Effect of Additional N-Glycosylation Signal in the N-Terminal Region on Intracellular Function of the Human Gonadotropin α-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. The α-subunit has two N-glycosylation sites at Asn^52 and Asn^78. To obtain more information on the relationship between the structure and function of the α-subunit, we introduced a novel N-glycosylation site in the N-terminal region by mutating Asp^5 and Gln^9 into Asn and Thr, respectively. Glycosylation mutants were expressed alone or with hCGβ-subunit in Chinese hamster ovary cells. New N-linked oligosaccharides were efficiently added to the wild-type and mutant α-subunits lacking N-glycan at Asn^78 (αΔAsn^1), Asn^78 (αΔAsn^2), and both (αΔAsn^1+2). The new sugar chain did not affect secretion and assembly except that 1) it increased the intracellular degradation of αΔAsn^1+2, and 2) it augmented the assembly of αΔAsn^1 with hCGβ-subunit. Amino acid changes generated the attachment of O-glycosylation in free α-subunit but not in assembled form. These data indicate that the newly introduced N-glycosylation consensus sequence is functional, and that the N-terminal region of the α-subunit is flexible and can be modified without affecting the intracellular function. Furthermore, amino acid sequences in the N-terminus are involved in the O-glycosylation in free α-subunit.

Key words: Gonadotropin, α-Subunit, Amino acid change, N-Linked glycosylation, 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]. Both α and hCGβ have two N-glycosylation sites at Asn^52 and Asn^78, and Asn^13 and Asn^90, respectively. The intracellular function of the individual oligosaccharide chains has been well studied by site-directed mutagenesis. In α-subunit, N-glycosylation at Asn^78 plays a role in the integrity of the hormone, while N-glycosylation at Asn^52 is important for the assembly of intact hCG molecules [3].

Protein glycosylation has been shown to be significant in secretion, stability, clearance, interactions with receptors or ligands, and oligomerization of a variety of proteins [4]. As a glycoprotein can change glycosylation pattern if expressed in a non-native cell system and that this may in turn introduce changes in function, expression rates, and oligomerization, glycosylation has been recognized to have increasing implications for the biotechnology industry [5]. Extensive efforts have been undertaken to modify the characteristics of the protein by introduction of N-glycosylation sequence(s) into selected proteins [6-10]. Based on data that the N-terminal region of the α-subunit is not involved in assembly with the β-subunit or the biological activity of the heterodimers, we previously constructed...
an analog with the carboxyl-terminal peptide (CTP) of the hCGβ-subunit bearing four O-linked sugars at the N-terminal region of the α-subunit, between amino acid residues 3 and 4 [11]. The resulting chimeric α was secreted and combined with hCGβ-subunit comparable to wild-type (WT) α-subunit. Little is known, however, about the effect of the introduction of N-glycosylation in that region on intracellular function. To obtain more information on the relationship between the structure and function of the α-subunit, we introduced a novel N-glycosylation site by mutating Asp³ and Gin⁵ into Asn and Thr, respectively.

In this context, we aimed to find out 1) whether the newly introduced N-glycosylation site is in fact glycosylated, and 2) whether the additional N-glycosylation influences the independent role of each potential N-glycosylation site of the α-subunit.

Materials and methods

Site-directed mutagenesis

Construction of the hCGα minigene, pM\(\alpha\)WT, pM\(\alpha\)ΔAsn1, pM\(\alpha\)ΔAsn2, and pM\(\alpha\)ΔAsn(1 + 2) has been described previously [3]. Exon III of the α-subunit contains a unique XbaI restriction site within the sequence encoding amino acid residues 34 and 35. Because the expression vector pM² also contains a single XbaI site, XbaI-XbaI fragment of pM²-αWT was inserted into the XbaI site of the M13mp18 phage for mutagenesis. Oligonucleotide of 27-mers (5'-TGCCGCAATCCGTCACATTAGGACCGGA-3') for mutations of Asp³ and Gin⁵ to Asn and Thr, respectively, were prepared at the Equipment Center for Research and Education, Nagoya University School of Medicine (Nagoya, Japan). The reason why the Asn-Xaa-Thr sequence but not the Asn-Xaa-Ser one was used as an N-glycosylation signal is that the former is more glycosylated than the latter [12]. Site-directed mutagenesis was performed using the Muta-Gen M13 in vitro mutagenesis kit (Bio-Rad, Richmond, CA, USA). The modified DNA was transformed into competent Escherichia coli K12JM109 and the mutant was screened by hybridization/washing in tetramethylammonium chloride [13]. The entire coding regions were sequenced to verify that mutations were correct and that there were no other sequence alterations. XbaI-XbaI fragments bearing the mutations were ex-

