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An acetylation site in lectin domain modulates the biological activity of polypeptide GalNAc-transferase-2

Abstract: Polypeptide GalNAc-transferases (ppGalNAc-Ts) are a family of enzymes that catalyze the initiation of mucin-type *O*-glycosylation. All ppGalNAc-T family members contain a common $(QXW)_3$ motif, which is present in the R-type lectin group. The acetylation site K521 is part of the QKW motif of β-trefoil in the lectin domain of ppGalNAc-T2. We used a combination of acetylation and site-directed mutagenesis approaches to examine the functional role of K521 in ppGalNAc-T2. Binding assays of non-acetylated and acetylated forms of the mutant ppGalNAc-$T2^{K521Q}$ to various naked and αGalNAc-glycosylated mucin peptides indicated that the degree of interaction of lectin domain with αGalNAc depends on the peptide sequence of mucin. Studies of the inhibitory effect of various carbohydrates on the interactions of ppGalNAc-T2 with MUC1αGalNAc indicate that point K521Q mutation enhance the carbohydrate specificity of lectin domain for αGalNAc. K521Q mutation resulted in an enzyme activity lower than that of the wild-type ppGalNAc-T2, similar to the acetylation of ppGalNAc-T2. We conclude that an acetylation site in the QKW motif of the lectin domain modulates carbohydrate recognition specificity and catalytic activity of ppGalNAc-T2 for partially preglycosylated acceptors and a certain naked peptide. Posttranslational modifications of ppGalNAc-Ts, such as acetylation, may play key roles in modulating the functions of the R-type lectin domains in cellular homeostasis.

Keywords: acetylation site; carbohydrate specificity; glycosyltransferase activity; lectin domain; posttranslational modification.

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Introduction

Lectins are glycan-binding proteins containing a defined carbohydrate recognition domain (CRD). They are frequently classified based on common amino acid sequences or on the three-dimensional structure and function of CRDs. Several families of the intracellular lectins play key functions in eukaryotic cells (Varki et al., 2009). The calnexin family is a prototype of the endoplasmic reticulum (ER)-resident chaperone proteins that are able to recognize glucose $(Glc)_1Man_9GlcNAc_2$ oligosaccharide, which assists in the quality control of the protein-folding state (Takeda et al., 2009). The L-type lectin family, homologous with the leguminous lectins, is involved in glycoprotein sorting in the lumenal compartments of animal cells. ERGIC-53, the mammalian member of the L-type family, is localized at the ER-Golgi intermediate compartment, while VIP-36 is found in the Golgi apparatus and secretor pathway (Fiedler and Simons, 1994; Itin et al., 1996). The P-type lectins are mannose 6-phosphate receptors (MPRs) that recognize Man6P residues on the oligosaccharides of hydrolases and direct them from the Golgi to their lysosomal destination (Taylor and Drickamer, 2006). The R-type lectin family has a protein conformation similar to that

of the galactose-binding B chain of the plant toxin ricin, consisting of three lobes arranged as a β-trefoil around a threefold axis (Rutenber and Robertus, 1991). The $(QXW)_3$ motif, present in lectins belonging to the R-type family, is termed the ricin-like lectin domain (Hazes and Read, 1995). It has been characterized as a flexible lectin scaffold, present in all the polypeptide GalNAc-transferases (ppGalNAc-Ts) (Hazes, 1996). The ppGalNAc-Ts are a family of enzymes that catalyze the initiation of the mucin-type *O*-glycosylation by covalent bonding of αGalNAc to Thr or Ser residue of the acceptor peptide. All ppGalNAc-T members in mammals are type II transmembrane proteins having a Golgi lumenal region that contains a catalytic domain with glycosyltransferase activity and a C-terminal R-type lectin domain. The lectin domain of ppGalNAc-T modulates the GalNAc-transferase enzyme activity on the partially glycosylated peptide acceptors (Hassan et al., 2000).

Posttranslational modifications (PTMs) involve the covalent bonding of the chemical residues on the proteins following translation. PTMs change the physical and chemical properties, such as folding and biological activity and, consequently, the functions of the target proteins (Lee et al., 2006). The processes of the cellular homeostasis are determined by PTMs such as phosphorylation in signal transduction, attachment of fatty acids in membrane anchoring, glycosylation in changes of protein half-life or targeting of substrates, and acetylation in chromatin regulation and gene expression (Fukuda et al., 2006; Yang and Seto, 2008). We recently described the modification of the biological properties of ppGalNAc-T2 by acetylation (Zlocowski et al., 2011). The presence of acetyl residues significantly reduces the enzyme activity of ppGalNAc-T2 and modifies the carbohydrate recognition form of the lectin domain. Five acetylated amino acids (K103, S109, K111, K363, and S373) were identified in the catalytic domain, but only two (S529 and K521) in the lectin domain. K521 is part of the QKW motif of β-trefoil in the lectin domain of ppGalNAc-T2 and a crucial feature in the lectin domain scaffolds in the R-type lectin family.

We, therefore, investigated the significance of the K521 acetylation site on the biological activity of ppGalNAc-T2, using a combination of acetylation and site-directed mutagenesis approaches. The mutagenesis approach has been used previously to mimic a single PTM, e.g., aspartate mutation for the phosphorylation on Ser/Thr (Chang et al., 2010) and glutamine mutation for the acetylation on Lys (Costantini et al., 2007; Watanabe et al., 2010).

