Biochemical and Biophysical Research Communications 410 (2011) 140–145

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Catalytic and glycan-binding abilities of ppGalNAc-T2 are regulated by acetylation

Natacha Zlocowski [a], Victor G. Sendra [a], Virginia Lorenz [a], Marcos A. Villarreal [a], Alberto Jorge [b], Yolanda Núñez [b], Eric P. Bennett [c], Henrik Clausen [c], Gustavo A. Nores [a], Fernando J. Irazoqui [a,*]

[a] Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC, UNC–CONICET), Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina
[b] Centro de Biología Molecular "Severo Ochoa", CSCIC, Universidad Autónoma de Madrid, Spain
[c] Faculty of Health Sciences, University of Copenhagen, Blegdamsvej 3, Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 16 May 2011
Available online 27 May 2011

Keywords:
Acetylation effect
Glycosyltransferase activity
Lectin domain

ABSTRACT

Post-translational acetylation is an important molecular regulatory mechanism affecting the biological activity of proteins. Polypeptide GalNAc transferases (ppGalNAc-Ts) are a family of enzymes that catalyze initiation of mucin-type O-glycosylation. All ppGalNAc-Ts 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 ("ricin-like") lectin domain. We investigated the effect of acetylation on catalytic activity of glycosyltransferase, and on fine carbohydrate-binding specificity of the R-type lectin domain of ppGalNAc-T2. Acetylation effect on ppGalNAc-T2 biological activity *in vitro* was studied using a purified human recombinant ppGalNAc-T2. Mass spectrometric analysis of acetylated ppGalNAc-T2 revealed seven acetylated amino acids (K103, S109, K111, K363, S373, K521, and S529); the first five are located in the catalytic domain. Specific glycosyltransferase activity of ppGalNAc-T2 was reduced 95% by acetylation. The last two amino acids, K521 and S529, are located in the lectin domain, and their acetylation results in alteration of the carbohydrate-binding ability of ppGalNAc-T2. Direct binding assays showed that acetylation of ppGalNAc-T2 enhances the recognition to αGalNAc residue of MUC1αGalNAc, while competitive assays showed that acetylation modifies the fine GalNAc-binding form of the lectin domain. Taken together, these findings clearly indicate that biological activity (catalytic capacity and glycan-binding ability) of ppGalNAc-T2 is regulated by acetylation.

1. Introduction

Mucin-type O-glycosylation is initiated when GalNAc is α-linked to threonine or serine (GalNAcαThr/Ser) of the acceptor peptide. UDPαGalNAc is the sugar donor in reactions catalyzed by the enzyme uridine diphospho-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase (ppGalNAc-T) [1]. The mammalian ppGalNAc-T family contains more than 14 members, having confirmed transferase activity, different peptide substrate specificities, and dissimilar patterns of expression [2]. Distinct biological functions, without overlapping redundancy, have been described for all members of the ppGalNAc-T family [3]. Mutations in members of the pgant family (pgant35A) of *Drosophila* were shown to abrogate enzyme activity that initiates O-glycosylation, resulting in recessive lethality [4]. Based on studies of structure–function relationships of ppGalNAc-Ts, several amino acid (a.a.) domains with different biological activities have been identified. ppGalNAc-Ts are type II transmembrane proteins with a short N-terminal cytoplasmic tail (4–24 a.a.s), followed by a small transmembrane anchor (15–25 a.a.s) and a Golgi lumenal region [5]. The Golgi region of the protein includes a stem section, catalytic domain, and C-terminal end. The C-terminal domain has sequence and predicted structural homology to a lectin in the plant toxin ricin [6].

Lectins are glycan-binding proteins that play important roles in mammalian cellular homeostasis. They are classified into families based on structural and functional homology [7]. In many cases, the characteristics that define a lectin family are protein conformation of glycan-binding pockets, and carbohydrate specificity. Members of one intracellular lectin family, the R-type lectins, display a protein conformation similar to that of the galactose-binding B chain of ricin, i.e., three lobes arranged as a β-trefoil around a 3-fold axis [8]. The $(QXW)_3$ motif has been observed in several lectins within the R-type family, and is termed the "ricin-like lectin domain" [9]. This motif is essentially a flexible lectin scaffold, and is present in the C-terminal domain of all ppGalNAc-Ts [10]. The lectin domain of ppGalNAc-T4 was reported to play an active role in GalNAc-transferase effect on glycopeptide acceptors [11]. The C-terminal R-type domains of ppGalNAc-T4 and ppGalNAc-T2 direct lectin binding to GalNAc-glycopeptide substrates required for high density GalNAc-O-glycosylation [12].

* Corresponding author. Address: Haya de la Torre s/n, Ciudad Universitaria, 5000 Córdoba, Argentina. Fax: +54 351 4334074.
E-mail address: irazoqui@dqb.fcq.unc.edu.ar (F.J. Irazoqui).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.05.125

Genome-wide correlative studies of loci that influence plasma lipid levels in humans identified ppGalNAc-T2 as one of several genes associated with variations in high density lipoprotein levels [13]. ppGalNAc-T2 variants may contribute to the heritable component of lipid profiles by glycosylation of proteins involved in lipid metabolism. Therefore, studies of O-linked glycosylation provide a new approach for therapeutic regulation of lipid levels, and for treatment of cardiovascular diseases.

