Processing of O-linked Glycosylation in the Chimera Consisting of α-Subunit and Carboxyl-terminal Peptide of the Human Chorionic Gonadotropin β-Subunit is Affected by Dimer Formation with Follicle-stimulating Hormone β-Subunit

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Abstract. hCG, LH, FSH, and TSH are a family of heterodimeric glycoprotein hormones that contain a common α-subunit, but differ in their hormone-specific β-subunits. hCG is unique among β-subunits due to a carboxyl-terminal peptide (CTP) bearing four O-linked oligosaccharides. We previously reported that there were differences in O-glycosylation between two chimeras consisting of α-subunit and CTP, i.e. a variant with CTP at the N-terminal region (Cα) and another analog with CTP at the C-terminus (αC) of the α-subunit. To address whether O-glycosylation is influenced by the heterodimer formation, Cα and αC were expressed alone or with FSHβ-subunit in Chinese hamster ovary cells. The O-linked glycosylation was assessed by continuous labeling with [35S]methionine/cysteine, immunoprecipitation with anti-α or anti-FSH serum, serial digestion with endoglycosidase-F and neuraminidase, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The decrease in molecular weight of dimeric chimeras digested with endoglycosidase-F was greater in Cα than that in αC after treatment with neuraminidase, revealing that both chimeras have different numbers of sialic acids on O-linked carbohydrates. By treating with endoglycosidase-F, the dimeric αC migrated faster than its free form, whereas the mobility difference between assembled and unassembled forms of Cα was very little. These data indicate that processing of O-glycosylation is affected by the backbone polypeptide chain(s).

Key words: hCGβ-subunit, α-subunit, Carboxyl-terminal peptide, O-Linked glycosylation

THE family of glycoprotein hormones includes pituitary TSH, LH, FSH, and placental hCG. These hormones are noncovalently associated heterodimers consisting of dissimilar polypeptide chains, designated α and β. The unique β-subunit determines biological specificity, whereas the α-subunits of these hormones have identical amino acid sequences within the same species except for heterogeneity at their amino termini [1, 2]. The crystal structure of hCG revealed that the glycoprotein hormones are members of the superfamily of cystine-knot growth factors [3]. Both α and hCGβ have two N-glycosylation sites at Asn52 and Asn78, and Asn13 and Asn30, respectively. hCGβ is unique among β-subunits due to a carboxyl-terminal peptide (CTP) bearing four O-linked oligosaccharides attached to Ser121, Ser127, Ser132, and Ser138 [4] (Fig. 1). It has been shown that the CTP does not play roles in the assembly, secretion and in vitro bioactivity of hCG [5–7], but is important for maintaining the longer half-life of hCG [6]. The structures of O-linked carbohydrates in hCGβ-subunit have been elucidated using the urine of patients with trophoblastic diseases and healthy pregnant women [8], and the choriocarcinoma cell line, BeWo [9]. The major backbone structures identified are galactose (Gal) β1→3GalNAc, and Galβ1→4N-acetylglucosamineβ1→6 (Galβ13) GalNAc, each of which has zero, one or...
two N-acetylneuraminic acids (Fig. 2). Assembly of O-linked oligosaccharides is initiated by the transfer of GalNAc from UDP-GalNAc to a core protein acceptor. This step is catalyzed by UDP-GalNAc:polypeptide N-acetylgalactosaminyl transferase (GalNAc-T) in the cis-Golgi compartment [10].

Previously, we fused the CTP to the α-subunit between amino acid residues 3 and 4 in the amino terminal region (Cα) or at the carboxyl end (αC) [11]. When the chimeras were expressed with FSHβ-subunit in Chinese hamster ovary (CHO) cells and terminal sialylation of O-linked carbohydrates in the CTP of Cα and αC were analyzed by SDS-PAGE. Here we report that 1) the number of sialic acids on O-glycosylation differs between heterodimer-forming Cα and αC, and 2) association with FSHβ-subunit alters the maturation of O-glycosylation in the CTP fused to the α-subunit.