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\begin{align*}
\alpha\text{WT} & : \text{H}N \quad \text{N} \quad \text{N} \quad \text{COOH} \\
\alpha\Delta\text{Asn1} & : \text{H}N \quad \text{N} \quad \text{COOH} \\
\alpha\Delta\text{Asn2} & : \text{H}N \quad \text{N} \quad \text{COOH} \\
\alpha\Delta\text{Asn(1+2)} & : \text{H}N \quad \text{N} \quad \text{COOH} \\
\alpha\text{WT+S} & : \text{N} \quad \text{N} \quad \text{N} \quad \text{COOH} \\
\alpha\Delta\text{Asn1+S} & : \text{N} \quad \text{N} \quad \text{COOH} \\
\alpha\Delta\text{Asn2+S} & : \text{N} \quad \text{N} \quad \text{COOH} \\
\alpha\Delta\text{Asn(1+2)+S} & : \text{N} \quad \text{N} \quad \text{COOH}
\end{align*}
\]

Fig. 1. Schematic depiction of WT and mutant α-subunits. N denotes the N-linked oligosaccharides. Deglycosylation was introduced by converting Asn⁵ to Asp [3]. A new N-glycosylation site was introduced by replacing Asp³ and Gin⁵ to Asn and Thr, respectively.

Cells and transfection

Chinese hamster ovary (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 mutant α were transfected alone or with pMhcCGβ into the cells by the calcium phosphate method [14]. 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

For continuous labeling experiments, 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 ([3S]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). For tunicamycin treatment, cells were washed twice, preincubated for 1.5 h in labeling medium containing 2 µg/ml tunicamycin (Sigma Chemical Co., St. Louis, MO, USA), and labeled for 18 h in labeling medium containing 2 µg/ml tunicamycin and 25 µCi/ml of Tran35S-label. For pulse-chase experiments, stable clones of CHO cells were plated in 12-well tissue culture plates and grown to near confluence. The cells were washed twice with phosphate-buffered saline and preincubated for 1.5 h with labeling medium, followed by a 20-min pulse of 100 µCi/ml of Tran35S-label. Labelled cells were washed twice with chase medium (labeling medium containing 1 mM unlabeled methionine and cysteine) and incubated in the chase medium for the indicated times. Media and cell lysates were precleared with normal rabbit serum and immunoprecipitated (GIBCO, Grand Island, NY, USA). Supernatants were precipitated with subunit-specific antisera and the proteins were resolved by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described [16]. Polyclonal antisera against α- and hCGβ-subunit were gifts from Dr. Irving Boime, Washington University School of Medicine, St. Louis, MO, USA.

Enzymatic digestion

Endoglycosidase treatment has been described previously [17]. 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, 0.1% SDS, 12.5 mM 2-mercaptoethanol, pH5.5) at 37°C for 24 h with 0.1 U endoglycosidase-F (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). For neuraminidase and O-glycanase serial digestion, samples were first digested with 7 mU neuraminidase in 18 µl O-buffer (1 mM calcium acetate, 15 mM sodium phosphate, pH7.3) at 37°C for 24 h, then 1 mU O-glycanase was added, followed by incubation for another 24 h. Samples were subjected to 15% SDS-PAGE [3].