Post-translational modification (PTM) is an important molecular regulatory mechanism in proteins. It involves modification of chemical and physical properties of proteins, e.g., folding and biological activity, and consequent changes in function [14]. Examples of biological targets of PTM include phosphorylation in signal transduction, attachment of fatty acids for membrane anchoring, glycosylation for changing protein half-life or targeting substrates, and acetylation in chromatin regulation and gene expression. The regulatory scope of lysine acetylation is broad, and comparable with that of other major post-translational modifications [15]. Post-translational acetylation has also been shown to regulate the biological activity of histone and non-histone proteins such as p53 and NFkB [16].

Here, we studied effects of acetylation on catalytic activity of ppGalNAc-T2, and on carbohydrate-binding specificity of the ricin-like lectin domain of ppGalNAc-T2 as a regulatory control of glycosyltransferases.

2. Materials and methods

2.1. Expression of ppGalNAc-transferases

Human soluble ppGalNAc-T2 cDNA was cloned into Baculovirus expression vector pAcGP67 as described previously [12]. The construct was inserted into pAcGP67-His downstream containing a 6xHis-T7 tag (-SSHHHHHHSSGLVPRGSHMASMTGGQQMD-; T7 tag shown in bold), generating pAcGP67-His-T7-ppGalNAc-T2 expression vector. Sf9 cells were grown at 27 °C in Grace's insect medium containing 10% fetal calf serum. Plasmid pAcGP67-His-T7-ppGalNAc-T2 was co-transfected with Baculo-Gold DNA (BD Biosciences). Recombinant baculovirus was obtained after two successive amplifications in Sf9 cells grown in serum-containing medium, and virus titers were estimated by titration in 24-well plates and monitoring of enzyme activity. The amplified virus was infected in High Five$_{TM}$ cells grown in serum-free medium (Invitrogen, Carlsbad, CA), in upright roller bottles in shaking water bath (140 rpm) at 27 °C. Secreted, soluble recombinant proteins were harvested by centrifugation at 2000*g*, and supernatants were subjected to affinity chromatography.

2.2. Purification of recombinant ppGalNAc-T2

Proteins were purified from supernatant of High Five medium after centrifugation at 2000*g* for 30 min at 4 °C. The supernatant was dialyzed against PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl), and re-centrifuged at 2500*g* for 30 min at 4 °C. Proteins were purified by iminodiacetic acid metal affinity chromatography (IMAC) on Ni^{2+}-charged resin (Qiagen), and eluted with 250 mM imidazole in 50 mM sodium phosphate, pH 8.0, 500 mM NaCl. Eluted proteins were dialyzed three times against PBS, and concentrated by a centrifuge filter device (Millipore; 10,000 kDa cut off). Homogeneity of recombinant ppGalNAcT2 was confirmed by SDS–PAGE and Coomassie Brilliant Blue (CBB) staining.

2.3. Acetylation of ppGalNAc-T2

Protein acetylation was performed as described previously [17]. ppGalNAc-T2 was diluted to 4 μM with 25 volumes of 100 mM H_3BO_3 (pH 9.0). To each 20 μl sample, 2 μl freshly prepared 10 mM acetic anhydride was added (molar ratio 1:250 ppGalNAc-T2 to acetic anhydride), the mixture was incubated 60 min at 4 °C, and reaction was terminated by addition of 40 μl 100 mM NH_4HCO_3. Resulting acetylated ppGalNAc-T2 was dialyzed against PBS and stored at −18 °C until use. Samples were analyzed by SDS–PAGE (7.5% gel) and stained with CBB. Acetylation of ppGalNAc-T2 resulted in altered electrophoretic mobility.

2.4. Identification of acetylated a.a.s by MS

Acetylated vs. non-acetylated ppGalNAc-T2 were compared in order to identify acetylated a.a.s. Protein bands were excised from CBB-stained SDS–PAGE, and digested with trypsin *in situ* as described previously [18], with modification. The digest was analyzed by a MALDI-TOF (matrix-assisted laser desorption/ionization time of flight) MS using an Autoflex spectrometer equipped with reflector, 2.5-dihydroxybenzoic acid as matrix and Anchor-Chip surface target (Bruker Daltonics, Bremen, Germany). Peak identification and monoisotopic peptide mass were assigned automatically using Flex Analysis software (Bruker Daltonics). Assignments were verified by analyzing the peptide by RP-LC/MS (reversed-phase liquid chromatography coupled to MS), using a Deca XP mass spectrometer and a ThermoHypersil (0.18 ×150 mm) C18 column. MS/MS spectra were analyzed by assigning fragments to the candidate sequence, after calculation of the series of theoretical fragmentations, according to the nomenclature of a previously-described series. Finally, the acetyl residues in a.a.s K103, S109, K111, K363, S373, K521, and S529 of human ppGalNAc-T2 were identified.

2.5. ppGalNAc-transferase assays

Standard assays were performed on 25–50 μl of total reaction mixture containing 25 mM cacodylate (pH 7.4), 10 mM $MnCl_2$, 0.25% Triton X-100, 50 μM UDP-[^{14}C]GalNAc (1–3000 cpm/nmol) (Amersham Biosciences), 0.01–0.5 mU ppGalNAc-transferase, and 20–100 μM acceptor peptide. MUC1 (HGVTSAPDTRPAPGSTAPPA), MUC2 (PTTTPISTTTMVTPTPTPTC), and MUC5B (VLTTTATTPTA), synthesized by Neosystem (Strasbourg), or partially pre-glycosylated peptides (∼1.2 αGalNAc residue/peptide molecule), were the assayed acceptors of glycosyltransferase. [^{14}C]GalNAc incorporation in acceptor peptides, catalyzed by ppGalNAc-T2, was determined by scintillation counting following Dowex-1 formic acid chromatography [19]. Enzyme activity of ppGalNAc-T2 was expressed as specific activity (U/mg) relative to the specific activity for each acceptor peptide, defined as 100%. Following acetylation, the residual specific activity of ppGalNAc-T2 was calculated as a percentage of the value for non-acetylated enzyme.

