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Alteration of N-linked oligosaccharide structures of human chorionic gonadotropin β-subunit by disruption of disulfide bonds

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The human chorionic gonadotropin β-subunit (hCGβ) is a glycoprotein in which 12 cysteine residues pair to form six intramolecular disulfide bonds. In order to elucidate the effect of each disulfide bond on glycosylation of the molecule, we analysed structures of asparagine-linked oligosaccharides of various recombinant hCGβ produced in Chinese hamster ovary (CHO) cells: wild-type hCGβ (βWT) and mutants in which any one of the six intramolecular disulfide bonds had been disrupted by site-directed mutagenesis. SDS-PAGE analysis of βWT and these mutants before and after digestion with endoglycosidase F and H revealed structural changes in the oligosaccharide moieties of some mutants. In addition, structural analysis of oligosaccharides obtained from metabolically labeled βWT and a mutant showed that the mutant contained additional high mannose type oligosaccharides. These results suggest that elimination of a specific disulfide bond, resulting in a change in the protein conformation, disturbs the normal assembly of the mature complex type oligosaccharides in the hCGβ molecule.

***Keywords*: human chorionic gonadotropin, hCGβ-subunit, disulfide bond, N-linked oligosaccharide processing**

***Abbreviations*: hCGβ, human chorionic gonadotropin β-subunit; βWT, wild type hCGβ; CHO, Chinese hamster ovary; Endo-H, endoglycosidase H; Endo-F, endoglycosidase F**

Introduction

Human chorionic gonadotropin consists of two non-covalently joined α and β subunits, as do the other glycoprotein hormones, luteinizing hormone, follicle stimulating hormone, and thyroid stimulating hormone [1]. The α-subunit of all four hormones has an identical amino acid sequence, whereas the β-subunit is unique to each and determines biological specificity. There is, however, more than 40% amino acid homology among the various β-subunits, which is most apparent at the conserved positions of the 12 cysteine (Cys) residues which form six disulfide bonds in the molecule [1].

In an earlier study, we examined the effects of the disulfide bonds in hCGβ on key intracellular events, such as assembly with the α-subunit, secretion from cells, and intracellular stability, using site-directed mutagenesis of each Cys residue and transfection of the gene into Chinese hamster ovary (CHO) cells [2]. From these analyses, it was demonstrated that each disulfide bond in hCGβ has a different role to play in intracellular events. In addition, we also found that the migration patterns of radioisotope-labeled hCGβ on SDS-PAGE were altered by some Cys mutations. This suggested the possibility that disruption of certain disulfide bonds in hCGβ results in changes of the oligosaccharide structure.

HCGβ possesses two N-linked and four O-linked oligosaccharide chains [3, 4]. Although hCGβ contains mainly biantennary complex type N-linked oligosaccharides [5], various subtypes of oligosaccharide chains are found in the hCGβ of placenta as well as in that of molar and choriocarcinoma tissues [5–7]. In order to elucidate the role of disulfide bonds on glycosylation, in the present study, we compared the structures of N-linked oligosaccharides between the wild type hCGβ (βWT) and mutants in which any one disulfide bond was disrupted by converting the Cys residue to alanine (Ala).

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Materials and methods

Site-directed mutagenesis

A fragment containing exons II and III of the *hCGβ* gene [8] was inserted into M13mp19, and single stranded DNA was isolated for site-directed mutagenesis. Mutagenesis of Cys residues to Ala was performed as previously described [2]. After reconstruction of the entire *hCGβ* gene containing the mutation, the genes were inserted into the eukaryotic expression vector, pM^2 [9]. Construction of a vector containing the *βWT* gene has been described previously [10].

DNA transfection, clone selection, and cell culture

The plasmids were transfected into CHO cells as described previously [9, 10]. Cells were selected for insertion of plasmid DNA by growing in culture medium containing 0.25 mg ml^{-1} of the active form of the neomycin analogue, G418 (Gibco, Life Technologies Inc., Gaithersburg, MD). Transfected colonies resistant to G418 were screened for the expression of *hCGβ* mutants as previously described [2]. The mutants with an alteration at a Cys residue were as follows: *β*34, *β*88, *β*9, *β*57, *β*38, *β*90, *β*23, *β*72, *β*93, *β*100, *β*26, and *β*110 (*eg* *β*34 denotes a change of ^{34}Cys to ^{34}Ala). Cells producing *β*WT were selected as described previously [10].

