Site-specific Processing of the N-Linked Oligosaccharides of the Human Chorionic Gonadotropin \( \alpha \) Subunit

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Two forms of the gonadotropin \( \alpha \) subunit are synthesized in placenta and in human chorionic gonadotropin (hCG)-producing tumors: an uncombined (monomer) form and a combined (dimer) form. These forms show differences in their migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The slower migration of the monomeric form on sodium dodecyl sulfate-polyacrylamide gel electrophoresis has been attributed to a different glycosylation pattern. Previous studies demonstrated different roles of each of the two \( \alpha \) N-linked glycosylation sites (Asn-52 and Asn-78) in secretion of the uncombined subunit and the biologic activity of hCG dimer. To assess the influence of formation of dimer on the processing pattern at the individual sites, we characterized the N-linked oligosaccharides of monomer and dimer forms of recombinant human choriogonadotropin \( \alpha \) subunit. Two approaches were employed. First, site-directed mutagenesis was used to alter the two N-linked oligosaccharide attachment sites, thus allowing the expression of \( \alpha \) subunits containing only one glycosylation site. Second, tryptic glycopeptides of the wild-type subunits were examined. Concanavalin A (ConA) binding and sialic acid content indicated that the oligosaccharides at each glycosylation site of the uncombined \( \alpha \) subunit are processed differently. Oligosaccharides present at Asn-52 are almost exclusively ConA-unbound and contain three sialic acid residues. The majority of Asn-78-linked oligosaccharides are ConA-bound and disialylated. Both sites are processed independently because no significant differences were observed between the oligosaccharides at the same sites in wild-type and mutant monomeric \( \alpha \) subunits. By contrast, the majority of the oligosaccharides at both glycosylation sites of the dimer \( \alpha \) are bound to ConA. Thus, combination primarily affects the processing pattern of the Asn-52-linked species. Because glycosylation at this site is essential for hCG assembly and signal transduction, these data imply a critical link between the site-specific processing and hormone function.

Human chorionic gonadotropin (hCG) is a heterodimer composed of noncovalently bound \( \alpha \) and \( \beta \) subunits secreted by the placenta in early pregnancy (1). In addition to the biologically active dimer, the placenta secretes an uncombined form of the \( \alpha \) subunit and its synthesis persists throughout gestation even after synthesis of the \( \beta \) subunit ceases (2, 3). This monomeric \( \alpha \) subunit has a slower mobility on SDS-PAGE than the combined \( \alpha \), and the larger molecular weight is apparently due to differences in the processing of its N-linked oligosaccharides (4, 5). The \( \alpha \) subunit contains two N-linked oligosaccharides attached to asparagine (Asn) 52 and 78. Because the combination of \( \alpha \) and \( \beta \) subunits occurs early in the secretory pathway (6, 7), further processing steps of the N-linked oligosaccharides of dimer \( \alpha \) are influenced by the presence of the \( \beta \) subunit (8, 9). Previous studies from our laboratory suggested a different role of the N-linked oligosaccharides at each of the two glycosylation sites of the subunit in the stability and secretion of the monomeric form (10), and in assembly and steroidogenic activity of dimer hCG (10, 11). To assess if these site-specific functions are reflected by different processing patterns of the individual oligosaccharide units and how \( \alpha \) and \( \beta \) combination influences this processing, we characterized the two glycosylation sites present on the monomer and dimer \( \alpha \) forms. This was done by analysis of tryptic glycopeptides derived from wild-type subunits and oligosaccharides released by endoglycosidase F from \( \alpha \) glycosylation mutants. These \( \alpha \) mutants were generated by site-directed mutagenesis and were expressed in Chinese hamster ovary cells transfected with vectors bearing the \( \alpha \) and \( \beta \) genes (10). This approach allows the synthesis of \( \alpha \) subunits containing only one glycosylation site by abolishing the glycosylation recognition sequence at the other. Here we show that the oligosaccharide processing pattern of the uncombined \( \alpha \) subunit is site-specific. Moreover, \( \alpha \) dimerization primarily influences processing of the Asn-52-linked oligosaccharides which may be an important determinant in the formation of the biologically active hormone.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media were obtained from the Washington University Center for Basic Cancer Research. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-trypsin was from Worthington, aconitine from Fisher, and trifluoroacetic acid and triethylamine from Pierce Chemical Co. All other reagents, unless stated otherwise were purchased from Sigma.