2.6. Lectin binding assay

Polystyrene microtiter plates (Maxisorb, Nunc) were coated with peptides or glycopeptides 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 or acetylated ppGalNAc-T2 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 reaction was stopped by addition of 0.5 N H_2SO_4, 50 μl/ well. Absorbance was read at 490 nm with a microplate reader [20]. Optical densities of products of interactions of ppGalNAcT-2 vs. acetylated ppGalNAcT-2, with naked peptide vs. glycosylated peptide, are shown.

2.7. Competitive lectin binding assay

Optimal concentrations of acetylated and non-acetylated ppGalNAc-T2 showing optical density value 1.0 against MUC1α GalNAc were determined in regular lectin binding assay experiments as above. The procedure for competitive lectin binding assay was the same as for regular lectin binding assay except that ppGalNAc-T2 or acetylated ppGalNAc-T2 was pre-incubated with carbohydrate for 1 h at RT before being added to the well [20].

2.8. Molecular conformations

The ppGalNAc-T2 conformation was obtained from PDB (codes 2FFU and 2FFV), and was visualized using AutoDock 4.0 software (Scripps Research Institute, La Jolla, CA, USA). Atoms of acetylated a.a.s are shown as spheres, while the rest of the molecule appears as ribbons. Stereochemical positions of Mn^{++}, UDP, and acceptor peptide in the catalytic domain are shown.

Initial structures of the GalNAc glycosides PhlαGalNAc and UDPαGalNAc, and the glycoconjugates GST(αGalNAc)AP (MUC1α GalNAc) and GalNAcα3(Fucα2)Gal (blood group A trisaccharide), were generated by a molecular editor. Glycan conformations were obtained by energy minimization of the structures, using MM2 energy function [21].

3. Results and discussion

3.1. Effect of acetylation on glycosyltransferase activity of ppGalNAc-T2

Members of the ppGalNAc-T family display unique spatial and temporal gene expression patterns during both mammalian and *Drosophila* development [22]. The particular substrate performance of each member, and the hierarchy of action within this family, suggest that acquisition of mucin-type O-glycans is governed by a complex and highly regulated process [2]. The conformation of isoform 2 of ppGalNAc-T (ppGalNAc-T2) was studied previously using X-ray crystallography (Protein Data Bank (PDB) codes 2FFU and 2FFV) [23,24]. This protein catalyzes incorporation of αGalNAc at three different acceptor sites every 20 a.a. tandem of MUC1 [11]. D458H mutation in the lectin domain of ppGalNAc-T2 was shown to affect binding to MUC1αGalNAc, and to suppress high density biosynthesis of αGalNAc O-glycosylation [12].

A recombinant protein was generated in order to study the biological effect of acetylation on ppGalNAc-T2. Recombinant human ppGalNAc-T2 was expressed in insect cells as a soluble protein by removing the cytosolic tail and transmembrane domain from the N-terminal region. It was constructed with 6xHis motif for affinity-chromatography purification, and with T7 tag for detection. Next to the stem region, the construct had an active catalytic GalNAc-transferase domain, and a C-terminal ricin-like lectin domain (Fig. 1A). The recombinant ppGalNAc-T2, purified to homogeneity by affinity chromatography (Fig. 1B-a), was acetylated *in vitro*, and displayed electrophoretic mobility different from that of non-acetylated protein (Fig. 1B-b). Samples of non-acetylated and acetylated ppGalNAc-T2, run on SDS–PAGE and CBB stained gel (Fig. 1B), were used for study by mass spectrometry (MS). Comparison of acetylated vs. non-acetylated ppGalNAc-T2 revealed the a.a. sites where acetyl residues were covalently bound (Suppl. Doc. 1). Seven acetyl residues bound to ppGalNAc-T2 were detected by MS (Fig. 1C). Acetyl residues were found in a.a.s K103, S109, K111 (*m/z* 1604-N**K**FNQVE**S**D**K**LR), K363, S373 (*m/z* 1934-Q$_{\mathbf{K}}$HPYTFPGG**S**GTVFAR), K521, and S529 (*m/z* 1700-Q**K**WEQIEGN**S**KLR). Of these a.a.s, K521 and S529 were in the R-type lectin domain, and the rest were in the catalytic domain.