Metabolic labeling and immunoprecipitation

Cells were labeled with 25 μCi ml^{-1} [^{35}S]Cys (ICN Radiochemicals Co., Irvine, CA) for the endoglycosidase experiment, or with 200 μCi ml^{-1} [1-^{3}H]galactose or 200 μCi ml^{-1} *N*-acetyl[1-^{3}H]glucosamine (Amersham, Arlington Heights, IL) for oligosaccharide analysis, as previously described [2]. Media were immunoprecipitated with polyclonal antiserum against hCG*β* as described previously [9].

Digestion with endoglycosidase

Digestion of oligosaccharides in hCG*β* was performed with endoglycosidase H (Endo-H) and F (Endo-F) (Boehringer Mannheim Biochemicals, Indianapolis, IN) as described previously [10, 11]. The samples were analysed by SDS-PAGE before and after digestion [12].

Analyses of N-linked oligosaccharides of hCG*β*

Metabolically tritium-labeled hCG*β* samples (*β*WT and *β*57) were subjected to gas-phase hydrazinolysis for 3 h at 90 °C using Hydraclub S204 (Honen Corporation, Tokyo) followed by *N*-acetylation to quantitatively liberate the radioactive N-linked oligosaccharides of hCG*β* [13]. Analysis of hCG*β* oligosaccharides based on their anionic charges was performed by ion exchange HPLC on a COSMOGEL DEAE column (0.75 × 7.5 cm, Nacalai Tesque Inc., Kyoto). The radioactivity in each fraction was measured by liquid scintillation spectrometry. In order to analyse the size of the oligosaccharides, the neutral oligosaccharide mixture was obtained by exhaustive sialidase treatment of the oligosaccharide fraction derived from each hCG*β* sample, subjected to $NaBH_4$ reduction, and then to HPLC on a Bio-Gel P-4 (-400 mesh) column (1 × 130 cm) [13].

Results and discussion

As we have reported previously [2], the secreted forms of *β*WT (Figure 1, lane 1) and hCG*β* with mutations at positions 110 (lane 4), 26, 93, 100, 90, and 23 from CHO cells appeared as two bands on SDS-PAGE which differ from each other in the N-linked oligosaccharide moiety (N-1, 32 kDa; N-2, 34 kDa). On the other hand, the secreted form of *β*72 (lane 7) displayed a single band, and the other 5 Cys mutants including *β*57 (lane 10) appeared as one broad band. This difference in band pattern suggests that N-linked oligosaccharide processing is affected by disruption of specific disulfide bonds. To confirm if this is so, the secreted hCG*β* molecules were digested with endoglycosidase. Endo-H releases high mannose type and hybrid type oligosaccharide but not the complex type, whereas Endo-F cleaves all

