Elucidation of the sugar recognition ability of the lectin domain of UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase 3 by using unnatural glycopeptide substrates

Yayoi Yoshimura1,2,3, Aaron S Nudelman4, Steven B Levery4, Hans H Wandall4, Eric P Bennett4, Ole Hindsdall5, Henrik Clausen4, and Shin-Ichiro Nishimura2

1Graduate School of Life Science and Research Center for Post-Genome Science and Technology, Hokkaido University, Sapporo 001-0021, Japan; 2Carlsberg Laboratory, Copenhagen DK-1799, Denmark; and 3Copenhagen Center for Glycomics, University of Copenhagen, Copenhagen DK-2200, Denmark

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Mucin-type glycosylation [α-N-acetyl-D-galactosamine (α-GalNAc)-O-Ser/Thr] on proteins is initiated biosynthetically by 16 homologous isoforms of GalNAc-Ts (uridine diphosphate-GalNAc:polypeptide N-acetylgalactosaminyltransferases). All the GalNAc-Ts consist of a catalytic domain and a lectin domain. Previous reports of GalNAc-T assays toward peptides and α-GalNAc glycopeptides showed that the lectin domain recognized the sugar on the substrates and affected the reaction; however, the details are not clear. Here, we report a new strategy to give insight on the sugar recognition ability and the function of the GalNAc-T3 lectin domain using chemically synthesized natural-type (α-GalNAc-O-Thr) and unnatural-type [β-GalNAc-O-Thr, α-Fuc-O-Thr and β-GlcNAc-O-Thr] MUC5AC glycopeptides. GalNAc-T3 is one of isoforms expressed in various organs, its substrate specificity extensively characterized and its anomalous expression has been identified in several types of cancer (e.g. pancreas and stomach). The glycopeptides used in this study were designed based on a preliminary peptide assay with a sequence derived from the MUC5AC tandem repeat. Through GalNAc-T3 and lectin-inactivated GalNAc-T3, competition assays between the glycopeptide substrates and product analyses (MALDI-TOF MS, RP-HPLC and ETD-MS/MS), we show that the lectin domain strictly recognized GalNAc on the substrate and this specificity controlled the glycosylation pathway.

Keywords: ETD-MS/MS / lectin domain / mucin-type O-glycosylation / polypeptide GalNAc-transferase / unnatural glycopeptide substrate

Introduction

O-Glycosylation is an important posttranslational modification on mucin-type proteins that plays many crucial roles in biological processes, e.g. specific cellular adhesion during differentiation, proliferation or malignant alteration in embryogenesis, organogenesis, carcinogenesis and cancer metastasis (Brockhausen 1999; Hollingsworth and Swanson 2004; Hattrup and Gendler 2008). Cancer-associated mucins are especially attractive as potential targets for diagnosis and therapeutic uses. For instance, glycan alterations on MUC1 are well known as a tumor-associated markers (Hanisch 2001; Taylor-Papadimitriou et al. 2002) for which immunogenic epitopes have been elucidated (Ohyabu et al. 2009) and utilized for vaccine synthesis (Tarp and Clausen 2008; Westerlind et al. 2008; Buskas et al. 2009; Kaiser et al. 2009). Mucin-type O-glycosylation is initiated by the addition of N-acetyl-D-galactosamine (GalNAc) residues to Ser/Thr amino acids catalyzed by members of the GalNAc-T family (polypeptide N-acetylgalactosaminyltransferases; EC 2.4.1.41) (Clausen and Bennett 1996; Hassan, Bennett, et al. 2000; Ten Hagen et al. 2003). O-Glycan structures are subsequently built up by the stepwise addition of monosaccharides through reactions catalyzed by a large array of glycosyltransferases. GalNAc-Ts are thus the limiting enzymes in mucin-type O-glycan biosynthesis. Sixteen distinct mammalian GalNAc-Ts have been cloned and characterized to date (Clausen and Bennett 1996; Ten Hagen et al. 2003; Tarp and Clausen 2008). GalNAc-T isoforms have different but partly overlapping substrate specificities, and although most of them utilize both peptide and glycopeptide substrates (Wandall et al. 1997; Takeuchi et al. 2002; Pratt et al. 2004), GalNAc-T4 (Bennett et al. 1998), GalNAc-T7 (Bennett, Hassan, Hollingsworth, et al. 1999), GalNAc-T10 (Ten Hagen et al. 2001) and GalNAc-T20 (Peng et al. 2010) work mainly with glycopeptides. The crystal structure for three isoforms (murine GalNAc-T1 and human GalNAc-T2 and -T10) have been solved (Fritz et al. 2004, 2006; Kubota et al. 2006), but
isoform-specific reaction mechanisms of GalNAc-Ts and consensus substrate sequence motifs remain unresolved. The large GalNAc-T family provides for complementary substrate specificities that in a regulated and coordinated fashion can produce diverse patterns of O-glycosylation on proteins, and the repertoire of GalNAc-Ts in cells determines how proteins are decorated with O-glycans.