Results

Secretion of WT and mutant α-subunits

To examine whether the newly synthesized N-linked sugar chain affect the secretion of the α-subunits, pulse-chase experiments were performed. As previously observed [3], αWT and αΔAsn1 were rapidly and efficiently secreted (Fig. 2, panels A and C). In-

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Fig. 2. Pulse-chase analysis of WT and mutant α-subunits. Clones expressing WT and mutant α-subunits were pulse labeled with [35S]methionine/cysteine for 20 min and chased for 1, 2, 4, and 6 h. Chase 0 h indicates a lysate sample prepared immediately after pulse labeling. Lysate and medium samples were immunoprecipitated with anti-α-serum and subjected to 15% SDS-PAGE.
tracellular αΔAsn2 disappeared with similar kinetics as αWT but the amount recovered in the medium after 6 h was less than 10% and much lower than αWT or αΔAsn1, showing an enhanced degradation (panel E). αΔAsn1(1 + 2) stayed longer in the cell; 60% of the synthesized αΔAsn1(1 + 2) was observed even after 6 h. Furthermore, <10% was recovered in the medium after 6 h (panel G). When the new glycosylation was introduced, no effect was shown on the kinetics of αWT, αΔAsn1, and αΔAsn2 (panels B, D, and F). However, αΔAsn1(1 + 2) + S disappeared faster from the cell (t½ = 80 min) than αΔAsn1(1 + 2) and little was secreted (panels G and H), indicating that the new sugar chain increased the intracellular degradation of αΔAsn1(1 + 2).

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

To study the assembly, WT and mutant α-subunits were cotransfected with the hCGβ gene and stable cell lines producing both subunits were selected. For each mutant, clones synthesizing excess hCGβ-subunit were isolated to ensure that it would not limit dimer formation. Antisera against α and hCGβ detect, in addition to heterodimer, free α and free hCGβ, respectively. Thus, the α-subunit immunoprecipitated with hCGβ antisera means only the dimerized α-subunit, although the immunoprecipitates with α antisera contained both free and dimerized α-subunits. Because hCGβ is synthesized in excess, there should be equal amounts of α-subunit precipitated by both antisera if complete dimerization has occurred. In all clones isolated, hCGβ exists as two forms which are due to the addition of one or two N-linked oligosaccharides [16]. As shown previously [3, 15], immunoprecipitation of WT hCG dimer (Fig. 3, lanes 1 and 2) and αΔAsn2/hCGβ (lanes 9 and 10) by α or hCGβ antisera resulted in the recovery of equal amounts of the α-subunit. This indicates that >95% of the αWT or αΔAsn2 is combined with the hCGβ in the dimeric form. In contrast, <25% of αΔAsn1 (lanes 5 and 6) and <40% of αΔAsn1(1 + 2) (lanes 13 and 14) were assembled into dimers. The efficiency of assembly of αWT + S (lanes 3 and 4), αΔAsn2 + S (lanes 11 and 12), and αΔAsn1(1 + 2) + S (lanes 15 and 16) with the hCGβ-subunit was comparable to αWT, αΔAsn2, and αΔAsn1(1 + 2), respectively. The data indicated that additional N-glycosylation at position 3 did not impair the assembly with the hCGβ-subunit. Analysis of clone secreting αΔAsn1 + S/hCGβ revealed that >95% of the total αΔAsn1 + S secreted was in the form of dimer (lanes 7 and 8), indicating that additional N-glycosylation at position 3 of αΔAsn1 increased the efficiency of heterodimer formation.