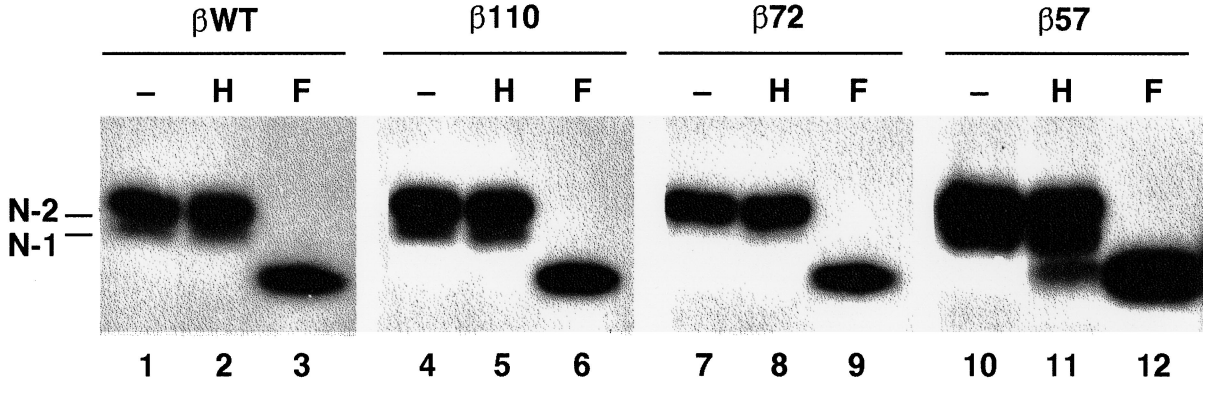

Figure 1. Enzymatic deglycosylation of *β*WT and the various Cys mutants with Endo-F or Endo-H. Metabolically [^{35}S]Cys-labeled *β*WT (lanes 1–3), *β*110 (lanes 4–6), *β*72 (lanes 7–9), *β*57 (lanes 10–12) were untreated (–) or treated with Endo-H (*H*) or with Endo-F (*F*). Samples were then subjected to SDS-PAGE and the gels were autoradiographed. N-1 and N-2 denote two secreted forms of hCG*β* with molecular weights of 32 and 34 kDa, respectively.

types of N-linked oligosaccharides [14, 15]. Thus, Endo-H and Endo-F treatment is useful for discriminating among the oligosaccharide structures and for determining whether these oligosaccharides are secreted through the normal pathway which allows sufficient processing to the complex type. As shown in Figure 1 and Table 1, Endo-F treatment of both βWT (Figure 1, lane 3) and all mutants of *hCGβ* (lanes 6, 9, and 12) generated a single identical band on SDS-PAGE, indicating that the different band patterns of *hCGβ* were caused by differences in N-linked oligosaccharide structures. On the other hand, Endo-H treatment did not affect the profiles of βWT (lane 2) of hCGβ with mutations at positions 110 (lane 5), 72 (lane 8), 26, 93, 100, and 23. However, the other six hCGβ with mutations at positions 57 (lane 11), 9, 34, 88, 38, and 90 were partially sensitive to Endo-H, indicating that these mutants contain high mannose and/or hybrid type oligosaccharide chains. Since a part of the endo-H digest migrated to the same position as the endo-F digest and since a part of hCGβ was not susceptible to endo-H (lane 11 and 12), it was assumed that three kinds of hCGβ, hCGβ with endo-H sensitive oligosaccharides, hCGβ with both endo-H sensitive and resistant oligosaccharides, and hCGβ with endo-H resistant oligosaccharides were produced by the mutations. It is proposed that the six disulfide assignments in hCGβ are 9–57, 23–72, 26–110, 34–88, 38–90, and 93–100 [16]. Therefore, these results suggest that elimination of disulfide bond 9–57, 34–88, or 38–90 in hCGβ will change the nature of its oligosaccharide structures.

In order to confirm the above, we analysed the oligosaccharide structures of βWT and β57, a mutant which was partially sensitive to Endo-H treatment and was likely to contain high mannose type and/or hybrid type oligosaccharides. Both βWT and β57 were metabolically labeled with [1-^{3}H]galactose or *N*-acetyl[1-^{3}H]glucosamine. The radioactive oligosaccharide mixtures were prepared from each subunit and the oligosaccharide structures were compared as described in Materials and methods.

To analyse the changes in oligosaccharides of βWT and β57, the radioactive oligosaccharide mixtures were subjected to ion exchange HPLC with a COSMOGEL DEAE column. As shown in Figure 2, all four oligosaccharide mixtures derived from [1-^{3}H]galactose-labeled or *N*-acetyl[1-^{3}H]glucosamine-labeled βWT and β57 were separated into four oligosaccharide fractions, one neutral (N) and three acidic (A1, A2, and A3) at different ratios. Since all the acidic fractions were converted to neutral oligosaccharides by sialidase treatment, and since the elution positions of A1, A2, and A3 corresponded to those of authentic mono-, di-, and tri-sialylated complex-type oligosaccharides, respectively (data not shown), it was suggested that the acidic nature of oligosaccharides A1, A2, and A3 was due to the presence of 1, 2, and 3 sialic acid residues in the oligosaccharides, respectively. As shown in Figure 2A

Table 1. Susceptibility of βWT and the various Cys mutants to endoglycosidase H and F.

hCGβ	Susceptibility to	
	Endo-H	Endo-F
βWT	No	Yes
β9	Partially sensitive	Yes
β23	No	Yes
β26	No	Yes
β34	Partially sensitive	Yes
β38	Partially sensitive	Yes
β57	Partially sensitive	Yes
β72	No	Yes
β88	Partially sensitive	Yes
β90	Partially sensitive	Yes
β93	No	Yes
β100	No	Yes
β110	No	Yes