Results

Expression and purification of recombinant human ppGalNAc-T2 and ppGalNAc-T2^{K521Q}

The human soluble ppGalNAc-T2 and ppGalNAc-T2^{K521Q} cDNAs were cloned into baculovirus expression vector pAcGP67 for expression in insect cells. The constructs were characterized by the removal of the N-terminal cytosolic and transmembrane domains and the incorporation of 6xHis and T7 tags (Figure 1A). The vectors were

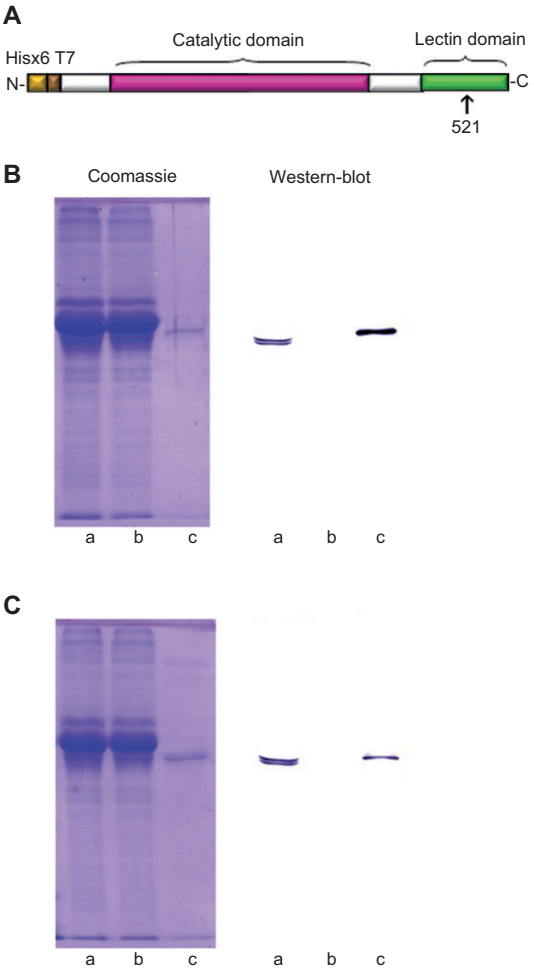

Figure 1 Expression and purification of ppGalNAc-T2s. (A) Schematic representation of the soluble recombinant ppGalNAc-T2, showing two tags (Hisx6, T7), the catalytic domain, and the C-terminal R-type lectin domain. The point mutation is located in the β-motif of lectin domain of ppGalNAc-T2. The purification processes of ppGalNAc-T2 (B) and ppGalNAc-T2^{K521Q} (C) were assessed by SDS-PAGE with CBB staining and Western blot detected with anti-T7 tag antibody. The samples obtained during the purification process correspond to the supernatant (a), flow-through of Co^{2+} column (b), and Co^{2+} column eluted (c).

Fraction	Volume (ml)	Proteins (mg/ml)	Glycosyltransferase activity[a]		Purification (-fold)
			Total (U)	Specific (U/mg protein)	
ppGalNAc-T2 sup	9	6.5	4.86	0.083	1
Co^{2+}-eluate	2.5	0.04	1.73	17.3	208
ppGalNAc-$T2^{K521Q}$ sup	9	6.5	2.45	0.041	1
Co^{2+}-eluate	2.1	0.05	0.65	6.2	150

Table 1 Purification of soluble recombinant ppGalNAc-T2 and ppGalNAc-$T2^{K521Q}$.
[a]Measured using $MUC1_{24}$ acceptor peptide.

amplified, and the recombinant proteins were expressed in Sf9 cells. The secreted, soluble proteins were harvested from the supernatant, dialyzed against PBS, centrifuged, and purified through the 6xHis residue using Co^{2+}-charged resin. The purified proteins were evaluated by SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. ppGalNAc-T2 and ppGalNAc-$T2^{K521Q}$ had high degrees of purity after affinity-chromatography purification (Figure 1B–C). The purification process of the recombinant wild-type and mutant ppGalNAc-T2 was shown by Western blots developed with the anti-T7 tag antibody. The specific GalNAc-transferase activities of ppGalNAc-T2 and ppGalNAc-T2K^{521Q} were increased 208-fold and 150-fold higher, respectively, by affinity-chromatography purification (Table 1).

Effect of K521Q mutation on binding of ppGalNAc-T2s to mucins

Lectin binding assays were used to study the effect of K521Q point mutation on ppGalNAc-T2 interaction. The mutant ppGalNAc-$T2^{K521Q}$, in comparison to the wild-type ppGalNAc-T2, showed a similar capacity to bind with the naked $MUC1_{24}$ or with $MUC1_{24}$αGalNAc (Figure 2A–B). The acetylation of ppGalNAc-$T2^{K521Q}$ further reduced its binding ability for these mucins (Figure 2C). Both ppGalNAc-$T2^{K521Q}$ and acetylated ppGalNAc-$T2^{K521Q}$ bound preferentially to $MUC1_{24}$αGalNAc over the naked $MUC1_{24}$, indicating the importance of GalNAc in the binding.