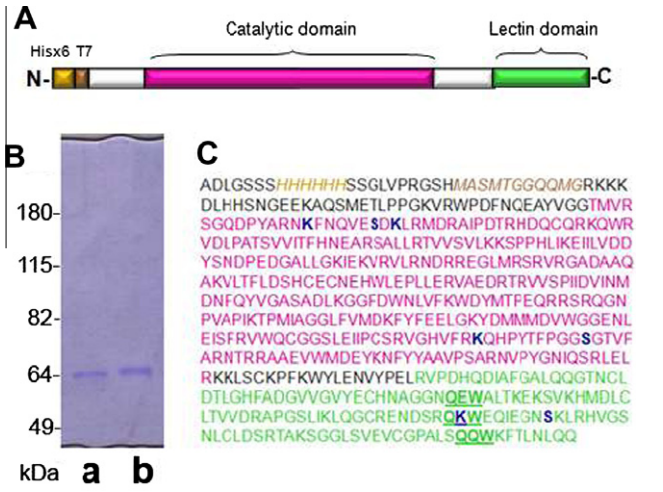

Fig. 1. Acetylated recombinant ppGalNAc-T2. A: Scheme of soluble recombinant ppGalNAc-T2 showing two tags (Hisx6 and T7), the catalytic domain, and C-terminal R-type lectin domain. B: CBB staining SDS–PAGE of purified recombinant ppGalNAc-T2 without (a) or with (b) acetylation, showing different electrophoretic mobilities. C: a.a. sequence of recombinant ppGalNAc-T2, indicating tags (brown, italic), catalytic domain (fuchsia), lectin domain (green), and $(QXW)_3$ motif characteristic of the R-type lectin family (bold, underline). MS of acetylated ppGalNAc-T2 determined the presence of acetyl residues in seven a.a.s (blue, bold) of ppGalNAc-T2 (K103, S109, K111, K363, S373, K521, S529). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Acetylation affected the enzymatic properties of ppGalNAc-T2 (Fig. 2A). The presence of acetyl residues reduced the catalytic specific activity of ppGalNAc-T2 by >95% in assays of naked MUC1, MUC2, and MUC5B acceptor peptides. Identical result was observed when partially pre-glycosylated acceptor peptides were assayed (Fig. 2B). Fig. 2C shows positions of acetylated a.a.s in the crystallographic conformation of ppGalNAc-T2, and magnification of the catalytic domain in the presence of acceptor peptide, UDP, and Mn^{++}, indicating location of the catalytic pocket (PDB code 2FFU) (Fig. 2D). Of the five acetylated a.a.s in the catalytic domain, K363 and S373 were found to be located in flexible "loop B" (a.a.s 361–377), adjacent to the catalytic pocket. The evolutionarily conserved residue W331 appears to act as a gatekeeper, controlling the conformational transition of loop B adjacent to the catalytic active site, from closed (unable to accept ligand; PDB code 2FFU) to open (able to accept ligand; PDB code 2FFV) conformation [23,24]. Loop B may be involved in positioning of substrates into the active site. Acetylation of K363 and S373 presumably alters normal function of loop B, and consequently activity of the catalytic site of ppGalNAc-T2.

3.2. Effect of ppGalNAc-T2 acetylation on direct binding to glycan

Presence of $(QXW)_3$ motif, the flexible scaffold characteristic of members of the R-type lectin family, in the C-terminal domain of ppGalNAc-T2, accounts for the lectin properties of this protein [6]. The lectin domain of ppGalNAc-T2 was shown recently to have active glycan-binding ability [12], and this ability is altered by acetylation. Dose/response curves for several concentrations of non-acetylated and acetylated ppGalNAc-T2, interacting with naked peptides (MUC1 and MUC2) and with glycosylated peptides (MUC1αGalNAc and MUC2αGalNAc), are shown in Fig. 3. The degree of interaction of acetylated ppGalNAc-T2 with naked MUC1 and MUC2 was lower than that of non-acetylated ppGalNAc-T2. Similarly, acetylation significantly reduced the glycosyltransferase activity of ppGalNAc-T2 on MUC1 and MUC2 acceptor peptides.