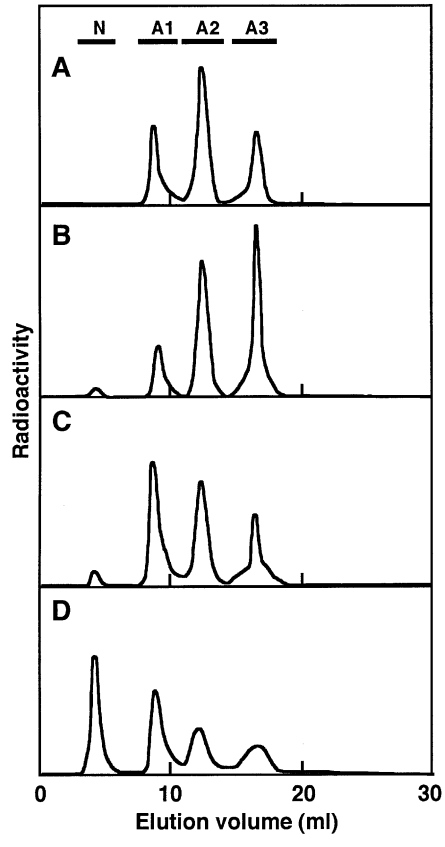

Figure 2. Ion exchange HPLC profiles of oligosaccharides obtained from metabolically labeled βWT and β57. βWT (A and B) and β57 (C and D) were metabolically labeled with [1-^{3}H]galactose (A and C) or with *N*-acetyl[1-^{3}H]glucosamine (B and D) and the radioactive oligosaccharide fraction obtained from each subunit was analysed by ion exchange HPLC on a COSMOGEL DEAE column as described in Materials and methods.

and 2B, both oligosaccharide mixtures from [1-^{3}H]galactose-labeled βWT and from *N*-acetyl[1-^{3}H]glucosamine-labeled βWT gave three major acidic peaks (A1 to A3). Levels of radioactivity detected in the neutral oligosaccharide fractions were 0 and 2% of the total, respectively. Since βWT was resistant to Endo-H treatment (Figure 1), it was presumed that almost all of the oligosaccharides in βWT were mono-, di-, and tri-sialylated complex type oligosaccharides.

On the other hand, different elution profiles were shown between the oligosaccharides of β57 labeled with [1-^{3}H]galactose or with *N*-acetyl[1-^{3}H]glucosamine. Although the oligosaccharide mixture obtained from [1-^{3}H]galactose-labeled β57 gave three major acidic peaks (Figure 2C) as in the case of βWT (Figure 2A), that from *N*-acetyl[1-^{3}H]glucosamine-labeled β57 gave a major neutral peak in addition to three acidic peaks (Figure 2D). The amount of the radioactivity in the neutral oligosaccharide fraction was 31% of the total, while that from [1-^{3}H]galactose-labeled β57 (Figure 2C) was only 3%. Therefore, it was suggested that β57, in contrast to βWT, contained a considerable amount of neutral oligosaccharides without a galactose residue, as well as mono-, di-, and tri-sialylated oligosaccharides with galactose residues. Since a galactose residue is present in complex type and hybrid type oligosaccharides but not in the high mannose type and since an *N*-acetylglucosamine residue is found in all types of N-linked oligosaccharides [17–19], most of the radioactivity in the neutral oligosaccharide fraction of the *N*-acetyl[1-^{3}H]glucosamine-labeled β57 could be attributed to the high mannose type oligosaccharides in the fraction.