GalNAc-Ts have a characteristic structure, which consists of two separate domains, a catalytic domain and a lectin domain. The lectin domain is located in the C-terminal structure and consists of three subunits (α, β, and γ) resembling R-type lectins. Since the initial reports on the existence of the lectin domain (Hazes 1996; Imberty et al. 1997), several studies have focused on the function of such lectin domains in the GalNAc-T catalyzed reaction. The main experimental strategy used in these studies has been in vitro assays using mutated GalNAc-T lectins with a (α-GalNAc glyco) peptide substrate. Five isoforms, GalNAc-T1, -T2, -T3, -T4 and -T10, have been studied in some detail, and with all tested isoforms, the lectin domain has been shown to be required for one or more α-GalNAc glycopeptide substrate specificities (Hanisch et al. 2001; Hassan, Reis et al. 2000; Tenno et al. 2002; Fritz et al. 2006; Kubota et al. 2006; Wandall et al. 2007; Raman et al. 2008; Pedersen et al. 2011). This feature of GalNAc-Ts appears to provide a mechanism for a follow-up or filling-in function to obtain high density of O-glycans in mucins. Analysis of the carbohydrate-binding specificity of the lectin domains by direct binding and inhibition assays of GalNAc-T reactions have indicated that at least the lectins on GalNAc-T1, T2, T3 and T4 preferably bind GalNAc. However, problems associated with the previous studies is α-GalNAc glycopeptide substrates were designed and utilized without considering the original glycosylation pathway of each GalNAc-T.

To elucidate the function of the lectin domain, here we report an adaptable strategy for the GalNAc-T assay using glycopeptides modified with α-GalNAc or other sugars [β-GalNAc, α-Fuc and β-GlcNAc]. As described above, most of previous studies utilized α-GalNAc glycopeptides as substrates for GalNAc-T assays to reveal the effect of the lectin domain in the reactions (Hanisch et al. 2001; Hassan and Reis et al. 2000; Tenno et al. 2002; Fritz et al. 2006; Kubota et al. 2006; Wandall et al. 2007; Raman et al. 2008; Pedersen et al. 2011). Here, we have used a peptide sequence derived from the MUC5AC mucin (GTTPSPVPTTSTSAP; Guyonnet Duprat et al. 1995) and human recombinant GalNAc-T3 (Bennett et al. 1996). MUC5AC has in total nine potential glycosylation sites (six Thr and three Ser residues) and is widely employed in GalNAc-T assays including GalNAc-T3 (Tetaert, Ten Hagen, et al. 2001; Tetaert, Richet, et al. 2001; Cheng et al. 2004; Fritz et al. 2006; Raman et al. 2008). GalNAc-T3 is expressed in a wide range of organs, e.g. pancreas, skin, kidney, testis, prostate, ovary, intestine and colon (Bennett et al. 1996). It exhibits aberrant expression in cancer cells that affects growth properties (Sutherlin et al. 1997; Bennet, Hassan, Mandel, et al. 1999; Marcos et al. 2003). Moreover, its specific glycosylation of HIV gp120 (Bennet, Hassan, Mandel, et al. 1999) and FGF23 (Kato et al. 2006) has been identified. By subjecting unnatural MUC5AC glycopeptides to enzymatic reaction, we confirmed that the differences of the pre-existing sugar moiety on the substrate affected the GalNAc-T3 reaction, especially that the GalNAc-modified substrate specifically accelerated further glycosylation and that the substrate selectivity was derived from the sugar recognition ability of the lectin domain.