Materials and Methods

Vector construction

The construction of the vectors has been described previously [11]. In brief, the nucleotide sequence encoding the CTP was inserted in the frame between amino acid residues 3 and 4 or at the carboxyl end of the human hCGα-subunit sequence using polymerase chain reaction (PCR). The PCR product was inserted into the eukaryotic expression vector, pM2 [12]. Expression vectors for αWT (pMα), FSHβWT (pMFαFSHβ), and hCGβWT (pMhCGα) were also described previously [13, 14]. The WT and chimeric subunits are depicted in Fig. 1.

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 for chimeric α were transfected alone or with pMFαFSHβ into the cells by the calcium phosphate method [5]. The stable clones were selected with
0.25 mg/ml of the neomycin analog G418 (Life Technologies, Gaithersburg, MD, USA) as described previously [15]. Single colonies were isolated and maintained in culture in the presence of 0.125 mg/ml G418.

Metabolic labeling and immunoprecipitation

Stable clones were plated onto 60-mm petri dishes and grown to near confluence. CHO cells were labeled for 18 h with 25 μCi/ml of Tran35S-label ([35S]methionine/cysteine, ICN, Irvine, CA, USA; SA, >1000 Ci/mmol) in labeling medium (conditioned medium minus methionine, cysteine and G418, but supplemented with 7.5% dialyzed FBS). Polyclonal antisera against α- and hCGβ-subunits were gifts from Dr. Irving Boime, Washington University School of Medicine, St. Louis, MO, USA. Polyclonal antiserum directed against FSH dimer was obtained from the National Hormone and Pituitary Distribution Program (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, USA).

Enzymatic digestion

Endoglycosidase treatment has been described previously [16]. Endoglycosidase-F digestion was performed in 18 μl F1-buffer (50 mM sodium acetate, 50 mM EDTA, 10 mM sodium azide, 0.5% n-octyl-glucoside, 0.1% SDS, 12.5 mM 2-mercaptoethanol, pH 5.5) at 37°C for 24 h with 0.1 U endoglycosidase-F (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). For neuraminidase and endoglycosidase-F serial digestion, samples were first digested with 7 mU neuraminidase (Boehringer Mannheim Biochemicals) in 18 μl N-buffer (50 mM sodium acetate, 1 mM calcium chloride, pH 5.5) at 37°C for 24 h, and then 0.1 U endoglycosidase-F and F2-buffer (50 mM sodium phosphate, 50 mM EDTA, 0.5% Triton X-100, 0.1% SDS, 12.5 mM 2-mercaptoethanol, pH 7.2) were added, followed by incubation for another 24 h. Samples were subjected to 15% SDS-PAGE as previously described [13].

Results

Difference of O-linked oligosaccharides between assembled forms of Ca and αC with FSHβ-subunit

We previously observed that the apparent molecular weight of secreted Ca was greater than that of αC. The difference was due to O-linked oligosaccharides because the electrophoretic mobilities were still different between the chimeras after removing N-linked oligosaccharides. However, the difference in mobility of endoglycosidase-F treated chimeras was not evident after digestion with neuraminidase. Thus, the data indicated that the number of sialic acids on O-linked oligosaccharides in Ca was greater than that in αC [11]. To examine the O-linked oligosaccharides on heterodimer-forming α-chimeras, clones expressing FSHβ WT along with αWT (Fig. 3, lanes 1, 4, and 7), Ca (lanes 2, 5, and 8), and αC (lanes 3, 6, and 9) were continuously labeled with [35S]methionine/cysteine for 18 h. The medium from each clone was immunoprecipitated with FSH dimer-specific antiserum. Thus, the immunoprecipitable subunits were not unassembled but assembled ones. They were treated with endoglycosidase-F alone or both endoglycosidase-F and neuraminidase, followed by SDS-PAGE. The assembled αC with or without endoglycosidase-F treatment migrated faster than the assembled Ca (lanes 2, 3, 5, and 6). The migration of assembled Ca after digestion with both endoglycosidase-F and neuraminidase was identical with that of assembled αC (lanes 8 and 9). The difference in mobility of endoglycosidase-F treated chimeras before and after digestion with neuraminidase was greater in Ca than that in αC (lanes 5, 6, 8, and 9). Although sialic acid can attach to both the N- and O-linked oligosaccharides, the increased electrophoretic mobility of endoglycosidase-F treated chimeras by neuraminidase digestion reflects the loss of sialic acids on O-glycosylation. The data showed that, similar to free forms, assembled Ca contained more sialic acids on O-linked oligosaccharides than assembled αC.