In the experiments with naked $MUC2_{33}$ and $MUC2_{33}$αGalNAc, the binding ability of ppGalNAc-$T2^{K521Q}$

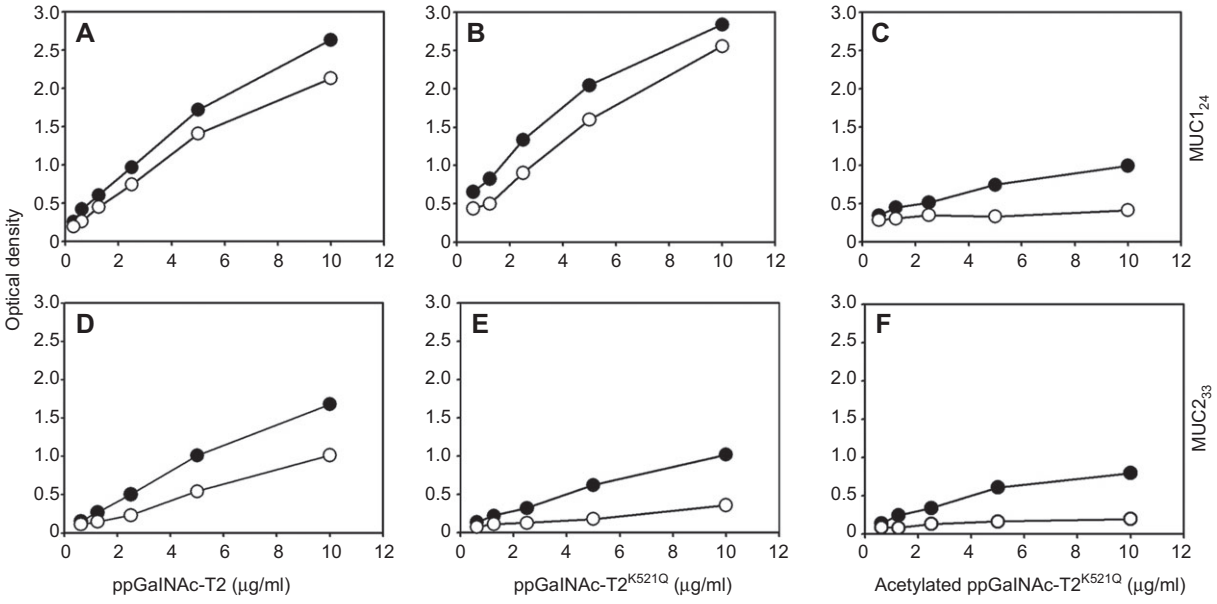

Figure 2 Reactivity of ppGalNAc-T2 and ppGalNAc-$T2^{K521Q}$ with peptides and glycopeptides. The binding of ppGalNAc-T2, ppGalNAc-$T2^{K521Q}$, and acetylated ppGalNAc-$T2^{K521Q}$ was assayed against MUC1 (top) and MUC2 (bottom) using naked (open circle) or αGalNAc glycosylated (solid circle) peptide. Adsorbed non-acetylated and acetylated ppGalNAc-T2s were detected with HRP-labeled anti-T7 tag antibody, as described in the Materials and methods section.

was lower than that of the wild-type ppGalNAc-T2 (Figure 2D–E), and the binding in both cases was lower than the binding to $MUC1_{24}$ and $MUC1_{24}$αGalNAc. The degree of binding of ppGalNAc-$T2^{K521Q}$ to $MUC2_{33}$ and $MUC2_{33}$αGalNAc was not significantly different from that of the acetylated ppGalNAc-$T2^{K521Q}$ (Figure 2E–F). These findings indicate the influence of the mucin peptide sequence on the recognition of αGalNAc *O*-glycans by ppGalNAc-$T2^{K521Q}$.

Carbohydrate specificity of interaction of ppGalNAc-T2s

Competitive lectin binding assays were used to study carbohydrate recognition specificity of ppGalNAc-T2, ppGalNAc-$T2^{K521Q}$, and their acetylated forms (Figure 3). These ppGalNAc-T2s were incubated with $MUC1_{24}$αGalNAc under optimal interaction conditions, in the presence of potential sugar competitors (Glc, GalNAc) and α-anomers of GalNAc glycosides [benzyl residue (Bzl)αGalNAc, UDPαGalNAc] (Table 2). The IC50 value (concentration required for 50% inhibition of interaction) of GalNAc was high (400 mM) for non-acetylated ppGalNAc-T2, but low (i.e., binding inhibition was stronger) for the other three ppGalNAc-T2s. The results for ppGalNAc-$T2^{K521Q}$ were quite different from those for the wild-type ppGalNAc-T2. The binding of ppGalNAc-$T2^{K521Q}$ was strongly inhibited by GalNAc (IC50: 15 mM), whereas Glc and UDPαGalNAc had little or no effect. BzlαGalNAc had a strong inhibitory effect (IC50: 8 mM) on the binding of ppGalNAc-$T2^{K521Q}$, but much less effect on the binding of ppGalNAc-T2. The results for the acetylated ppGalNAc-T2 were very similar to those for ppGalNAc-$T2^{K521Q}$. The binding of the acetylated ppGalNAc-T2 was strongly inhibited by GalNAc (IC50: 20 mM) and BzlαGalNAc (IC50: 10 mM), but not by Glc or UDPαGalNAc. The results for the acetylated ppGalNAc-$T2^{K521Q}$ were also similar or identical to those for the non-acetylated ppGalNAc-$T2^{K521Q}$, indicating that additional acetyl residues did not greatly affect GalNAc recognition of the acetylated mutant lectin domain.