**Results**

**MUC5AC peptide assay for the design of glycopeptide probes**

To design an adequate glycopeptide probe for elucidating the sugar recognition ability of the lectin domain, we initially carried out the MUC5AC peptide assay with a wild-type enzyme (GalNAc-T3) and a lectin domain-inactivated form bearing a point mutation of the Asp519 residue to His in its α-subdomain (GalNAc-T3D519H). This Asp519 is presumed to be a key residue for sugar recognition according to previous studies about GalNAc-T1, GalNAc-T2 and GalNAc-T4 (Tenno et al. 2002; Wandall et al. 2007; Pedersen et al. 2011). The assays were performed using a peptide substrate (20 nmol) and only one molar equivalent of a glycosyl donor (UDP-GalNAc) to restrict the study to the initial part of the glycosylation reaction. After incubation for 24 h at 37°C, the reactions were initially analyzed by MALDI-TOF MS (Figure 1A). GalNAc-T3 incorporated 1–3 GalNAc residues per peptide substrate; however, the GalNAc-T3D519H mutant produced principally monoglycosylated and a small amount of diglycosylated products (Figure 1A). These resulting products were analyzed by RP-HPLC (Figure 1B) and the sites of GalNAc incorporation determined by Orbitrap-ETD-MS/MS analysis (McAlister et al. 2008; see Supplementary data for MS/MS spectra). Based on these results, a map of the glycosylation pathways is proposed (Figure 1C). GalNAc-T3 exhibited a stepwise reaction pattern to produce the tri-glycosylated form 3G (10% yield, glycosylated sites were Thr3, Thr12 and Thr13). Meanwhile, GalNAc-T3D519H accumulated monoglycosylated product 1G (44% yield, the glycosylated site was only Thr3) and a minor amount of diglycosylated product 2G (10% yield). These data identified Thr1 as the substrate site for GalNAc-T3 independent of its lectin domain, and also the subsequent site Thr13, since GalNAc-T3D519H could yield the GalNAc-modified products, 1G and 2G. On the other hand, GalNAc-T3 generated lectin-domain-dependent products 2G’ (the glycosylated sites were Thr1 and Thr13) and 3G (the glycosylated sites were Thr3, Thr12 and Thr13). These results suggest that the lectin recognized α-GalNAc on Thr1 and promoted the following glycosylation on Thr12, which is separated from the initial site by nine amino acid residues (Figure 1D).

**Application of unnatural MUC5AC glycopeptide substrates to probe the carbohydrate specificity of the lectin domain**

We prepared MUC5AC glycopeptide substrates substituted at Thr1 by various sugars, natural-type (α-GalNAc) and other sugar moieties (β-GalNAc, α-Fuc and β-GlcNAc). This Thr1 residue was selected for modification because it was shown to be a substrate site independent of the lectin domain, whereas subsequent sites Thr12,13 were dependent. A control peptide
with Thr\textsuperscript{3} substituted by Ala was also prepared (Figure 2A). These substrates were subjected to GalNAc-T3 or GalNAc-T3\textsuperscript{D519H} reactions under the same conditions as the previous peptide assay. As shown in Figure 2B, both GalNAc-T3 (left) and GalNAc-T3\textsuperscript{D519H} (right) transferred 1 or 2 equivalent of α-GalNAc to each substrate. Subsequently, to identify the α-GalNAc-attached products, RP-HPLC analysis (Figures 3A–C, 4 and 5) and ETD-MS/MS (Figure 3D and E and Supplementary data) were performed.