Methods—The construction of glycosylation mutants has been described previously (10). Briefly, the mutated gonadotropin \( \alpha \) subunit genes were generated by convering asparagine 52 or asparagine

1 The abbreviations used are: hCG, human chorionic gonadotropin; CHO, Chinese hamster ovary cells; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; ConA, concanavalin A; Endo F, endoglycosidase F.
78 codons to aspartic acid codons. Expression vectors containing the wild-type or mutant \( \alpha \) genes were transfected into Chinese hamster ovary (CHO) cells, and stable lines were selected. For hCG dimer expression, vectors bearing \( \alpha \) and hCG\( \beta \) genes were co-transfected into the CHO cells (10).

Cell Labeling and Subunit Isolation—The transfected cells were grown in Ham’s F-12 medium containing 100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine supplemented with 5% fetal calf serum in the presence of 125 \( \mu \)g/ml of G418 (Geneticin; Gibco). Near-confluent cultures were washed twice with the above medium containing 5% of the normal glucose concentration (90 mg/liter). Subsequently, cells were incubated with 50 \( \mu \)g/ml of \(^3\)H glutamine (ICN Radiochemicals or American Radiolabeled Chemical Inc., St. Louis, MO; specific activity = 30 Ci/mmol) for 16 h in a low glucose medium supplemented with 5% dialyzed calf serum (Gibco) without G418. Incubation with 50 \( \mu \)g/ml of \(^{35}\)S-cysteine (ICN; specific activity > 1000 Ci/mmol) was performed in F-12 medium without cysteine. After incubation, media were collected and preabsorbed with normal rabbit serum followed by precipitation with Pansorbin (Calbiochem) (4). Supernatants were precipitated with polyclonal \( \alpha \) or \( \beta \) antisera (the latter was used to precipitate dimer \( \alpha \) subunit) overnight at 4°C. After incubation with Pansorbin, the immunoprecipitates were subjected to SDS-PAGE and fluorography (4). Radioactive bands corresponding to monomer and dimer \( \alpha \) subunit were eluted with distilled water containing 0.02% NaN3, dialyzed, lyophilized, and reduced and carboxymethylated (12).

N-Linked Oligosaccharide and Glycopeptide Isolation—The N-linked oligosaccharides were isolated by endoglycosidase F (Du Pont-New England Nuclear) digestion. The reduced and carboxymethylated subunits were dissolved in 200 \( \mu \)l of 50 mM cacodylate buffer (pH 6.0) and incubated with 0.1 unit of Endo F (Du Pont-New England Nuclear) overnight at 37°C.

Tryptic glycopeptides were resolved by reverse-phase HPLC as described (12), and \(^3\)H-glucosamine or \(^{35}\)S-cysteine containing peaks were collected for further analysis. Concanavalin A-Sepharose Chromatography—Endo F-released oligosaccharides or tryptic glycopeptides were divided into two aliquots. One was applied to a 3-ml concanavalin A-Sepharose (Pharmacia Fine Chemicals AB,Upsala, Sweden) column, and the bound material was not eluted, reduced, and carboxymethylated. The other was eluted, reduced, and carboxymethylated. The reduced and carboxymethylated subunits were applied to a 3-ml concanavalin A-Sepharose column, and the bound material was eluted with distilled water containing 0.02% NaN3, dialyzed, lyophilized, and reduced and carboxymethylated (12).

Ion-exchange HPLC—A MicroPak AX-10 column (Varian) was used and the oligosaccharides were separated with a gradient of KH2PO4 (pH 4.0) (14).