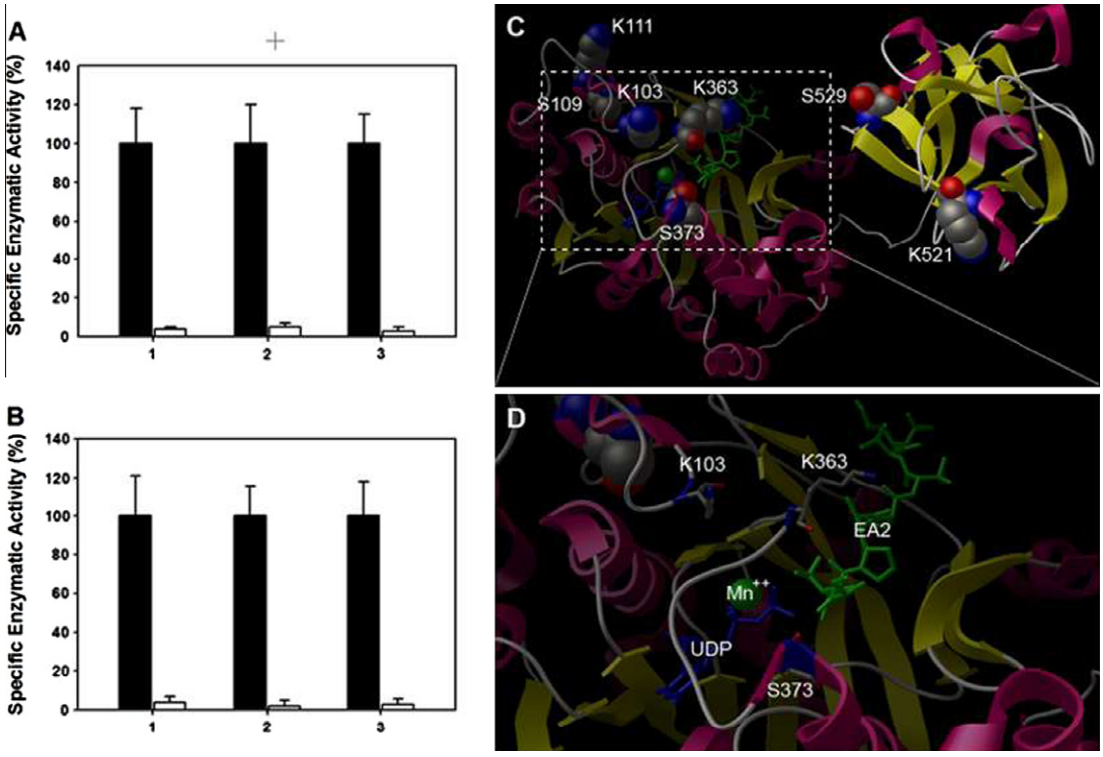

Fig. 2. Effect of acetylation on catalytic activity of ppGalNAc-T2. Specific enzymatic activity of non-acetylated ppGalNAc-T2 (black) and acetylated ppGalNAc-T2 (white), assayed with peptide acceptors MUC1 (1), MUC2 (2), and MUC5B (3) (A) or MUC1αGalNAc (1), MUC2αGalNAc (2), and MUC5BαGalNAc (3) (B). Conformation of human ppGalNAc-T2 (PDB code 2FFU). The seven acetylated a.a.s are shown as spheres (**C**). Magnification of the catalytic domain reveals that K103, K363, and S373 are in the environment of the catalytic pocket (D). Stereochemical positions of Mn^{++} (green), UDP (red), and acceptor peptide EA2 (green) are indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Although interactions of non-acetylated and acetylated ppGalNAc-T2 with MUC1αGalNAc were similar, acetylated ppGalNAc-T2 showed higher recognition to αGalNAc on MUC1 because its interaction with naked MUC1 was lower. Acetylation of ppGalNAc-T2 clearly affected its carbohydrate-binding ability to MUC1αGalNAc. However, this binding ability is not enhanced when αGalNAc is covalent linked to MUC2 (Fig. 3C–D) or FGF23 (data not shown), reflecting the importance of the environment in binding of acetylated ppGalNAc-T2. Neither non-acetylated nor acetylated ppGalNAc-T2 showed binding to blood group A trisaccharide (GalNAcα3(Fucα2)Gal-) conjugated to BSA, or to terminal lactosamine conjugated to BSA (data not shown). The influence of environment of the sugar ligand on interaction differs for each lectin. We previously described several examples in which not all terminal GalNAc residues were recognized by a GalNAc-binding lectin [20].

The fact that acetylated ppGalNAc-T2 showed major recognition for αGalNAc of MUC1αGalNAc could suggest a positive effect on high density of αGalNAc O-glycosylation, however glycosyltransferase activity on partially pre-glycosylated acceptor peptide was not observed (Fig. 2B).

3.3. Fine glycan-binding recognition of acetylated ppGalNAc-T2

Fine carbohydrate-binding specificity of non-acetylated vs. acetylated ppGalNAc-T2 was studied by competitive assay as described previously [20]. The two forms of ppGalNAc-T2 were incubated with MUC1αGalNAc under optimal interaction conditions, in the presence of various potential inhibitors, i.e., free sugars, and α- or β-anomers of GalNAc/GlcNAc glycosides. The majority of carbohydrates and glycosides had no inhibitory effect on binding of non-acetylated ppGalNAc-T2 to MUC1αGalNAc (Fig. 4A). Only GalNAc produced 50% binding inhibition, at high concentration (IC50: 400 mM). Assay results for acetylated ppGalNAc-T2 were quite different (Fig. 4B). Of the free monosaccharides, GalNAc strongly inhibited acetylated ppGalNAc-T2 binding (IC50: 20 mM), whereas Glc, GlcNAc, Gal, MeαGal, and lactose had no effect. The binding-inhibitory effect of GalNAc on acetylated ppGalNAc-T2 was 20-fold higher than on non-acetylated ppGalNAc-T2 (400 compared to 20) (Suppl. Table 1). Clearly, acetylation influences the form of ppGalNAc-T2 lectin domain that recognizes GalNAc residue of MUC1αGalNAc. That the lectin domain, rather than the catalytic region, is involved in this recognition process was also evidenced by the very weak inhibitory effect of UDPαGalNAc. UDPαGalNAc functions as the sugar donor for enzyme activity of ppGalNAc-T2, which has an UDPαGalNAc binding pocket on the catalytic domain (Fig. 2D). If the ppGalNAc-T2 catalytic domain were involved in interaction with the sugar residue of MUC1αGalNAc, then UDPαGalNAc would be the strongest binding inhibitor, in contrast to the results obtained. A weak inhibitory effect of UDPαGalNAc was observed previously for interaction of ppGalNAc-T2 lectin domain with MUC1αGalNAc [12], consistent with our findings. We conclude that interaction of acetylated ppGalNAc-T2 with MUC1αGalNAc takes place through the lectin domain.

Direct binding and competitive assays also provided information regarding the glycan-binding site of acetylated ppGalNAc-T2. GlcNAc glycosides (Bzl, pNP), and free GlcNAc, had no inhibitory effect, indicating that this monosaccharide or its aglycons *per se* are not recognized by acetylated ppGalNAc-T2. In contrast, GalNAc glycosides (Phl, pNP, Bzl) had a stronger inhibitory effect than free GalNAc on binding of acetylated ppGalNAc-T2 (Suppl. Table 1). That is aglycon residues have an effect on binding, but only in the case of GalNAc. PhlαGalNAc had an inhibitory effect 3-fold higher than that of GalNAc on acetylated ppGalNAc-T2 binding.