**Differences in molecular weight between free and dimerized α-subunits**

Uncombined α-subunit is more heterogeneous and

![Fig. 3. Assembly of WT and mutant α-subunits with hCGβ-subunit. Clones expressing α-subunit with hCGβ/WT were continuously labeled with [35S]methionine/cysteine for 18 h. The medium was immunoprecipitated with anti-α serum (lanes 1, 3, 5, 7, 9, 11, 13, and 15) or anti-hCGβ serum (lanes 2, 4, 6, 8, 10, 12, 14, and 16), followed by 15% SDS-PAGE. Molecular weight markers (kDa; Amersham) are shown at the left. The extent of assembly was determined as a percent of the dimer form of the α-subunit precipitated by anti-hCGβ serum to the total α-subunit pool (noncombined and dimer) collected with anti-α serum.](#)
migrates slower on SDS-PAGE than the dimer form of α-subunit due to alterations in the processing of the N-linked oligosaccharides [18, 19]. Asn^2-linked glycan is responsible for the heterogeneity of the free α-subunit [3]. To address the molecular weight shift by additional N-glycosylation and by assembly with hCGβ-subunit, free and combined forms of α-glycosylation mutants were co-migrated in the gel. Free αWT + S, αΔAsn1 + S, αΔAsn2 + S, and αΔAsn(1 + 2) + S contained slower migrating forms than αWT, αΔAsn1, αΔAsn2, and αΔAsn(1 + 2), respectively; i.e., N-3 (triglycosylated) form of WT + S, N-2 (doubly glycosylated) form of αΔAsn1 + S and αΔAsn2 + S, and N-1 (monoglycosylated) form in αΔAsn(1 + 2) + S. (Fig. 4, lanes 1, 3, 5, 7, 9, 11, 13, and 15). This indicates that a new N-linked sugar chain was attached by the introduction of the N-glycosylation consensus sequence. The apparent molecular weight shift by dimerization with hCGβ-subunit was observed in αWT + S, αΔAsn1 + S, αΔAsn2 + S, and αΔAsn(1 + 2) + S (lanes 3, 4, 7, 8, 11, 12, 15, and 16).

**Endoglycosidase-F treatment of α mutants**

Electrophoretic mobility of N-2 form of free αWT + S and N-1 form of free αΔAsn1 + S was slower than that of corresponding free αWT and free αΔAsn1 (Fig. 4, lanes 1, 3, 5, and 7). Furthermore, the molecular weight shift of αWT + S, αΔAsn1 + S, αΔAsn2 + S, and αΔAsn(1 + 2) + S by dimerization with hCGβ was much greater than expected from αWT, αΔAsn1, αΔAsn2, and αΔAsn(1 + 2), respectively (Fig. 4). These data suggest that the processing of N-linked oligosaccharides might be altered, or that another type of glycan may be created. To address this issue, free and assembled forms of WT and mutant α-subunits were treated with endoglycosidase-F, which cleaves all forms of N-linked oligosaccharides (Fig. 5). Endoglycosidase-F treatment of free αWT and αΔAsn1 generated one band (panel A, lanes 2 and 6), whereas αWT + S and αΔAsn1 + S showed two bands (lanes 4 and 8). This is not due to incomplete digestion because the apparent molecular weight of the
Fig. 5. Endoglycosidase-F treatment of WT and mutant α-subunits. Clones expressing α alone (panel A) and with hCGβ WT-subunit (panel B) were continuously labeled with [35S]methionine/cysteine. The medium from each clone was immunoprecipitated with anti-α (panel A) or anti-hCGβ serum (panel B). Immunoprecipitable α-subunits were treated with (lanes 2, 4, 6, and 8) or without endoglycosidase-F (lanes 1, 3, 5, and 7), followed by 15% SDS-PAGE. Arrowheads indicate O-glycosylated forms of α-subunits.

Fig. 6. O-glycanase treatment of WT and mutant α-subunits. Clones expressing αWT (lanes 1–3), αWT + S (lanes 4–6), αΔAsn1 (lanes 7–9), and αΔAsn1 + S (lanes 10–12) were continuously labeled with [35S]methionine/cysteine for 18 h in the presence of tunicamycin. Immunoprecipitable α-subunits were treated with neuraminidase alone (lanes 2, 5, 8, and 11), both neuraminidase and O-glycanase (lanes 3, 6, 9, and 12), or no enzyme (lanes 1, 4, 7, and 10). The digested samples were then run on 15% SDS-PAGE. Molecular weight markers (KD; Amersham) are shown at the left.

slower migrating form was much less than that of the undigested form of αΔAsn1 (lane 5), which contains one N-linked sugar. If the difference in electrophoretic mobility among αWT, αWT + S, αΔAsn1, and αΔAsn1 + S is caused by the number and/or processing of N-linked oligosaccharides, they should have one form after digestion with endoglycosidase-F. The difference in the molecular weight of these compo-
ments could be due to addition of O-glycan. In contrast, assembled form of $\alpha WT + S$ and $\alpha\Delta Asn1 + S$ showed an identical migration to that of $\alpha WT$ (panel B).