Influence of K521Q mutation on catalytic activity of ppGalNAc-T2

The products of the glycosyltransferase catalytic activity of the ppGalNAc-T2 as above were analyzed by MS using soluble acceptor peptides. The GalNAc-transferase activity of the ppGalNAc-T2s was assayed using $MUC1_{24}$, $MUC2_{33}$, and $MUC5B_{27}$ acceptor peptides (Figure 4). When $MUC1_{24}$ was analyzed in the presence of ppGalNAc-T2, two

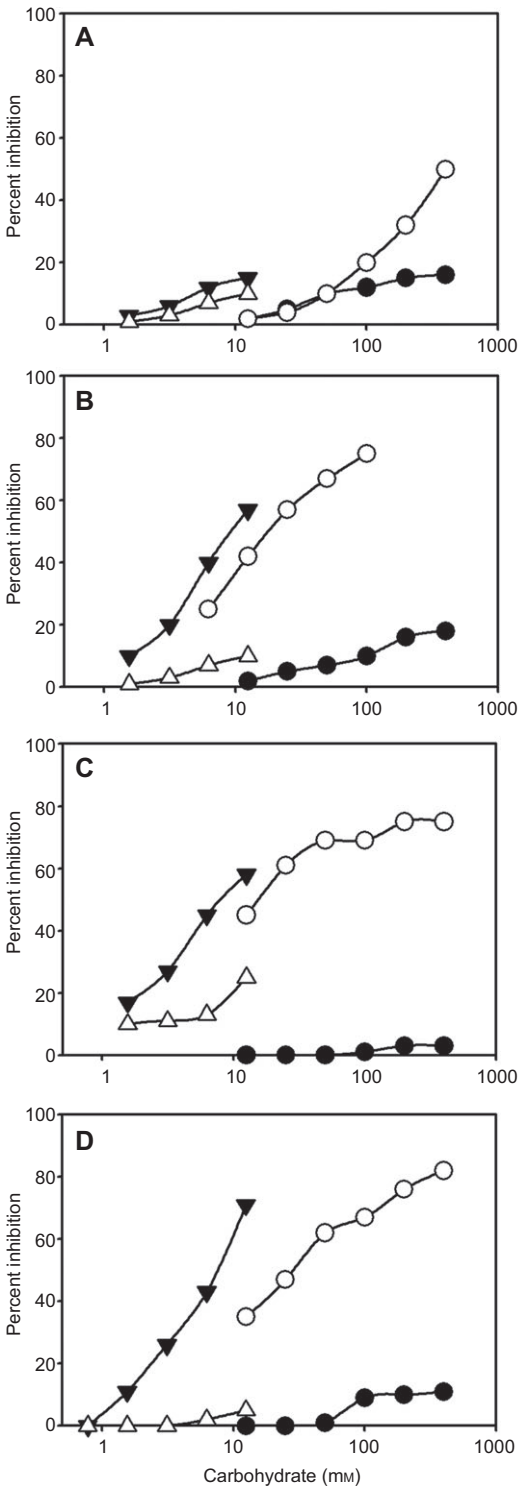

Figure 3 Competitive lectin binding assay, using carbohydrates as inhibitors of ppGalNAc-T2 interaction. Wells coated with MUC1αGalNAc were incubated with ppGalNAc-T2 (A), ppGalNAc-$T2^{K521Q}$ (B), acetylated ppGalNAc-T2 (C), or acetylated ppGalNAc-$T2^{K521Q}$ (D), followed by incubation with various carbohydrates: Glc (solid circle), GalNAc (open circle), BzlαGalNAc (solid inverted triangle), and UDPαGalNAc (open triangle). Adsorbed ppGalNAc-T2s were detected with HRP-labeled anti-T7 tag antibody, as described in the Materials and methods section.

Carbohydrate	ppGalNAc-T2[a]		ppGalNAc-T2^{K521Q}[a]	
	Non-acetylated	Acetylated	Non-acetylated	Acetylated
Glc	>500[b]	>500	>500	>500
GalNAc	400	20	15	30
BzlαGalNAc	>25	10	8	8
UDPαGalNAc	>25	>25	>25	>25

Table 2 Inhibitory effect of carbohydrates on interaction of ppGalNAc-T2s with $MUC1_{24}$αGalNAc.
[a]Binding interaction detected using HRP-labeled anti-T7 tag antibody.
[b]Concentration (mM) required for 50% inhibition (IC50).

αGalNAc were covalently bound to the $MUC1_{24}$ peptide. A weak inhibitory effect on the incorporation of a second αGalNAc on $MUC1_{24}$ was observed using the ppGalNAc-T2^{K521Q} enzyme. A reduced catalytic activity was observed on the acetylated forms of ppGalNAc-T2 and ppGalNAc-T2^{K521Q}. When $MUC2_{33}$ was used as an acceptor peptide, the GalNAc-transferase activity was lower for ppGalNAc-T2^{K521Q} than for ppGalNAc-T2, and incorporation of the third, second, and first αGalNAc on the $MUC2_{33}$ peptide was reduced. This reduction in mutant GalNAc-transferase activity was similar to that produced by the acetylation of ppGalNAc-T2. When $MUC5B_{27}$ was used as an acceptor peptide, the acetylated ppGalNAc-T2s displayed a lower catalytic activity than the non-acetylated forms, and the effect of K521Q mutation was similar to that of the acetylation of ppGalNAc-T2 in terms of the inhibitory effect. The K521Q mutation reduced the enzyme's capacity to transfer the fourth, third, and second GalNAc, but had no effect on the first αGalNAc.