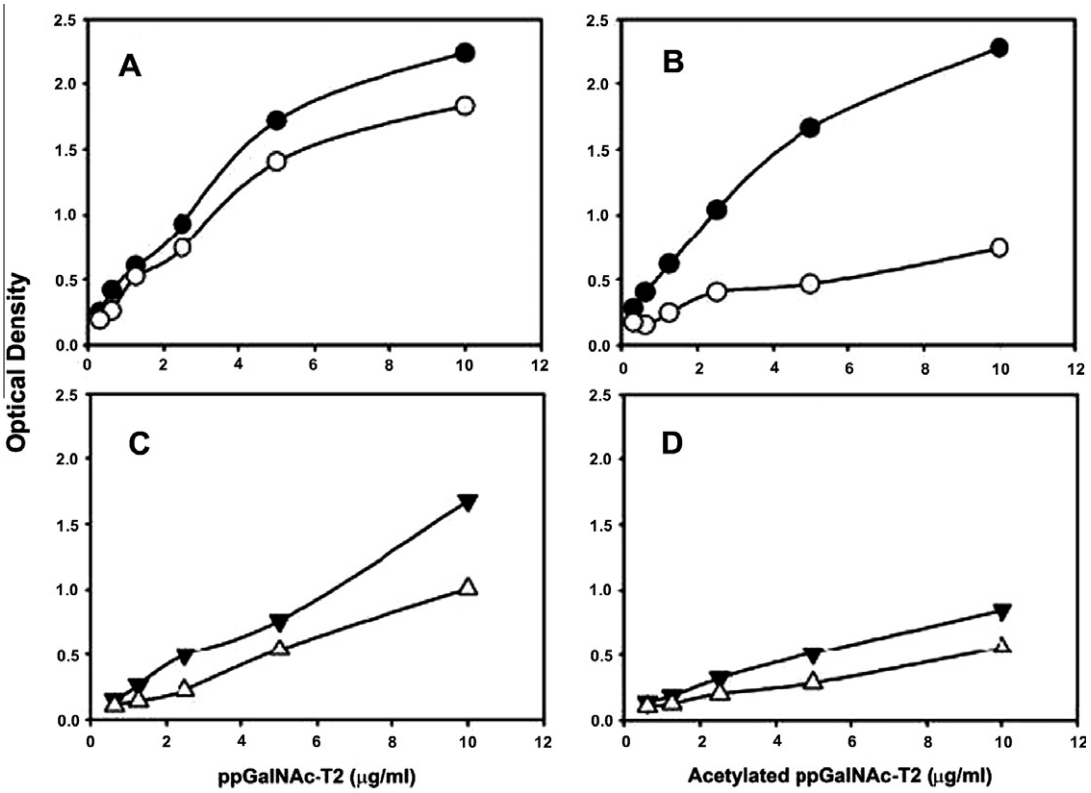

Fig. 3. Reactivity of non-acetylated and acetylated ppGalNAc-T2 with MUC1αGalNAc, MUC2αGalNAc, MUC1 and MUC2 by lectin binding assay. Interaction of non-acetylated ppGalNAc-T2 (A) and (C) and acetylated ppGalNAc-T2 (B) and (D) with MUC1αGalNAc (solid circles), naked MUC1 (open circles), MUC2αGalNAc (inverted solid triangle), and naked MUC2 (open triangle) is indicated as optical density. Adsorbed (acetylated) ppGalNAc-T2 was detected with HRP-labeled anti-T7 tag antibody, as described in M&M.

The Phl residue, because of its hydrophobicity, has a potential capacity for interacting with non-polar residues, suggesting the presence of hydrophobic loci adjacent to the GalNAc-binding site of acetylated ppGalNAc-T2. Stereochemical analysis of PhlαGalNAc and MUC1αGalNAc revealed a common non-polar subsite adjacent to the GalNAc residue (Fig. 4Ca-c), consistent with the observed