RESULTS

Previous studies from our laboratory (4) and others (5) showed that, in transfected heterologous cells expressing hCG, the uncombined \( \alpha \) subunit migrates slower on SDS-PAGE than the dimer \( \alpha \); this was attributed to differences in the processing of the N-linked oligosaccharides (4, 5). Similar differences in N-linked oligosaccharides between dimer and monomer \( \alpha \) subunits have also been observed in subunits isolated from urine of pregnant women (15). These data together with the observed hormone-specific glycosylation of the glycoprotein hormone family (8, 9) led to the conclusion that \( \alpha \beta \) subunit dimer formation determines the \( \alpha \) subunit glycosylation pattern. To study the influence of dimerization on the processing of \( \alpha \) subunit oligosaccharides, we characterized the oligosaccharide population present at a particular glycosylation site on monomeric and dimer \( \alpha \) subunits using two different approaches. First, we analyzed concanavalin A binding properties and extent of sialylation of oligosaccharides cleaved by Endo F from \( \alpha \) subunit mutants which contain only one glycosylation site. Second, these results were verified by similar analysis of tryptic glycopeptides derived from wild-type \( \alpha \) subunits containing both glycosylation sites. This was necessary because we could not exclude the possibility that the lack of one oligosaccharide in a mutant may influence the processing pattern of the remaining one.

Characterization of Oligosaccharides from Glycosylation Mutants of Gonadotropin \( \alpha \) Subunit—Glycosylation mutants of the gonadotropin \( \alpha \) subunit were generated by site-directed mutagenesis (10). Substitution of either the asparagine or threonine residues in the Asn-X-Thr recognition sequence at either of the two \( \alpha \) glycosylation sites abolishes glycosylation, and the structures present on each individual site can be studied separately. The mutant and wild-type monomeric \( \alpha \) subunits and hCG dimer were expressed in CHO cells transfected with vectors containing either \( \alpha \) alone or both \( \alpha \) and \( \beta \) genes (10). Glycosylation mutants are easily distinguishable from the native forms by an enhanced migration on SDS-PAGE (Fig. 1).

To isolate the N-linked oligosaccharides, the CHO cells expressing mutants of uncombined \( \alpha \) or dimer hCG\( \alpha \) were labeled with \(^3\)H-glucosamine. After SDS-PAGE, the subunits were eluted, reduced, and carboxymethylated. N-Linked oligosaccharides were released with Endo F which cleaves complex, hybrid-, and high mannose-type oligosaccharides (16). \(^3\)H-glucosamine-labeled oligosaccharides derived from the individual glycosylation sites were analyzed by ConA-Sepharose chromatography according to Cummings and Kornfeld (13). This method allows a classification of the oligosaccharides based on lectin binding properties. In general, biantennary complex-type structures are weakly bound and elute with 10 mM \( \alpha \)-methylglucoside. More extensively processed highly branched or bisected forms do not bind to the lectin and are recovered in the buffer wash. The least processed structures, such as high mannose- or hybrid-type, are tightly bound to the column and are eluted with warm 200 mM \( \alpha \)-methylmannoside.

When the Asn-52-linked oligosaccharides from the site 78 mutant were subjected to ConA-Sepharose chromatography, more than 90% of \(^3\)H-glucosamine-labeled material was not bound to the lectin, and only a small fraction eluted with \( \alpha \)-methylglucoside (Fig. 2, panel A). This suggested that the Asn-52 glycosylation site contains structures larger than biantennary. Oligosaccharides derived from the mutant containing...
only the Asn-78 glycosylation site showed a more heterogeneous ConA binding profile (Fig. 2, panel B). The ConA unbound fraction was reduced to 25–30% of the recovered radioactivity, whereas 70% was eluted with a-methylglucoside with the remainder tightly bound to the lectin. Therefore, oligosaccharide processing is different at each of the glycosylation sites in monomeric α subunits.