Another assay method utilizing the acceptor peptides adsorbed to a surface confirmed the reduction of the catalytic activity by K521Q mutation of ppGalNAc-T2

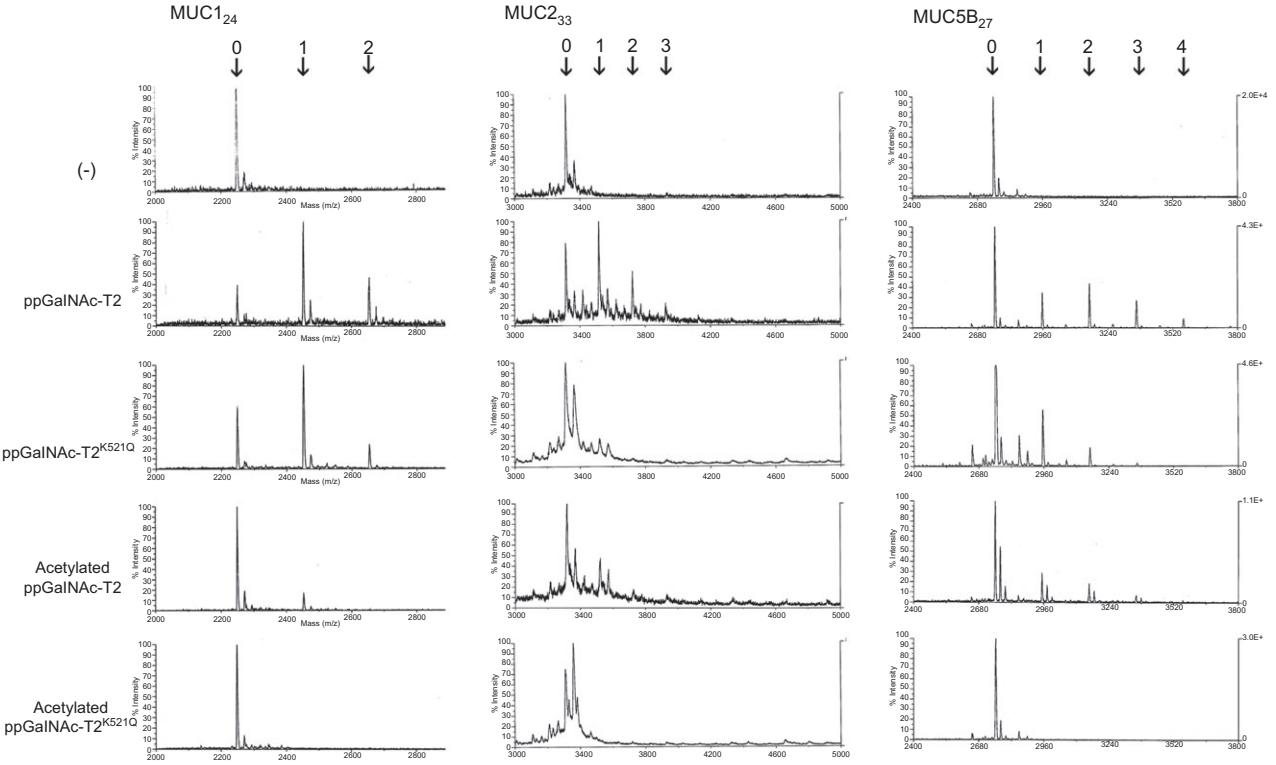

Figure 4 Glycosyltransferase activity measured by MS. The catalytic activities of ppGalNAc-T2, ppGalNAc-T2^{K521Q}, acetylated ppGalNAc-T2, and acetylated ppGalNAc-T2^{K521Q} were assayed using the soluble acceptor peptide MUC1, MUC2, or MUC5B. The arrows indicate the number of incorporated GalNAc on each acceptor peptide.

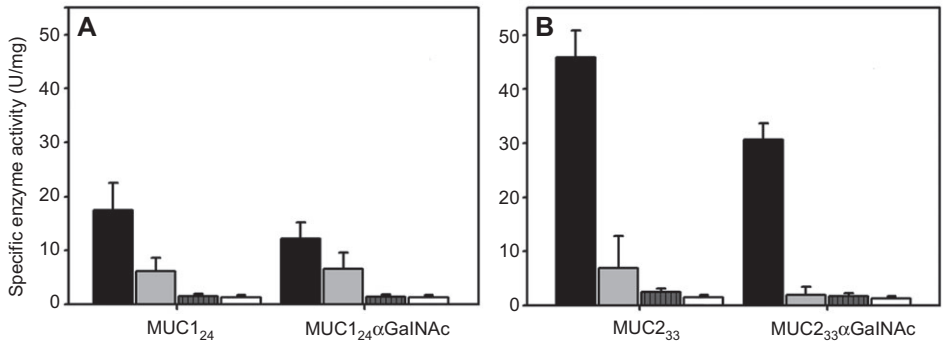

Figure 5 Glycosyltransferase activity measured by colorimetric assay. The catalytic activities of ppGalNAc-T2 (black), ppGalNAc-T2^{K521Q} (gray), acetylated ppGalNAc-T2 (gray-vertical lines), and acetylated ppGalNAc-T2^{K521Q} (white) were measured using surface adsorbed naked and partially preglycosylated MUC1 (A) or MUC2 (B). GalNAc incorporated in mucins was detected with HRP-HPA, as described in the Materials and methods section.

