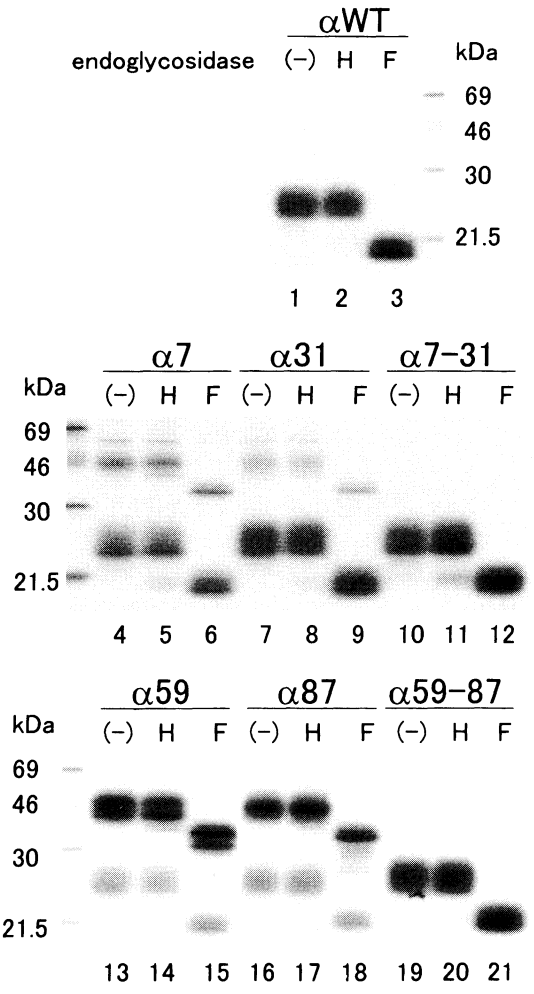

Fig. 3. Endoglycosidase treatment of WT and mutant α-subunits. Clones expressing α-WT or -mutants were continuously labeled with [^{35}S]methionine/cysteine for 24 h, and medium were immunoprecipitated with α antiserum before digestion with endoglycosidase-H (lanes 2, 5, 8, 11, 14, 17, and 20), endoglycosidase-F (lanes 3, 6, 9, 12, 15, 18, and 21), or no enzyme (lanes 1, 4, 7, 10, 13, 16, and 19). M_r markers (kilodaltons; Amersham) are shown.

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-

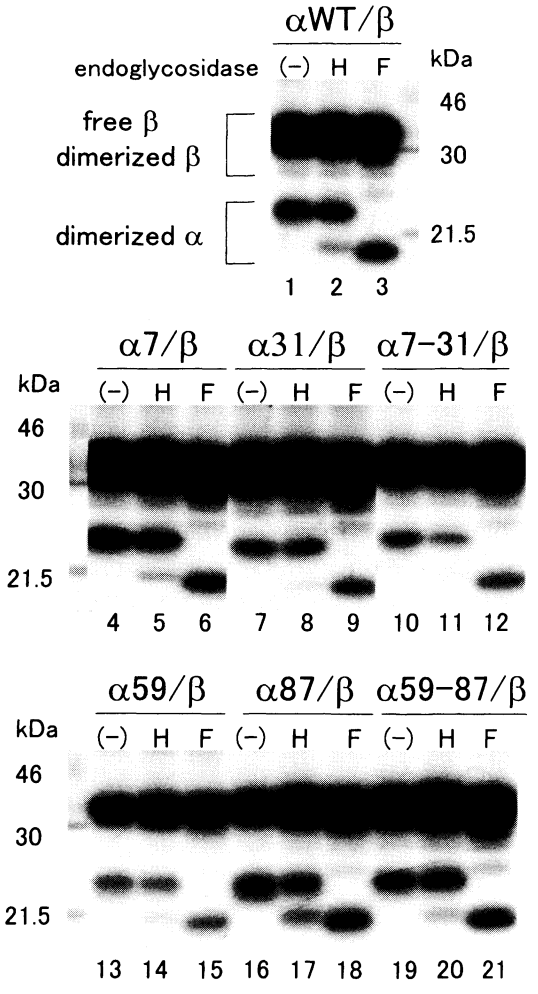

Fig. 4. Endoglycosidase treatment of WT and mutant α-subunits dimerized with hCGβ-subunit. Clones expressing both hCGβWT and either αWT (lanes 1–3), α7 (lanes 4–6), α31 (lanes 7–9), α7–31 (lanes 10–12), α59 (lanes 13–15), α87 (lanes 16–18), and α59–87 (lanes 19–21) were continuously labeled with [^{35}S] methionine/cysteine for 24 h, and medium were immunoprecipitated with hCGβ antiserum before digestion with endoglycosidase-H (H), endoglycosidase-F (F), or no enzyme (−). M_r markers (kilodaltons; Amersham) are shown.