We also studied the Asn-52-linked oligosaccharides of the mutant dimer hCGα. The ConA binding profile of this oligosaccharide population from dimer (Fig. 2, panel C) was completely different from that observed for the free α subunit (Fig. 2, panel A). The majority of the oligosaccharides at this site bound to ConA, suggesting the presence of structures not larger than biantennary or an unusual hybrid previously described for hCG dimer (17, 18). The unbound fraction amounted to only 20% of the recovered radioactivity. The ConA binding pattern of Asn-52-linked oligosaccharides derived from the dimer containing mutant α subunit was therefore more similar to the oligosaccharides derived from Asn-78 than to the Asn-52 oligosaccharides in the monomeric α mutant. Thus, the presence of the β subunit alters the processing at the Asn-52 glycosylation site of the α dimer resulting in less complex structures.

Characterization of Tryptic Glycopeptides of Free and Dimer α Subunits—As discussed above, the removal of one glycosylation site from the α subunit may influence the processing pattern of the other site due to steric changes in the mutated subunit. To address this point, the ConA binding properties of each of the glycosylation sites in the wild-type monomer and dimer α subunits were examined. CHO cells were labeled in the presence of [3H]glucosamine or [35S]cysteine, and tryptic peptides were separated by reverse-phase HPLC. The elution times of Asn-52- and Asn-78-containing peptides have been established previously (12). Both glycopeptides were subjected to ConA chromatography.

The ConA binding properties of [3H]glucosamine-labeled glycopeptides of monomer α subunit were almost identical to that observed for oligosaccharides isolated from analogous glycosylation sites in monomer α mutants (Fig. 3). Very little of the Asn-52-containing glycopeptide bound to ConA (panel A), similar to the oligosaccharides released by Endo F from the Asn-52 site in the Asn-78 mutant. The Asn-78-containing glycopeptide was resolved into an unbound fraction (26%) and a weakly bound fraction (70%) with a small amount tightly bound to the lectin (panel B). These results confirm the data obtained from the analysis of the mutants that the oligosaccharide processing at the two glycosylation sites is very different. The similar ConA profiles of Asn-52- and Asn-78-linked oligosaccharides from mutants and wild-type monomeric α suggest that absence of the oligosaccharide from one glycosylation site did not influence the general processing pattern at the remaining site.

In contrast, the ConA binding pattern of the two glycopeptides derived from the dimer hCGα are very similar; about 80% of [3H]glucosamine counts were recovered in the a-methylglucoside-eluted fraction (Fig. 3, panels C and D). Presumably, these oligosaccharides correspond to biantennary or unusual hybrid-type structures (17). About 8% does not bind ConA, and the remainder binds tightly to the lectin. The difference in oligosaccharide processing after αβ assembly is manifested by a reduction in the amount of the ConA-unbound species, mainly at the Asn-52 site. The processing of N-linked oligosaccharides of the α subunit is less extensive when the subunit traverses the secretory pathway bound to the β subunit.

The Asn-78-containing glycopeptides of uncombined α subunit elutes from reverse-phase HPLC close to the peptide which contains a potential O-linked glycosylation site (4). The heterogeneous ConA binding of oligosaccharides linked to Asn-78 in uncombined α suggested that a fraction of the [3H]glucosamine-labeled material not bound to ConA is due to contamination from an O-linked glycosylated peptide. To address this point, ConA binding of glycopeptides derived from [35S]cysteine-labeled α subunits was examined. Analysis of [35S]cysteine glycopeptides also provides better comparison of the distribution of ConA-separated fractions at a single glycosylation site. Depending on the extent of processing, varying amounts of [3H]glucosamine can be incorporated into different oligosaccharides present at the same glycosylation site; however, each oligosaccharide is linked to a peptide containing the same number of cysteines. The cysteine-containing tryptic peptides bearing the N-linked glycosylation sites do not elute in the position of the peptide containing the putative O-linked glycosylation site (12). The ConA binding profiles of [35S]cysteine-labeled tryptic glycopeptides in both uncombined and dimer α were similar to [3H]glucosamine-labeled peptides (data not shown). The results are summarized
TABLE 1

Concanavalin A binding of [35S]cysteine-labeled glycopeptides

The values are expressed as a percentage of total counts recovered from the column. aMG, 10 mM α-methylglucoside eluted fractions; αMM, 200 mM α-methylmannoside eluted fractions.