O-glycosylation in the mutant $\alpha$-subunits

To confirm that O-linked sugar chain is attached to the mutant $\alpha$-subunit and to clarify whether it is due to the newly synthesized N-glycosylation or to the amino acid change at positions 3 and 5, the cells were incubated in the presence of tunicamycin, which inhibits the synthesis of N-linked oligosaccharides. $\alpha WT$, $\alpha WT + S$, $\alpha\Delta Asn1$, and $\alpha\Delta Asn1 + S$ secreted were digested with neuraminidase and O-glycanase, which remove sialic acid and galactose (Gal)-N-acetylgalactosamine (GalNAc), respectively. Electrophoretic mobility of $\alpha WT + S$ and $\alpha\Delta Asn1 + S$ became faster by serial digestion with neuraminidase and O-glycanase (Fig. 6, lanes 4, 5, 6, 10, 11, and 12), demonstrating that the Gal-GalNAc sugar chains were O-glycosidically linked. Because the secreted forms of $\alpha WT + S$ and $\alpha\Delta Asn1 + S$ in the presence of tunicamycin are devoid of N-linked sugars, the addition of O-linked oligosaccharides is due to the amino acid change at positions 3 and 5.

Discussion

It has been shown that N-glycosylation at Asn$^{52}$ and Asn$^{78}$ of the $\alpha$-subunit plays an important role in assembly of intact hCG molecules and the folding of the $\alpha$-subunit, respectively [3]. The present study showed that $\alpha\Delta Asn2 + S$ dimerized with hCG$\beta$-subunit comparable to $\alpha\Delta Asn2$, and that $\alpha\Delta Asn1 + S$, as well as $\alpha\Delta Asn1$, were secreted. These results imply that the additional sugar chain at Asn$^{3}$ does not impair the independent roles of N-glycosylation at Asn$^{52}$ and Asn$^{78}$. Furthermore, the assembly of $\alpha\Delta Asn1 + S$ was more efficient than that of $\alpha\Delta Asn1$ and was comparable to $\alpha WT$. This finding indicates that N-glycosylation at Asn$^{3}$ compensates for the role of N-glycosylation at Asn$^{52}$. Considering the fact that the assembly of $\alpha\Delta Asn(1 + 2) + S$ was not substantial, however, cooperation with N-glycosylation at Asn$^{78}$ is mandatory to achieve the compensatory effect of N-glycosylation at Asn$^{3}$.

Pfeiffer et al. [20] reported that a newly introduced N-glycosylation in human uterine tissue plasminogen influenced the processing of a potential N-glycosylation. Furthermore, it has been shown that the assembly with hCG$\beta$-subunit changed the glycosylation pattern of $\alpha$-subunit [21]. Therefore, it can be assumed that the creation of new N-glycosylation at Asn$^{3}$ influenced the processing of other N-glycosylations. Because endoglycosidase-H sensitivity distinguishes the complex type from high mannose, noncomplex, and hybrid type oligosaccharides, the secretory forms of WT and all glycosylation mutants were treated with endoglycosidase-H and then subjected to SDS-PAGE (data not shown). Unexpectedly, there were no differences in endoglycosidase-H sensitivity between WT and each mutant; >95% of the secreted materials were resistant to endoglycosidase-H. Although we did not analyze the sequence of each N-linked carbohydrate sugar, the new N-glycosylation at Asn$^{3}$ does not seem to affect the N-glycosylations at Asn$^{52}$ and Asn$^{78}$. The N-terminal region is far from Asn$^{52}$ and Asn$^{78}$ in the three-dimensional structure [22], hence it is reasonable to assume that the N-linked carbohydrate sugar chain at Asn$^{3}$ is at some distance from and thus avoids steric hindrance of the functional domains created by N-glycosylation at Asn$^{52}$ or Asn$^{78}$.