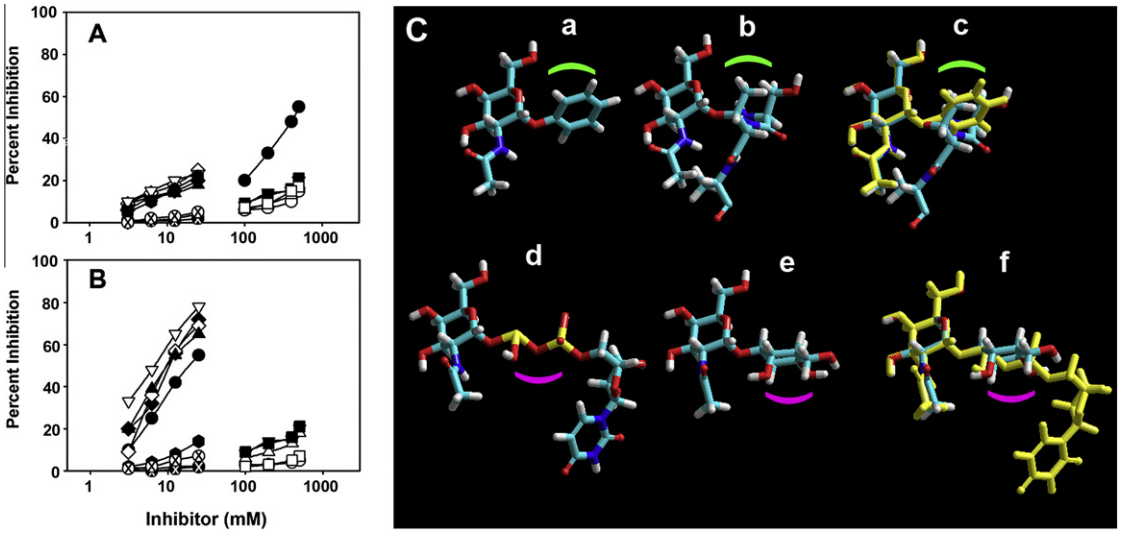

Fig. 4. Competitive lectin binding assay, using carbohydrates as inhibitors of non-acetylated or acetylated ppGalNAc-T2 interaction. Wells coated with MUC1αGalNAc were incubated with non-acetylated (A) or acetylated (B) ppGalNAc-T2, followed by incubation with various carbohydrates: GlcNAc (open square), Glc (open triangle), Gal (solid square), MeαGal (open circle), lactose (inverted solid triangle), GalNAc (solid circle), PhlαGalNAc (inverted open triangle), pNPαGalNAc (solid diamond), pNPβGalNAc (open diamond), BzlαGalNAc (solid triangle), UDPαGalNAc (solid hexagon), UDPαGlcNAc (X), BzlαGlcNAc (open circle with black X), pNPαGlcNAc (solid circle with white X). Adsorbed (acetylated) ppGalNAc-T2 was detected with HRP-labeled anti-T7 tag antibody, as described in M&M. Glycan recognition of acetylated ppGalNAc-T2 (C). Minimum energy conformation of glycans is shown by element colors in tube form: PhlαGalNAc (a), MUC1αGalNAc (b), overlapping PhlαGalNAc (yellow) + MUC1αGalNAc (c), UDPαGalNAc (d), blood group A trisaccharide (e), overlapping UDPαGalNAc (yellow) + blood group A trisaccharide (f). In the MUC1αGalNAc (GST(αGalNAc)AP) structure, G and P a.a.s are hidden. Fucose of blood group A trisaccharide ((GalNAcα3(Fucα2)Gal) is also hidden to allow clear visualization of the atoms. Rotated green parentheses indicate common hydrophobic loci on PhlαGalNAc and MUC1αGalNAc adjacent to GalNAc, both of which are recognized by acetylated ppGalNAc-T2. Rotated fuchsia parentheses indicate the polar region close to terminal GalNAc in structures that are not recognized by acetylated ppGalNAc-T2.

binding to acetylated ppGalNAc-T2. UDPαGalNAc and blood group A residue had no such hydrophobic region, but did have a polar region adjacent to α-linked GalNAc, which accounts for their inability to interact with acetylated ppGalNAc-T2 (Fig. 4Cd-f).

Covalent bonding of biotin to protein (biotinylation) produced significant changes in glycan-binding recognition of ppGalNAc-T2, again indicating major GalNAc specificity [12]. The biotinylation strategy (using biotinyl-*N*-hydroxysuccinimide) and PTM acetylation strategy both involve molecules that are chemically conjugated to protein through the ε-amine of lysine [25]. This results in neutralization of the positive charge of primary amines, thereby reducing polarity of the lysine side chain. Changes in protein conformation and stability as a consequence of acetylation have been described previously. MS analysis of acetylated ppGalNAc-T2 revealed two acetyl residues, K521 and S529, on the lectin domain. K521 is part of the QKW motif, which constitutes the R-type lectin domain scaffold of ppGalNAc-T2. Neutralization of the positive charge of ε-amine of K521 by acetylation may affect folding of the carbohydrate-binding pocket in the ppGalNAc-T2 lectin domain. Acetyl residues from acetylation are commonly involved in hydrophobic lectin-glycan interaction [26]. We conclude that the two acetyl residues (K521 and S529) in the lectin domain of acetylated ppGalNAc-T2 cause changes in protein conformation, and also contribute to hydrophobic interactions in the environment of GalNAc-binding site of the lectin domain of non-acetylated ppGalNAc-T2, which accounts for the novel carbohydrate-binding recognition form of acetylated ppGalNAc-T2.

Finally, acetylation reduces catalytic activity of ppGalNAc-T2, but enhances recognition to the αGalNAc residue of MUC1αGalNAc, as shown by direct binding assays. The specific GalNAc-binding form of the lectin domain is affected by acetylation, as demonstrated by competitive assays. These finding clearly indicate that biological activity of ppGalNAc-T2 is regulated by acetylation. The role of acetylated ppGalNAc-Ts remains to be clarified in future studies.

Acknowledgments

This study was supported by funding (to FJI) from the following Argentinean institutions: CONICET, SeCyT (UNC), MinCyT (Cba), ANPCyT-PICT 967. The authors are grateful to Dr. Stephen Anderson for English-language editing of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.125.