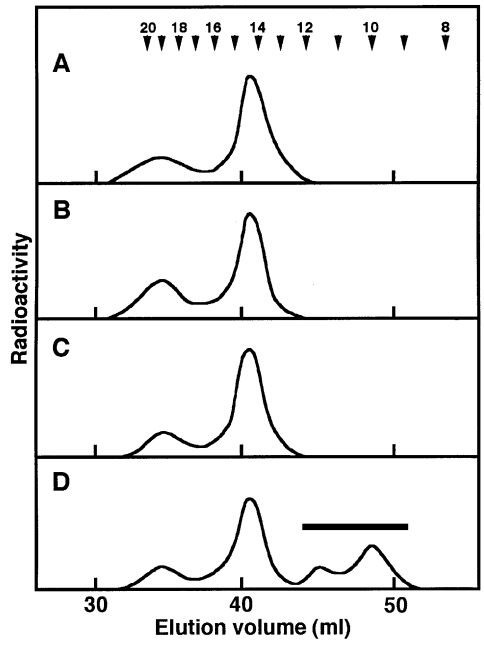

Figure 3. Bio-Gel P-4 column chromatogram of neutral oligosaccharides obtained from metabolically labeled βWT and β57. The radioactive neutral oligosaccharides of βWT and β57 were subjected to HPLC on a Bio-Gel P-4 column (1×130 cm) as described in Materials and methods. (A) [1-^{3}H]galactose-labeled βWT; (B) *N*-acetyl[1-^{3}H]glucosamine-labeled βWT; (C) [1-^{3}H]galactose-labeled β57; (D) *N*-acetyl[1-^{3}H]glucosamine-labeled β57. Arrows indicate eluting positions of glucose oligomers added as internal standards and numbers indicate glucose units. A bar indicates the region where a series of high mannose type oligosaccharides are eluted [20]. The radioactivity in each tube was measured by liquid scintillation spectrometry.

For further structural analysis of the oligosaccharide chains of hCGβ, the neutral oligosaccharide mixtures obtained by sialidase treatment of the oligosaccharide fraction containing N, A1, A2, and A3 were subjected to HPLC on a Bio-Gel P-4 column as described in Materials and methods. As shown in Figure 3A–C, the neutral oligosaccharide mixture obtained from βWT and [1-^{3}H]galactose-labeled β57 were eluted between glucose units 13 to 20 on the Bio-Gel P-4 column, a region where the authentic complex type oligosaccharides with two to four *N*-acetyllactosamine residues are also eluted [20]. The radioactive oligosaccharides in Figure 3A–C were not susceptible to α-mannosidase (data not shown). These results suggest that the radioactive oligosaccharides in Figure 3A–C are the complex type with two to four *N*-acetyllactosamine residues. On the other hand, the neutral oligosaccharide mixture from the *N*-acetyl[1-^{3}H]glucosamine-labeled β57 reveals the existence of additional oligosaccharides which elute in the region of glucose units 9 to 12 (bar in Figure 3D), a region where a series of high mannose type oligosaccharides are eluted [20]. The latter oligosaccharide fraction was degraded upon incubation with α-mannosidase (data not shown). It is, therefore, suggested that β57 contains a significant amount of high mannose type oligosaccharide chains that are not detected in the oligosaccharide fraction from βWT. This strongly supports the findings shown in Figure 1 and indicates that the elimination of certain disulfide bonds resulting in a change in protein conformation disturbed the normal processing of N-linked oligosaccharides to the mature complex type in hCGβ molecule.

It has been reported that the six disulfide bonds in the hCGβ molecule form sequentially in the order of 34–88, 9–57/38–90 (order not established), 23–72, 93–100, and 26–110 and that the first three disulfide bonds are essential to the folding of the subunit and its secretion [2, 21, 22]. Our present study demonstrates that elimination of an earlier forming disulfide bond, 34–88, or 9–57 or 38–90, interferes with the completion of glycosylation and that the elimination of a later forming disulfide bond, 23–72, 93–100, or 26–110, does not influence the complete oligosaccharide assembly. It is therefore suggested that early disulfide bond formation involved in hCGβ folding, which is essential for assembly with the α-subunit, secretion, and stability [2], is important for the normal processing of N-linked oligosaccharides and that the maintenance of protein conformation by disulfide bonds is crucial in oligosaccharide processing.

The high mannose type oligosaccharides that are attached to peptide and are processed in the rough endoplasmic reticulum are further transformed into the mature complex type *via* the hybrid type by various enzymes in the Golgi [17]. The hCGβ molecule with altered conformation caused by elimination of a specific disulfide bond may not be susceptible to these enzymes or may enter an irregular pathway. The structural differences observed in N-linked oligosaccharides of the mutants suggest that each of the disulfide bonds may play a specific role in oligosaccharide processing. In conclusion, the present study indicates that protein folding is a key factor in ensuring correct oligosaccharide formation.

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