Whether the new N-glycosylation at Asn$^{3}$ affects the biological function is a question of interest. As stated in the Introduction, peptide heterogeneity has been reported at the amino terminus of $\alpha$-subunit in different species, suggesting that N-terminal region is not involved in biological activity. In the crystal structure of hCG, the carboxy-terminal region, but not the amino terminus, was found to be located within a putative receptor-binding domain of the hormone [22]. We previously showed that fusing the CTP of the hCG$\beta$-subunit to the N-terminal region of the $\alpha$-subunit did not affect the receptor binding and signal transduction of the hCG [11]. Furthermore, our preliminary data showed that the receptor binding of hCG containing $\alpha WT + S$ was the same as that of hCGWT. Taken together, it is unlikely that the new N-glycosylation at Asn$^{3}$ is related to the biological function of hCG.

We did not address the site where the O-linked carbohydrate chain was attached. Free bovine $\alpha$-subunit has been shown to have O-linked sugar at Thr$^{91}$ (corresponding to Thr$^{99}$ in human $\alpha$-subunit) [23]. In human, free $\alpha$-subunit expressed in the choriocarcinoma cell line, JAR, has an O-linked sugar at Thr$^{99}$ [24]. But no other site of O-glycosylation in the $\alpha$-subunit has
been hitherto reported in any species. The associated α-subunit cannot have O-glycosylation due to steric hindrance of the glycosylation site by hCGβ-subunit [23, 24]. That the mutant, where Asp\(^3\) and Gln\(^5\) were replaced with Asn and Thr, respectively, contained O-glycosylation in free form but never in assembled form suggests that the O-glycosylation site should be within the interface between the α- and β-subunits, hence Thr\(^{59}\) is a possible glycosylation site. On the other hand, hydroxylamino acid of a predicted N-glycosylation tripeptide sequence (Asn-Xaa-Ser/Thr) has been shown to be O-glycosylated [25]. Although we cannot exclude the possibility that the replaced Thr\(^{5}\) might be an O-glycosylation site, it is unlikely because the N-terminal region is not a contact site with β-subunits.

Endoglycosidase-H-treated αWT + S and αΔAsn1 + S had two forms, i.e. with or without O-glycosylation, in almost equal amounts, whereas these mutants synthesized in the presence of tunicamycin demonstrated the O-glycosylated form alone. The data are similar to our previous report that the substitution of the Pro\(^{40}\)-Leu\(^{41}\)-Arg\(^{42}\) motif in the α-subunit by Ala-Leu-Ala generated the attachment of O-linked carbohydrate chain [17]. These findings indicate that the presence of N-linked sugars in the mutant-α partially inhibited O-glycosylation. Recent studies have shown that multiple UDP-GalNAc:polypeptide N-acetylglucosaminyltransferases (GalNAc-Ts) are expressed in any one cell type, and that these enzymes have distinct but overlapping acceptor specificities [26, 27]. Furthermore, O-glycosylation is a post-translational and post-folding event, whereas N-glycosylation occurs during translation and folding [27-29]. Thus, it is simply assumed that the steric fence by N-linked carbohydrate chain(s) can prevent one of the GalNAc-Ts from functioning and transferring GalNAc to the acceptor sites for O-linked sugars. In contrast, de Haan et al. [30] reported that the introduction of a functional N-glycosylation site adjacent to the site of O-glycosylation in the mouse hepatitis virus membrane protein did not significantly affect the addition of O-linked sugars. Also as stated above, both the Asn and Ser/Thr residues in the tripeptide Asn-Xaa-Ser/Thr could be glycosylated [25]. These reports indicate that the transferases are not sterically hindered by the prior addition of N-linked sugars. Differences in tertiary structures of acceptor proteins as well as cell-type specificity of GalNAc-Ts might explain this discrepancy.

Acknowledgments

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References

11. Furuhashi M, Shikome T, Fares FA, Sugahara T, Hsueh