(Figure 5). When the naked MUC1$_{24}$ or partially preglycosylated MUC1$_{24}$ was used as the acceptor peptide, the specific enzyme activity of ppGalNAc-T2^{K521Q} was ~50% lower than that of the wild-type ppGalNAc-T2, whereas in the acetylated versions, the reduction was ~95% (Figure 5A). When the naked MUC2$_{33}$ or the partially preglycosylated MUC2$_{33}$ was used, the K521Q mutation also had a major inhibitory effect (Figure 5B). ppGalNAc-T2^{K521Q}, compared to the wild-type ppGalNAc-T2, showed a 6.6-fold reduction of the specific enzyme activity with the naked MUC2$_{33}$ (6.9 vs. 45.8) and a 15-fold reduction of the specific activity with the partially preglycosylated MUC2$_{33}$ (2.0 vs. 30.7). The reduction of the specific enzyme activity in the acetylated versions of ppGalNAc-T2 and ppGalNAc-T2^{K521Q} was similar to that of the non-acetylated ppGalNAc-T2^{K521Q}.

Discussion

The eukaryotic cells contain a variety of endogenous lectins with important roles on cellular homeostasis. The C-terminal lectin domain of ppGalNAc-Ts has a carbohydrate recognition domain (CRD) with an amino acid homology to members of the R-type lectin family. The lectin domain of ppGalNAc-Ts promotes their catalytic GalNAc-transferase activity for full glycosylation of partially preglycosylated acceptor peptides (Wandall et al., 2007; Raman et al., 2008). The acetylation of ppGalNAc-T2 alters the CRD of the C-terminal domain and, thereby, reduces the catalytic activity (Zlocowski et al., 2011). Here, a mutation of the acetylation site K521 in the lectin domain of ppGalNAc-T2 was created, and its effect on the *O*-glycan biosynthetic pathway was studied. The K521Q mutation altered the results of the direct binding assays with the naked or glycosylated αGalNAc mucins. The binding of the mutant ppGalNAc-T2^{K521Q} to the naked or glycosylated MUC1 was slightly higher than that of the wild-type ppGalNAc-T2, whereas the binding of the mutant to the naked or glycosylated MUC2 was lower. These findings suggest that recognition of the lectin domain of ppGalNAc-T2 to glycosylated mucins is affected by the amino acid sequence of the acceptor peptide, consistent with another recent study (Pedersen et al., 2011). All four ppGalNAc-T2 forms studied (non-acetylated and acetylated wild-type and mutant) bound more strongly to the glycosylated mucins than to the naked mucins, indicating the importance of GalNAc for the interaction.

Competitive assays using Glc, GalNAc, BzlαGalNAc, and UDPαGalNAc helped clarify the carbohydrate specificity of the lectin domain of the four ppGalNAc-T2s. The GalNAc IC50 value was 26-fold lower for ppGalNAc-T2^{K521Q} than for the wild-type ppGalNAc-T2 (15 vs. 400; Table 2). The IC50 value for BzlαGalNAc in the ppGalNAc-T2^{K521Q} interaction was 8 mM, closer to the value for the acetylated ppGalNAc-T2 (10 mM) than that for the non-acetylated ppGalNAc-T2 (>25 mM). The lack of an inhibitory effect by UDPαGalNAc (all IC50 values >25) indicates that the interaction is not mediated by the catalytic domain of ppGalNAc-T2s. The similarity of the profiles for the non-acetylated ppGalNAc-T2^{K521Q} and the acetylated ppGalNAc-T2 in Table 2 shows that the K521Q mutation has an effect similar to that of the acetylation in enhancing the carbohydrate specificity of the lectin domain for αGalNAc. Furthermore, the similarity of the results between the non-acetylated and the acetylated ppGalNAc-T2^{K521Q} suggests that the addition of the acetyl residues does not contribute to the carbohydrate recognition of the mutant ppGalNAc-T2 lectin domain.

Previous studies have used a site-directed mutagenesis approach to study the sites involved in the biological activity of ppGalNAc-Ts. The DXH motif located in the catalytic domain of ppGalNAc-Ts is involved in the coordination bonding to manganese and the binding to the sugar nucleotide donors (Unligil et al., 2000). The point mutations at this site greatly reduced the GalNAc-transferase catalytic activity (Hassan et al., 2000). The point mutations at the lectin domain of the mouse ppGalNAc-T1 do not affect the enzyme activity *in vitro* (Hagen et al., 1999), whereas the point mutations at the lectin domains of ppGalNAc-T4 and ppGalNAc-T2 did alter enzyme activity. These lectin domain mutations reduce the high density of the mucin-type αGalNAc *O*-glycosylation and affect the catalytic activity only when the acceptor is partially preglycosylated (Hassan et al., 2000, Wandall et al., 2007).