In the case of GalNAc-T3, glycosylation of the α-GalNAc substrate resulted in two additional monoglycosylated (1G, 24%, Thr\textsuperscript{13} and 1G′, 13%, Thr\textsuperscript{12}) and diglycosylated (2G, 10%; Thr\textsuperscript{12} and Thr\textsuperscript{13}, Figure 3D) forms (Figure 3A), consistent with the previous peptide assay (Figure 1C, left). From the β-GalNAc substrate, the amount of product 1G (glycosylated site, Thr\textsuperscript{13}) was almost five times that of 1G′ (glycosylated site, Thr\textsuperscript{12}), in 34 and 7% yield, respectively (Figure 3B). Furthermore, the diglycosylated products (2G, 6%) consisted of two distinct products (glycosylated site, Thr\textsuperscript{2} and Thr\textsuperscript{17}/Thr\textsuperscript{12} and Thr\textsuperscript{13}, Figure 3E). The glycosylation to Thr\textsuperscript{2} was not observed in the case of the α-GalNAc substrate. The two glycosylated products derived from the β-GalNAc substrate were not possible to separate by HPLC and thus their relative amounts not possible to determine. The substrates carrying the sugars α-Fuc and β-GlcNAc suppressed glycosylation significantly, since mainly the monoglycosylated product 1G (glycosylated site, Thr\textsuperscript{5}) and a small amount of diglycosylated products 2* were produced (Figure 3C).

Examination of the activity of GalNAc-T3\textsuperscript{D519H} toward the same glycopeptide series, by both MALDI-TOF MS (Figure 2B, right panel) and HPLC, showed the similar results for all substrates (Figure 4). GalNAc-T3\textsuperscript{D519H} accumulated monoglycosylated products (1G in Figure 4;
glycosylated site, Thr\textsuperscript{13}). Thus, GalNAc-T3 appeared to have lost its selectivity toward glycopeptide substrates upon the inactivation of its lectin domain.

With the control substrate, the Ala-mutated peptide (Figure 5), both GalNAc-T3 and GalNAc-T3\textsuperscript{D519H} produced essentially only product 1G (glycosylated site, Thr\textsuperscript{13}). The similar phenomenon was observed in the GalNAc-T3 reaction toward α-Fuc and β-GlcNAc substrates (Figure 3C) and GalNAc-T3\textsuperscript{D519H} toward all glycopeptide substrates (Figure 4).

Competition assay to evaluate sugar preference of the lectin domain

The next question was whether GalNAc-T3 has a preferential reactivity for the GalNAc substrate, since all the glycopeptide substrates used in this study became GalNAc-modified. To address this point, we compared the reactivities of GalNAc-T3 and GalNAc-T3\textsuperscript{D519H} with the mixture of two substrates (20 nmol of α-GalNAc and α-Fuc glycopeptides) and 20 nmol of UDP-GalNAc. We chose these different substrates because the molecular weights of starting materials and resulting products are different, which makes it easy to distinguish them. The products of each substrate were examined by MALDI-TOF MS and RP-HPLC, and glycosylation positions subsequently determined by ETD-MS/MS (Supplementary data). Remarkably, GalNAc-T3 preferentially glycosylated the α-GalNAc substrate, whereas very little α-Fuc-peptide was glycosylated. The resulting profile of the wild-type GalNAc-T3 corresponded to the mono-GalNAc substrate case (Figure 3A). On the other hand, GalNAc-T3\textsuperscript{D519H} worked on both substrates at a similar rate and the GalNAc-attached sites were the same as on Thr\textsuperscript{13}. The competition assay between the two substrates demonstrated the directing effect of the lectin domain for GalNAc.