<table>
<thead>
<tr>
<th>ConA-unbound</th>
<th>ConA-bound</th>
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<tbody>
<tr>
<td></td>
<td>αMG</td>
</tr>
<tr>
<td>Monomer α Asn-52</td>
<td>91</td>
</tr>
<tr>
<td>Monomer α Asn-78</td>
<td>21</td>
</tr>
<tr>
<td>Dimer α Asn-52</td>
<td>8</td>
</tr>
<tr>
<td>Dimer α Asn-78</td>
<td>14</td>
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</tbody>
</table>

in Table I and are expressed as a percentage of [35S]cysteine counts of ConA-unbound and -bound fractions derived from an individual glycopeptide. No significant difference was observed in the amount of the ConA-unbound Asn-78 glycopeptide derived from the uncombined α when [35S]cysteine counts were compared to [3H]glucosamine counts (21 versus 26%). This confirms that the ConA-unbound fraction at the Asn-78 site is derived primarily from N-linked species. The data presented in Table 1 also confirm that αβ combination dramatically changes the ConA binding properties of the oligosaccharides at the Asn-52 glycosylation site and that the amount of ConA-unbound material from the other site is much less (14 versus 21%).

Sialylation at Individual Glycosylation Sites of α Subunit—Sialic acid has been shown to terminate the N-linked oligosaccharides units in hCG (17, 18). The hCG oligosaccharides are heterogeneous: neutral, monosialylated, and disialylated species are observed (14, 18). Different amounts of sialic acid at an individual site may influence the properties of the particular domains of the hormone by changes in the amount of negative charge. We analyzed sialylation of oligosaccharides at each glycosylation site using ion-exchange HPLC (14). The ConA-unbound fraction of the Endo F-cleaved oligosaccharides derived from the Asn-52 glycosylation site in the Asn-76 mutant eluted at the position corresponding to three sialic acid residues (Fig. 4, panel A). An identical elution profile was obtained when the Asn-52-containing glycopeptide isolated by reverse-phase HPLC from the wild-type uncombined α secreted by CHO cells was analyzed (panel B). The peptide alone did not bear any significant charge which could have altered the characteristic oligosaccharide profiles in this ion-exchange system. The majority of Asn-78 oligosaccharides contains two sialic acid residues, and, despite the presence of a significant amount of ConA-unbound material, none eluted in the position of 3 sialic acid residues (panel D). Thus, both glycosylation sites of the uncombined α subunit are uniformly sialylated. The single Asn-52 glycosylation site of the mutant dimer α showed a heterogenous pattern of sialylation (panel C). The major fraction eluted in the position corresponding to a structure containing 2 sialic acid residues, but a substantial amount of neutral and monosialylated species was also observed. Similar to the Asn-78 site of the free α, ConA-unbound fraction of the mutant dimer α does not contain trisialylated species. Processing at the individual glycosylation sites of uncombined and dimer α subunit differs not only in the ConA binding properties but also in the extent of sialylation.