In the present study, we combined the acetylation and mutagenic approaches to clarify the role of the acetylation site K521 in the catalytic activity of ppGalNAc-T2. Mutation at this site (ppGalNAc-T2^{K521Q}) resulted in an enzyme activity lower than that of the wild-type ppGalNAc-T2, similar to the effect of the acetylation of ppGalNAc-T2. This finding was confirmed using three acceptor peptides and two different analytical methods. The K521Q mutation of ppGalNAc-T2 greatly reduced the enzyme activity when the acceptor was partially preglycosylated, similar to the results with the D459H mutation (Wandall et al., 2007). The K521Q mutation strongly inhibited the catalytic effect when the naked MUC2 was used as the acceptor peptide, but not when the naked MUC1 or MUC5B was used. Thus, the lectin domain of ppGalNAc-T2 plays a role in the catalytic activity of the enzyme on the naked acceptor peptides (e.g., MUC2), depending on the amino acid sequence of mucin. In comparison to the wild-type ppGalNAc-T2, the mutant ppGalNAc-T2^{K521Q} had a markedly lower degree of interaction and catalytic activity with MUC2 and MUC2αGalNAc, but a similar interaction and only a slightly reduced catalytic activity with MUC1 and MUC1αGalNAc. Thus, the biological effect of an acetyl residue on K521 of ppGalNAc-T2 depends on the amino acid sequence of the acceptor peptide.

Both the acetylated ppGalNAc-T2 and mutant ppGalNAc-T2^{K521Q} specifically recognize the GalNAc residues (as shown by competitive assays), but their catalytic activity on the partially preglycosylated peptides is lower than that of the wild-type ppGalNAc-T2. Posttranslational modifications (PTMs) of ppGalNAc-T2, such as acetylation, may be associated with the novel functions of the lectin domain on the *O*-glycan biosynthetic pathway, e.g., quality control in protein folding or glycan sorting during the pathway through Golgi. Functions of this sort in *N*-glycan biosynthesis have been described for other intracellular lectins such as calnexin, calreticulin, ERGIC53, and VIP36 (Taylor and Drickamer, 2006; Reiterer et al., 2010).

We conclude that an acetylation site in the QKW motif of the lectin domain modulates the carbohydrate recognition specificity and catalytic activity of ppGalNAc-T2 for the partially preglycosylated acceptors and certain naked peptides. The PTMs of ppGalNAc-Ts, such as acetylation, may therefore play key roles in modulating the functions of the R-type lectin domains in cellular homeostasis.

Materials and methods

Expression of ppGalNAc-T2 and mutant ppGalNAc-T2^{K521Q}

The human soluble ppGalNAc-T2 cDNA was cloned into the Baculovirus expression vector pAcGP67 as described previously (Bennett et al., 1996). The construct was inserted into the pAcGP67-His downstream containing a 6xHis-T7 tag (-SSHHHHHHSSGLVPRGSH**MASMTGGQQM**D-; T7 tag shown in bold), generating the pAcGP67-His-T7-ppGalNAc-T2 expression vector. The mutant ppGalNAc-T2^{K521Q} construct was made by introducing a single point mutation (A→C) into this vector, using a Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instruction, with 5′-ATGACAGCAGACAG**CAA**TGGGAACAGATC-3′, synthesized by TAG Copenhagen (S/A, Denmark) (underline=mutation site; boldface=modified codon), as the mutation primer. The PCR reaction consisted of 30 min of Hot Start at 95°C, 13 cycles of 45″ at 95°C, 60″ at 55°C, 15′ at 68°C, and a final elongation of 15′ at 68°C. The mutation was confirmed by sequencing, and the vector was amplified in X-Mega cells using streptomycin as the selective antibiotic. The plasmids were purified by Pure Yield Plasmid Midiprep System (Promega, Madison, WI, USA) and co-transfected with Baculo-Gold DNA (BD Biosciences, Franklin Lakes, NJ, USA). Recombinant baculoviruses were obtained after two successive amplifications in insect Sf9 cells grown at 27°C in Grace's insect medium containing 10% fetal calf serum, and the virus titers were estimated by titration in 24-well plates with monitoring of the enzyme activity. Secreted, soluble recombinant proteins were obtained from the culture medium and purified by affinity chromatography.

Purification of recombinant ppGalNAc-Ts

The proteins were purified from the supernatant of the Sf9 cell culture medium after centrifugation at 2000×*g* for 30 min at 4°C. The supernatant was dialyzed (membrane MWCO <10 kDa) against PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl), and re-centrifuged at 2500×*g* for 30 min at 4°C. The proteins were purified using HisPur™ Cobalt Resin (Thermo Scientific, Rockford, IL, USA) and eluted with 150 mM imidazole in 50 mM sodium phosphate, pH 8.0, 500 mM NaCl. The eluted proteins were dialyzed three times against TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) and concentrated by a centrifuge filter device (Millipore, Temecula, CA, USA; MWCO <10 kDa). The protein concentration was

measured using the bicinchoninic acid method (Micro BCATM Protein Assay Kit, Thermo Scientific). The purity of the recombinant ppGalNAc-Ts was assessed by SDS-PAGE and CBB staining.

Western blotting

The protein samples, in Laemmli sample buffer, were subjected to SDS-PAGE as described previously (Irazoqui et al., 2005). Electrotransferred membranes were blocked with 1% skim milk in PBS for 60 min at room temperature (RT) (~25°C). The HRP-labeled IgG anti-T7 tag antibody (Novagen, San Diego, CA, USA; 1/5000 dilution in 0.05% Tween 20 in PBS) was incubated for 2 h at RT and washed with PBS. The color reaction was developed using 0.1 mg/ml 4-chloro-1-naphthol and 0.02% H_2O_2 in methanol-TBS for 20 min and stopped by washing with distilled water. The purification method of ppGalNAc-Ts was evaluated by immunoblotting.