Table 1. Sensitivity of WT and mutant α-subunits to endoglycosidase-H

	free α-subunit	heterodimerized α-subunit
αWT	<5	21.7±6.2
α7	<5	16.3±7.0
α31	<5	8.3±2.2
α7–31	<5	11.3±4.2
α59	<5	14.4±3.8
α87	<5	32.5±4.8
α59–87	<5	17.0±4.9

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 surrounded by the seat belt is the hairpin loop 2 where no disulfide bond is participates. Thus, it is likely that assembly and seat belt locking occur during the scrambling of α disulfide bonds, which prevent the homodimeric formation of the α-subunit created by intermolecular disulfide bond. In this process, the hCGβ-subunit seems to function like a molecular chaperone because it prevents inappropriate interactions between α mutants by preferentially combining with the α mutants. Our data support the view that the α-subunit is flexible and can be molded by a β-subunit after dimmer formation [14].

α7 is an exceptional case where the α homodimer was seen even in the presence of hCGβ-subunit. The homodimeric formation in α7 may be more rapid and/or its structure may be more rigid. The hypothesis that the homodimer of α assembles with β-subunit and constructs a trimer is negligible because the homodimeric form was not observed after immunoprecipitation with hCGβ-subunit (Fig. 2, lane 6). The more rigid structure of the homodimer may reflect the lower efficiency of combination with hCGβ-subunit reported previously [4]. The question arises whether other β-subunits affect α7. Because the assembly of α mutants with LHβ-subunit was markedly reduced [15], the effect of FSHβ-subunit was assessed in the same manner. Homodimeric form of α7 was also observed in the presence of FSHβ-subunit (data not shown). The finding supports the above hypothesis that the structure of the α7 homodimer is more rigid.

Within each species, differences were found between the carbohydrate moieties of the α-subunits in each hormone, even though the amino acid sequences were the same [5]. It has been shown that gonadotropin β-subunits can influence the oligosaccharide processing of noncovalently bound α-subunit [16]. Another study revealed that the local structures around the glycosylation sites of α-subunit were apparently altered by β-subunits [7]. Such inter-

subunit effects and resultant conformational change may account for the difference in endoglycosidase-H sensitivity between free and heterodimerized α-subunits. Likewise, the different endoglycosidase-H sensitivity among heterodimerized α-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 α-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 α and hCGβ half-Cys mutants [4, 17]. All the half-Cys mutants of hCGβ-subunit were secreted but heterodimer formation was inhibited or much less efficient than hCGβWT. In contrast, five of ten α half-Cys mutants were not secreted but all secreted mutants assembled with hCGβ-subunit. Recently, we reported that the secreted forms of some hCGβ half-Cys mutants were partially sensitive to endoglycosidase-H [18]. Although free α half-Cys mutants demonstrated sensitivity to endoglycosidase-H as shown here, the extent was much less than those seen in hCGβ 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 α59 and α87. Although α-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 α59 and α87 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 α-subunit, free bovine α-subunit has been shown to have O-linked sugar at Thr[41] (corresponding to Thr[39] in human α-subunit) [19]. In human, α-subunit expressed in the choriocarcinoma cell line, JAR, has O-linked sugar [20]. But no O-linked sugar has been demonstrated in α-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 α-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 α59 where two bands are apparent before and after endoglycosidase-F digestion.

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

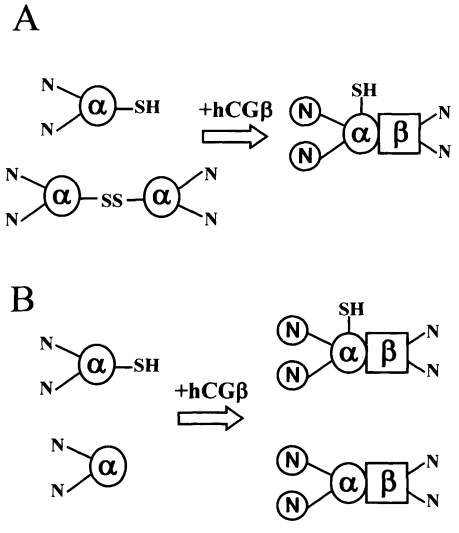

Fig. 5. Schematic diagram of effects of hCGβ-subunit on folding and glycosylation of α-subunit mutants. A. When α half-Cys mutants alone are expressed, both monomeric α containing free thiol and disulfide-linked homodimer exist. hCGβ-subunit rescues the α half-Cys mutants from the formation of homodimer by assembling with the α mutants. B. N-glycosylation of monomeric α is different from that of heterodimerized α. N denotes the asparagine-linked oligosaccharides.

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