Discussion

In the present study, we investigated the lectin-mediated function of GalNAc-T3 using a library of unnatural MUC5AC glycopeptides and demonstrated the selective requirement for GalNAc to prime the GalNAc glycopeptide substrate specificity of this enzyme.

Based on the earlier peptide assays, MUC5AC Thr\textsuperscript{3} model glycopeptides were designed considering the successive glycosylation process mediated by the lectin domain (Figure 1D). This presumption was in accordance with Fritz et al. (2006), who determined the crystal structure of murine GalNAc-T1, which shares 45% sequence similarity with GalNAc-T3. They proposed a simulation model, indicating the α-subunit of the lectin domain in GalNAc-T1 bound to the α-GalNAc moiety on Thr\textsuperscript{14} in the MUC1 glycopeptide.
Fig. 3. Products derived from the GalNAc-T3 reaction of MUC5AC glycopeptides. Each RP-HPLC peak was collected and characterized by ETD-MS/MS to identify the glycosylated sites. The pre-existing sugar on Thr$^3$ is (A) $\alpha$-GalNAc, (B) $\beta$-GalNAc and (C) $\alpha$-Fuc, or $\beta$-GlcNAc. The 1G/1G$^*$ and 2G/2* peaks correspond to the one and two $\alpha$-GalNAc-incorporated substrates. The glycosylated site of 2* peaks is unknown. ETD-MS/MS spectra represent diglycosylated products derived from (D) $\alpha$-GalNAc and (E) $\beta$-GalNAc substrates after GalNAc-T3 reaction.
Sugars = α-GalNAc, β-GalNAc, α-Fuc or β-GlcNAc

Fig. 4. Products derived from the GalNAc-T3D519H reaction of MUC5AC glycopeptide substrates. The pre-existing sugar on Thr³ is (A) α-GalNAc, (B) β-GalNAc and (C) α-Fuc, or β-GlcNAc. Product analyses were performed by RP-HPLC and ETD-MS/MS. S, 1G and 2* correspond to substrate, monoglycosylated products and diglycosylated products, respectively. In all substrates, main products were 1G (the glycosylated site was Thr¹³).

Fig. 5. Products derived from the GalNAc-T3/GalNAc-T3D519H reaction of the MUC5AC Ala-mutated peptide. Product analyses were performed by RP-HPLC and ETD-MS/MS. S, 1G and 2* correspond to substrate, monoglycosylated products and diglycosylated products, respectively.

(PAPGST2APPAHGVTT₁₈SAPDTR; *, α-GalNAc-Thr residue) and assisted the next GalNAc attachment to Thr⁶ located eight amino acid residues away. This supports our hypothesis, and therefore, we assumed that various sugar modifications at Thr³ on the MUC5AC sequence would yield appropriate tools to probe the actual sugar recognition ability of the lectin domain in GalNAc-T3.

The presence of α-GalNAc on the substrate, a natural glycoform, enhanced glycosylation by GalNAc-T3 on Thr¹² and it is presumed that this glycosylated product may be successively converted to diglycosylated product 2G (Figure 3A). β-GalNAc substrates exhibited a comparable glycosylation pattern to the α-GalNAc substrate (Figure 3B). In agreement with this, Wandall et al. (2007) previously found that both GalNAc-α-O-benzyl and GalNAc-β-O-benzyl had similar inhibitory activity on the binding between GalNAc-T2/-T4 lectins and MUC1 α-GalNAc glycopeptides. Two kinds of diglycosylated products were confirmed to be formed from the β-GalNAc substrate, whereas only one diglycosylated product was produced from the α-GalNAc substrate. For the consecutive glycosylation (Thr³→Thr¹²→Thr¹³) mediated by the lectin domain, the α-GalNAc substrate may be favorable for GalNAc-T3. On the other hand, modification with α-Fuc or β-GlcNAc did not induce the glycosylation of the Thr¹² residue, indicating GalNAc-specific sugar recognition of the lectin domain.