Acetylation of ppGalNAc-T2 and ppGalNAc-T2^{K521Q}

The proteins were acetylated by the chemical method described previously (Izumi et al., 2003). ppGalNAc-T2 and ppGalNAc-T2^{K521Q} were diluted to 4 μM and dialyzed against 100 mM H_3BO_3 (pH 9.0). Samples, 20 μl, were added with 2 μl freshly prepared 10-mM acetic anhydride (ppGalNAc-T2 to acetic anhydride molar ratio 1:250), incubated for 60 min at 4°C, and 40 μl of 100 mM NH_4HCO_3 was added to terminate the reaction. The acetylated ppGalNAc-T2s prepared in this way were dialyzed against TBS and stored at -18°C until use (Zlocowski et al., 2011).

Lectin binding assay

The polystyrene microtiter plates (Costar, Corning Inc., NY, USA) were coated with peptides $MUC1_{24}$ (TAPPAHGVTSAPDTRPAPGSTAPP) and $MUC2_{33}$ (PTTTPITTTTTVTPTPTPTGTQTPTTTPISTTC), or with glycopeptides (~5 GalNAc/peptide), in carbonate buffer (pH 9.6) overnight at 4°C, washed with PBS, blocked with 0.1% Tween 20 in PBS for 1 h at RT, incubated 2 h at RT with non-acetylated and acetylated ppGalNAc-T2 or ppGalNAc-T2^{K521Q} in PBS with 0.05% Tween 20, washed again with PBS, incubated 60 min at RT with HRP-labeled anti-T7 tag antibody (Novagen) diluted 1/5000 in PBS with 0.05% Tween 20, and washed again with PBS. Color was developed with 0.5 mg/ml *o*-phenylenediamine and 0.02% H_2O_2 in sodium citrate (pH 5.0) at RT, and the reaction was stopped by the addition of 0.5 N H_2SO_4, 50 μl/well. The absorbance was read by microplate reader at wavelength 490 nm (Irazoqui et al., 2005).

Competitive lectin binding assay

The optimal concentrations of the non-acetylated and acetylated ppGalNAc-T2s against $MUC1_{24}$αGalNAc were determined in the lectin binding assay experiments as above. The procedure for the competitive lectin binding assay was the same as for the lectin binding assay except that the non-acetylated or acetylated ppGalNAc-T2 or ppGalNAc-T2^{K521Q} was preincubated with carbohydrate for 1 h at RT before being added to the well (Irazoqui et al., 1999).

GalNAc-transferase assays

(A) Soluble assay with mass spectrometry

Standard enzymatic assay was performed on 50 μl total reaction mixture containing 25 mM cacodylate (pH 7.4), 10 mM $MnCl_2$, 0.25% Triton X-100, 200 μM UDPαGalNAc, 0.5 μg/ml ppGalNAc-T, and 20 μM acceptor peptide, incubated for 2 h at 37°C. The glycosyltransferase acceptor peptides assayed were $MUC1_{24}$, $MUC2_{33}$, and $MUC5B_{27}$ (SSTPGTTWILTELTTTATTTESTGSTA), synthesized by Neosystem (Strasbourg, France) or Cancer Research UK. Samples were purified by Poros 20 R2 nanoscale reversed-phase chromatography (Perceptive Biosystems, Applied Biosystems, Foster City, CA, USA) and applied directly to a probe for MALDI-TOF MS analysis (Wandall et al., 2007). The matrix used was 2,5-dihydroxybenzoic acid (25 mg/ml, Aldrich, St. Louis, MO, USA) dissolved in a mixture (2:1) of 0.1% trifluoroacetic acid in 30% aqueous acetonitrile (Rathburn Chemicals Ltd, Walkerburn, UK). The mass spectra were acquired on a Voyager-DE mass spectrometer equipped with delayed extraction (Perceptive Biosystems).

(B) Surface adsorbed assay with colorimetry

Polystyrene microtiter plates (Costar) were coated with 50 μl (4 μM) peptides or partially preglycosylated peptides (~1.2 GalNAc/peptide) in carbonate buffer (pH 9.6) overnight at 4°C, washed with PBS, and blocked with 0.1% Tween 20 in PBS for 1 h at RT. A catalytic reaction mixture containing 25 mM cacodylate (pH 7.4), 10 mM $MnCl_2$, 0.05% Tween 20, and 50 μMUDPαGalNAc was incubated 20 min at RT with 0.1 μg/ml non-acetylated or acetylated ppGalNAc-T2 or ppGalNAc-T2^{K521Q}, washed again with PBS, incubated 60 min at RT with HRP-*Helix pomatia* agglutinin (HPA) diluted 1/1000 in PBS with 0.05% Tween 20, and washed again with PBS. The color was developed with 0.5 mg/ml *o*-phenylenediamine and 0.02% H_2O_2 in sodium citrate (pH 5.0) at RT, and the reaction was stopped by the addition of 0.5 N H_2SO_4, 50 μl/well. The absorbance was read by a microplate reader at 490 nm.

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