References

[1] H. Clausen, E.P. Bennett, A family of UDP-GalNAc: polypeptide N-acetylgalactosaminyl-transferases control the initiation of mucin-type O-linked glycosylation, Glycobiology 6 (1996) 635–646.
[2] E. Tian, K.G. Ten Hagen, Recent insights into the biological roles of mucin-type O-glycosylation, Glycoconj. J. 26 (2009) 325–334.
[3] J.D. Marth, Complexity in O-linked oligosaccharide biosynthesis engendered by multiple polypeptide *N*-acetylgalactosaminyltransferases, Glycobiology 6 (1996) 701–705.
[4] K.G. Ten Hagen, D.T. Tran, A UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase is essential for viability in Drosophila melanogaster, J. Biol. Chem. 277 (2002) 22616–22622.
[5] T.A. Fritz, J.H. Hurley, L.B. Trinh, J. Shiloach, L.A. Tabak, The beginnings of mucin biosynthesis: the crystal structure of UDP-GalNAc:polypeptide alpha-N-acetylgalactosaminyltransferase-T1, Proc. Natl. Acad. Sci. USA 101 (2004) 15307–15312.
[6] A. Imberty, V. Piller, F. Piller, C. Breton, Fold recognition and molecular modeling of a lectin-like domain in UDP-GalNac:polypeptide N-acetylgalactosaminyltransferases, Protein. Eng. 10 (1997) 1353–1356.
[7] A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, M.E. Etzler, Essentials of Glycobiology, second ed., Cold Spring Harbor Laboratory Press, New York, 2009.
[8] E. Rutenber, J.D. Robertus, Structure of ricin B-chain at 2.5 A resolution, Proteins 10 (1991) 260–269.
[9] B. Hazes, R.J. Read, A mosquitocidal toxin with a ricin-like cell-binding domain, Nat. Struct. Biol. 2 (1995) 358–359.
[10] B. Hazes, The (QxW)3 domain: a flexible lectin scaffold, Protein Sci. 5 (1996) 1490–1501.
[11] H. Hassan, C.A. Reis, E.P. Bennett, E. Mirgorodskaya, P. Roepstorff, M.A. Hollingsworth, J. Burchell, J. Taylor-Papadimitriou, H. Clausen, The lectin domain of UDP-N-acetyl-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase-T4 directs its glycopeptide specificities, J. Biol. Chem. 275 (2000) 38197–38205.
[12] H.H. Wandall, F. Irazoqui, M.A. Tarp, E.P. Bennett, U. Mandel, H. Takeuchi, K. Kato, T. Irimura, G. Suryanarayanan, M.A. Hollingsworth, H. Clausen, The lectin domains of polypeptide GalNAc-transferases exhibit carbohydrate-binding specificity for GalNAc: lectin binding to GalNAc-glycopeptide substrates is required for high density GalNAc-O-glycosylation, Glycobiology 17 (2007) 374–387.
[13] S. Kathiresan, O. Melander, C. Guiducci, A. Surti, N.P. Burtt, M.J. Rieder, G.M. Cooper, C. Roos, B.F. Voight, A.S. Havulinna, B. Wahlstrand, T. Hedner, D. Corella, E.S. Tai, J.M. Ordovas, G. Berglund, E. Vartiainen, P. Jousilahti, B. Hedblad, M.R. Taskinen, C. Newton-Cheh, V. Salomaa, L. Peltonen, L. Groop, D.M. Altshuler, M. Orho-Melander, Six new loci associated with blood low-density lipoprotein cholesterol, high density lipoprotein cholesterol or triglycerides in humans, Nat. Genet. 40 (2008) 189–197.
[14] T.Y. Lee, H.D. Huang, J.H. Hung, H.Y. Huang, Y.S. Yang, T.H. Wang, DbPTM: an information repository of protein post-translational modification, Nucleic Acids Res. 34 (2006) D622–627.
[15] C. Choudhary, C. Kumar, F. Gnad, M.L. Nielsen, M. Rehman, T.C. Walther, J.V. Olsen, M. Mann, Lysine acetylation targets protein complexes and co-regulates major cellular functions, Science 325 (2009) 834–840.
[16] L.F. Chen, W.C. Greene, Assessing acetylation of NF-kappaB, Methods 36 (2005) 368–375.
[17] S. Izumi, H. Kaneko, T. Yamazaki, T. Hirata, S. Kominami, Membrane topology of guinea pig cytochrome P450 17 alpha revealed by a combination of chemical modifications and mass spectrometry, Biochemistry 42 (2003) 14663–14669.
[18] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, Mass spectrometric sequencing of proteins silver-stained polyacrilamide gels, Anal. Chem. 68 (1996) 850–858.
[19] H.H. Wandall, H. Hassan, E. Mirgorodskaya, A.K. Kristensen, P. Roepstorff, E.P. Bennett, P.A. Nielsen, M.A. Hollingsworth, J. Burchell, J. Taylor-Papadimitriou, H. Clausen, Substrate specificities of three members of the human UDP-N-acetyl-alpha-D-galactosamine:Polypeptide N-acetylgalactosaminyltransferase family, GalNAc-T1, -T2, and -T3, J. Biol. Chem. 272 (1997) 23503–23514.
[20] F.J. Irazoqui, M.M. Vozari-Hampe, R.D. Lardone, M.A. Villarreal, V.G. Sendra, G.G. Montich, V.M. Trindade, H. Clausen, G.A. Nores, Fine carbohydrate recognition of Euphorbia milii lectin, Biochem. Biophys. Res. Commun. 336 (2005) 14–21.
[21] G.I. Csonka, G.A. Schubert, A. Perczel, C.P. Sosa, I.G. Csizmadia, Ab initio conformational space study of model compounds of O-glycosides of serine diamide, Chemistry 8 (2002) 4718–4733.
[22] E. Tian, K.G. Ten Hagen, Expression of the UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase family is spatially and temporally regulated during Drosophila development, Glycobiology 16 (2006) 83–95.
[23] T.A. Fritz, J. Raman, L.A. Tabak, Dynamic association between the catalytic and lectin domains of human UDP-GalNAc:polypeptide alpha-N-acetylgalactosaminyltransferase-2, J. Biol. Chem. 281 (2006) 8613–8619.
[24] A.L. Milac, N.V. Buchete, T.A. Fritz, G. Hummer, L.A. Tabak, Substrate-induced conformational changes and dynamics of UDP-N-acetylgalactosamine:poly peptide N-acetylgalactosaminyltransferase-2, J. Mol. Biol. 373 (2007) 439–451.
[25] X.J. Yang, E. Seto, Lysine acetylation: codified crosstalk with other posttranslational modifications, Mol. Cell. 31 (2008) 449–461.
[26] W.I. Weis, K. Drickamer, Structural basis of lectin–carbohydrate recognition, Annu. Rev. Biochem. 65 (1996) 441–473.