In the case of GalNAc-T2, the glycosylation toward MUC5AC initially occurs at Thr⁹, next at Thr³ or Thr⁵ and thirdly at Thr¹³ (Cheng et al. 2004). We have previously confirmed that a pre-existing sugar (except α-GalNAc) on Thr⁹ did not affect the glycosylation by GalNAc-T2 (Yoshimura et al. 2010). Whether a pre-existing sugar on the initial glycosylation site influences the GalNAc-Ts reaction may depend on the glycosylation specificity of each isoform, related to the geometric constraints established by the interaction between
the already GalNAc-modified site and the lectin domain on the one hand and between the potential GalNAc site and the catalytic domain on the other.

In comparison with other GalNAc-T isoforms, it is hypothesized that GalNAc-T10 has a GalNAc-binding pocket in the catalytic domain, which induces the glycosylation of a Thr residue directly neighboring the previously GalNAc-modified site (Kubota et al. 2006; Raman et al. 2008; Perrine et al. 2009). GalNAc-T15 appears to exhibit a similar glycosylation behavior toward the MUC5AC peptide, adding GalNAc to Thrβ and then Thrα (Cheng et al. 2004). The initial glycosylation site (Thrα) is the same between GalNAc-T15 and -T3, but the subsequent reactions are specifically different. This suggests the possibility that the GalNAc pocket exists nearby the catalytic site of GalNAc-T15, and in the future, our method could confirm it.

To determine the sites of glycosylation, ETD-MS/MS was employed. The Edman degradation has generally been applied in the field (Sparrow et al. 2007); however, products after GalNAc-T reactions are frequently quite heterogeneous, and sequencing of individual glycoforms is needed to elucidate different patterns of GalNAc glycosylation. As a readily accessible alternative, tandem MS has been applied to identify the glycosylation sites (Conboy and Henion 1992; Huddleston et al. 1993; Wuher et al. 2007); however, the conventional CID-MS/MS methods are not perfect for glycosylated samples, since sugar moieties in glycopeptides (proteins) are easily cleaved during the fragmentation. In contrast to these conventional methods, the ETD-MS/MS (Syka et al. 2004; Pitteri et al. 2005) proved useful for revealing the exact glycosylated state and was applicable even to samples containing a mixture of several isomers (Figure 3D and E; Perdivara et al. 2009; Pedersen et al. 2011).

In summary, our new strategy utilizing unnatural glycopeptide substrates for GalNAc-T3 demonstrated that the GalNAc-specific sugar recognition of the lectin domain regulates further glycosylation. This method has the potential to contribute to understand the isoform-specific reaction mechanisms of the GalNAc-T family.

Materials and methods
All commercially available solvents and reagents were used without further purification. N-9-fluorenylmethyloxycarbonyl (Fmoc)-Pro-Novasyn TGT resin 0.20 mmol/g, Novabiochem, Germany was used for the synthesis of (glyco)peptides. The following protected amino acids were used: Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Pro-OH, Fmoc-Ser(O'Bu)-OH, Fmoc-Thr(O'-Bu)-OH and Fmoc-Val-OH (Novabiochem®). Glycosylated amino acid derivatives, Fmoc-Thr(α1,α1-Fuc)-OH (Elofsson et al. 1996) and Fmoc-Thr(α2,α1-d-GalNAc)-OH (Payne et al. 2007), were prepared as reported previously. The synthesis of the Fmoc-Thr(α2,β-1-d-GalNAc)-OH and Fmoc-Thr(α2,β-1-d-GlcNAc)-OH is described in Supplementary data. Uridine-5'-diphospho-N-acetylgalactosamine, 2Na (UDP-GalNAc), was purchased from Sigma (Germany).