Effect of dimer formation with FSHβ-subunit on O-linked oligosaccharides in chimeric α-subunits

It has been reported that there are some differences in N-glycosylation between assembled (dimer) and unassembled (free) α-subunits [17–19]. Previously, Ca
and αC were expressed with hCGβ-subunit in CHO cells [11]. Unfortunately the electrophoretic mobilities of the chimeric α-subunits were indistinguishable from that of hCGβ-subunit because the molecular weights of both subunits were very similar. Therefore, the difference between free and dimer forms of chimeric α-subunits could not be assessed by SDS-PAGE. To elucidate whether β-subunit affects O-glycosylation in chimeric α-subunits, clones expressing chimeric α alone or with FSHβWT were continuously labeled with $[^{35}S]$methionine/cysteine for 18 h. The medium from each clone was immunoprecipitated with FSH dimer-specific antiserum, followed by treatment with endoglycosidase-F (lanes 4–6), both endoglycosidase-F and neuraminidase (lanes 7–9), or no enzyme (lanes 1–3). The digested samples were then run on 15% SDS-PAGE. The asterisks, arrows, and arrowheads indicate the chimeric α-, FSHβWT-, and αWT-subunits, respectively. Molecular weight markers (kDa; Amersham) are shown at the left.

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<th>endoglycosidase-F (–)</th>
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**Fig. 3.** Glycosidase treatment of heterodimers consisting of chimeric α-subunit and FSHβWT-subunit. Clones expressing both FSHβWT and either αWT (lanes 1, 4, and 7), Cα (lanes 2, 5, and 8), and αC (lanes 3, 6, and 9) were continuously labeled with $[^{35}S]$methionine/cysteine for 18 h. The medium from each clone was immunoprecipitated with FSH dimer-specific antiserum, followed by treatment with endoglycosidase-F (lanes 4–6), both endoglycosidase-F and neuraminidase (lanes 7–9), or no enzyme (lanes 1–3). The digested samples were then run on 15% SDS-PAGE. The asterisks, arrows, and arrowheads indicate the chimeric α-, FSHβWT-, and αWT-subunits, respectively. Molecular weight markers (kDa; Amersham) are shown at the left.

**Fig. 4.** Effect of FSHβ-subunit on O-linked oligosaccharides in chimeric α-subunits. Clones expressing chimeric α alone or with FSHβWT were continuously labeled with $[^{35}S]$methionine/cysteine. The medium from each clone was immunoprecipitated with antiserum against α (lanes 1 and 3) or FSH dimer (lanes 2 and 4). Immunoprecipitable samples were treated with endoglycosidase-F and then were subjected to SDS-PAGE. Although the difference in mobility between unassembled and assembled Cα was very little (lanes 1 and 2), that of αC was more evident (lanes 3 and 4). These data demonstrated that dimer formation with FSHβ-subunit affected O-glycosylation in chimeric α-subunits.
Discussion

Several studies on N-glycosylation of gonadotropins have shown that 1) although the α-subunit is common, maturation of oligosaccharides is dependent on the formation of the different heterodimer-complexes [20–22], and 2) glycosylation is site-specific even in the same subunit [23–25]. These facts indicate that N-glycosylation is influenced by the surrounding polypeptide chain(s) at a given site. In contrast to N-glycosylation, few studies have been focused on the relationship between protein structure and O-glycosylation. In the present study we showed that the number of sialic acids in O-linked oligosaccharides in the CTP differed between dimer-forming Ca and αC, and that association with FSHβ-subunit altered the maturation of O-glycosylation in chimeric α-subunits, especially αC. Because O-glycosylation of Ca and αC was compared using the same cell system, the different maturation shown in the present study is not due to the different glycosylation enzyme levels. Rather, it is likely that, similar to N-glycosylation, the three-dimensional structure of the polypeptide portion of the molecule controls the maturation of O-linked sugar moieties.