DISCUSSION

The variety of oligosaccharide structures found on glycoproteins results from the action of many processing enzymes in the secretory pathway (19). For glycoproteins containing multiple glycosylation sites, each site can be processed differently resulting in heterogeneous structures both in terms of size and extent of sialylation (20, 21). Also the oligosaccharides at the same glycosylation site can be processed differently in tissues (22). It has been shown that in various dimeric glycoproteins, the oligosaccharide processing on one subunit differs depending on the nature of the complementary subunit; this suggests that the quaternary structure influences the processing pattern (9, 23) and that the influence can be site-specific (23). The importance of protein conformation on N-linked oligosaccharide processing has been recently discussed (24, 25). The glycoprotein hormone α subunit is a good model to study the determinants of oligosaccharide processing because it is expressed in two different tissues (placenta and pituitary) and is found uncombined and in a noncovalent association with four different β subunits (1). The oligosaccharides derived from bovine, ovine, and human pituitary glycoprotein hormones are heterogeneous, and the heterogeneity is manifested by variations in branching, sialylation, and/or sulfation patterns (26-28).
In this work, we characterized each of the two glycosylation sites in the uncombined and dimer forms of the human gonadotropin α subunit. This was achieved using glycosylation mutants generated by site-directed mutagenesis and by the analysis of tryptic glycopeptides derived from the wild-type subunits. Based on ConA binding properties and sialylation pattern, the oligosaccharides derived from the uncombined α subunit are similar to those described for the uncombined α isolated from urine of pregnant women (15) and from trophoblast cells in culture (29). However, in those studies, the oligosaccharides were characterized as a mixture derived from both glycosylation sites. Here we show that each site of monomer α subunit bears a different population of N-linked oligosaccharides. The Asn-52 site is occupied almost exclusively by ConA-unbound species containing 3 sialic acid residues. The second glycosylation site, Asn-78, contains primarily ConA-bound, disialylated structures. The oligosaccharides at the Asn-78 glycosylation site may be less accessible or differentially recognized by the processing enzymes. The ConA binding profile of oligosaccharides from each of the two glycosylation sites was identical whether derived from glycosylation mutants containing a particular site or from the corresponding glycopeptide isolated from the wild-type uncombined α. The absence of the Asn-78 oligosaccharides, which results in the poor secretion and increased degradation of the uncombined α subunit (10), does not change the processing pattern of the Asn-52 oligosaccharides.

Combination with hCG β subunit changes the processing of α oligosaccharides (4, 5). Our data indicate that the oligosaccharides at the Asn-52 site undergo more significant changes after combination than those at the Asn-78 site. The ConA binding profile showed that the unbound species present at the Asn-52 site in uncombined α are replaced in hCG dimer by lectin-bound, less sialylated structures. The changes at the Asn-78 glycosylation site are less pronounced, but, similar to the Asn-52 site, a reduction in the amount of ConA-unbound fraction is also observed. Thus, the processing at each glycosylation site is affected differently by αβ combination. By contrast, in bovine pituitary, the oligosaccharides present on uncombined α do not differ from those present in the luteinizing hormone dimer α (27). Asn-52 (Asn-56 in bovine α) is located in a highly conserved region of the α subunit, and only a few amino acid changes between species are observed near this glycosylation site. Although the differences in the oligosaccharide processing at this site in human and bovine α subunit are species-, or tissue-specific, conformational changes due to the differences in the primary structure of those two glycoproteins may represent a major determinant for these variations.

The mechanism whereby the β subunit affects the processing pattern of Asn-52 oligosaccharides is unclear. There is evidence that this site is in proximity to the β subunit in dimer hCG and that the conformation of the dissociated subunits differs from that found in dimer (30). The β subunit may hinder the action of the processing enzymes, or conformational changes between uncombined and dimer α may be differentially recognized by glycosidases and glycosyltransferases.

The ConA-unbound fraction of the Asn-52-linked oligosaccharides derived from mutant dimer α subunit (Fig. 2, panel C) is 2-fold greater than the amount of Asn-52 glycopeptide derived from the wild-type dimer α subunit. This suggests that the conformation of the dimer is different depending on whether one or two oligosaccharide units are present on the α subunit. In contrast, absence of one glycosylation site of the free α subunit does not influence the processing of the remaining one.

The site-specific processing of the oligosaccharides on uncombined α and dimer hCGs may be correlated with different functions of each of the two glycosylation sites. As discussed above, the uncombined α mutant which lacks the Asn-78 glycosylation site is poorly secreted. However, when hCG β is co-expressed with this α mutant, the secretion is increased (10). Glycosylation at Asn-78 appears to be necessary for stabilization and efficient secretion of uncombined α. In contrast, lack of the Asn-52 oligosaccharide does not influence secretion of the uncombined α subunit but does reduce dimer formation by altering the assembly (10). Importantly, the presence of the Asn-52 oligosaccharide in the hCG dimer is essential for the biological activity of the hormone (11). This provides a link between site-specific oligosaccharide processing and the bioactivity of hCG.

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