Preparation of GalNAc-T3 and GalNAc-T3D519H
Based on the previously reported secreted GalNAc-T3 construct, a histidine-tagged expression construct was generated by PCR using primer pairs T3HIS (5'-GCGGGATCCCCAC CATCACCACATCCGATGGAGAAGGATCAATG-3')/EBHIC215, pAcGP67-GalNAc-T3sol DNA as template and High Fidelity polymerase (Roche, Switzerland; Bennett et al. 1996). The BamHI-digested product was inserted into the BamHI site of pAcGP67 (Pharmingen, San Diego, CA) generating histidine-tagged secreted GalNAc-T3his-sol. The Quick Change kit (Stratagene, Germany) was utilized as previously described (Wandall et al. 2007) to generate the mutant constructs of GalNAc-T3: full-coding GalNAc-T3D519H using primers T3D519H (5'-AGCCTCTATGTCATGTGGAGGAACAC-3')/T3D519HR (5'-GTGTTCTCACCACATGCAGACATAGGAGCT-3') using full-coding pCDNA3-GalNAc-T3 as a template; secreted HIS-tagged GalNAc-T3D519H using T3D519HRF/T3D519HR and secreted HIS-tagged GalNAc-T3D277H using T3D273HR (5'-CGCTCAACTTTTATCAGTCATGTGAGAGCT-3')/T3H273HR (5'-CTCAGTGGCATGATGAAAATGGTGAAGC-3') using pAcGP67-GalNAc-T3his-sol as template. Baculovirus pAcGP67 expression constructs of truncated secreted human GalNAc-T3 or GalNAc-T3D519H were used to infect High Five cells grown in serum-free medium. The soluble enzymes were purified as previously described by successive sequential ion-exchange chromatography on Amberlite (IRA95, Sigma) and S-Sepharose Fast Flow (Pharmacia, Sweden; Wandall et al. 1997). Quantification of purified proteins was done by Coomassie stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Preparation of MUC5AC (glyco) peptide substrates
Synthesis of (glyco)peptide substrates was performed by a method for solid-phase synthesis under microwave irradiation as described in the previous paper (Matsushita et al. 2005; Yoshimura et al. 2010).

GalNAc-transferase assay
Standard assays were performed in 40 μL of total reaction mixtures containing: 10 mM MnCl₂, 0.1% Triton X-100, 0.75 ng GalNAc-T3 (or 0.75 ng GalNAc-T3D519H), 500 μM UDP-GalNAc and 500 μM acceptor (glyco)peptide in 25 mM Tris HCl buffer (pH 7.4). In the competition assay, 500 μM of α-GalNAc and α-Fuc glycopeptides were mixed in the above reaction mixture. After incubation at 37°C for 24 h, 200 μL of 0.1% trifluoroacetic acid (TFA) containing acetonitrile (ACN) was added to terminate the reaction and the reaction was checked by MALDI-TOF MS. The mixture was dried in a Speed-Vac and the residue was diluted with 20 μL of MilliQ and subjected to RP-HPLC.

MALDI sample preparation
MALDI-TOF MS spectra were recorded on Bruker Microflex instruments using 2,5-dihydroxybenzoic acid (DHB) as a matrix. The matrix solution was prepared by dissolving 10 mg of DHB in 1 mL of ACN/water (3:7, v/v) and 0.1% TFA. To prepare the sample targets, 1 μL of each of the enzyme reaction mixture and the matrix solution were combined together on a stainless steel MALDI plate and dried.
Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest
None declared.

Abbreviations
ACN, acetonitrile; CID, collision-induced dissociation; DHB, 2,5-dihydroxybenzoic acid; ESI, electrospray ionization; ETD, electron-transfer dissociation; Fmoc, 9-fluorenylmethoxy carbonyl; Fuc, L-fucose; GalNAc, N-acetyl-D-galactosamine; GalNAc-T, UDP-GalNAc-polypeptide N-acetylgalactosaminyltransferase; GlcNAc, N-acetyl-D-glucosamine; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; RP, reverse phase; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; UDP, uridine diphosphate.

References