Free but not combined bovine α-subunit has been shown to have O-linked sugar at Thr\textsuperscript{39} (corresponding to Thr\textsuperscript{39} in human α-subunit) [26]. In human, the free α-subunit expressed in the choriocarcinoma cell line, JAR, has O-linked sugar [27]. But no O-linked sugar has been demonstrated in α-subunit synthesized in the pituitary [21]. O-glycosylation is mainly a post-translation and post-folding event, hence only exposed serines and threonines will be glycosylated [28]. The reason why associated α-subunit cannot have O-glycosylation is considered to be steric hindrance of the glycosylation site by hCGβ-subunit [26, 27]. In the present study we have shown that free αC has an apparently higher molecular weight than the assembled form with FSHβ-subunit, and that the difference is due to O-glycosylation. Our recent study has demonstrated that the amino acid change of amino terminal region of the α-subunit generated the attachment of O-linked oligosaccharides [29], hence one may assume that the O-glycosylation occurred at Thr\textsuperscript{39} of αC. The lower molecular weight of assembled αC might be due to the lack of O-glycosylation at Thr\textsuperscript{39} because the region including Thr\textsuperscript{39} is also a contact site to FSHβ-subunit [30, 31]. However, the electrophoretic mobility after treatment with endoglycosidase-F and neuraminidase was identical between free and assembled αC (data not shown). If O-glycosylation occurred at Thr\textsuperscript{39} of αC, free αC should have migrated slower than the assembled form. Therefore, it is unlikely that the O-linked carbohydrate chain was created at Thr\textsuperscript{39} of αC.

It is of interest that FSHβ-subunit affected O-glycosylation in αC but less in Ca. The key to resolve this issue may be the position of α-subunit to which the CTP was fused. It is well known that free subunits are biologically inactive, and that dimer formation predicts biological activity of the CG, LH, FSH, and TSH [1]. The conformational change of the α-subunit occurs during dimer formation with β-subunits [32, 33]. Recently, a chimera consisting of a single chain construct of α- and hCGβ-subunits has been shown to have biological activity despite significant loss of quaternary and tertiary structure in several regions of the molecule [34, 35]. These findings imply that contact sites with the receptors are configured by the interaction between α- and β-subunits. Because the C-terminus, but not the N-terminus of the α-subunit is important for receptor binding [11, 36], it is simply assumed that the conformation of C-terminus of the α-subunit is much more modified by the β-subunit than the N-terminus. Thus, it appears that the conformational change of the C-terminus by assembly with FSHβ-subunit accounts for the change in O-glycosylation of αC.

The present study demonstrated that O-glycosylation in the CTP fused to the α-subunit was affected by the FSHβ-subunit, which in turn arouses interest as to whether O-glycosylation in the β-subunit is affected by the α-subunit. Because the hCGβ-subunit contains O-linked oligosaccharides, free and assembled hCGβ\textsubscript{WT} were similarly analyzed in SDS-PAGE (data not shown). Unexpectedly, the CTP in hCGβ-subunit was not influenced by the α-subunit. Although both subunits noncovalently combine and form glycoprotein heterodimers, the nature of their configuration seems to be different from each other. As stated above, the α-subunit associates with each of the four glycoprotein hormone β-subunits. Furthermore, the conformational change of the α-subunit occurs during dimer formation with β-subunits. This adaptability, taken together with the data presented here, leads to the view that the structure of the α-subunit is flexible and dominated by the β-subunit.

That the CTP sequence was successfully fused to
FSHβ- [37], TSHβ- [38], and α-subunits [11] indicated that all information for the recognition of O-glycosylation was contained within the CTP sequence. However, it remains unclear whether the information is identical in all CTP-containing polypeptides. Regarding the maturation of O-glycosylation in the CTP, it is unknown whether the determinant is included in the structure of the CTP. Our findings strongly suggest that sequences outside the CTP influence the maturation of O-glycosylation in the CTP probably due to differences in the conformation created in the different regions of the subunits. In such a case, the initial step of O-glycosylation by GalNAc-T and elongation of the sugar chain might be controlled by independent factors.

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