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A novel human UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase, GalNAc-T7, with specificity for partial GalNAc-glycosylated acceptor substrates

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Abstract A novel member of the human UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase gene family, designated GalNAc-T7, was cloned and expressed. GalNAc-T7 exhibited different properties compared to other characterized members of this gene family, in showing apparent exclusive specificity for partially GalNAc-glycosylated acceptor substrates. GalNAc-T7 showed no activity with a large panel of non-glycosylated peptides, but was selectively activated by partial GalNAc glycosylation of peptide substrates derived from the tandem repeats of human MUC2 and rat submaxillary gland mucin. The function of GalNAc-T7 is suggested to be as a follow-up enzyme in the initiation step of *O*-glycosylation.

Key words: *O*-Glycosylation; GalNAc-transferase; Glycosyltransferase; Mucin

1. Introduction

The initiation step of mucin-type *O*-linked protein glycosylation is controlled by a family of UDP-GalNAc: polypeptide *N*-acetylgalactosaminyltransferases (GalNAc-transferases) (EC 2.4.1.41), which transfer *N*-acetylgalactosamine to serine and threonine amino acid residues [1]. Six members of the mammalian GalNAc-transferase family have been reported [2–8] and additional members of the gene family have been identified (E.P. Bennett and H. Clausen, unpublished). Analysis of the in vitro kinetic properties of recombinant forms of these enzymes have revealed several characteristics: (a) the transferases show acceptor substrate specificities that differ but with some overlap, especially for substrates on mucin tandem repeats [4,5,7,9]; (b) the order in which individual sites in multiple site acceptor peptides are glycosylated by different enzymes differs markedly [9,10] and (c) the catalytic action of multiple GalNAc-transferases may be cooperative, as evidenced by the finding that glycosylation of some acceptor sites in the MUC1 tandem repeat by GalNAc-T4 requires prior glycosylation by GalNAc-transferases with different substrate specificities [5]. The latter phenomenon is poorly understood. Preceding GalNAc glycosylation at selected sites in an acceptor peptide may induce conformational changes that are required for some GalNAc-transferases to function. Alternatively, the attached GalNAc residue(s) may be directly involved in recognition of the acceptor substrate site by some GalNAc-transferases.

Here, we report on a novel GalNAc-transferase, designated GalNAc-T7, that exhibits a similar preference for GalNAc-glycosylated substrates as previously found for GalNAc-T4 with peptide substrates derived from the tandem repeat of MUC1 [5]. The kinetic properties of GalNAc-T7 were unique in that its GalNAc glycopeptide substrate specificity was different from that of GalNAc-T4, and in contrast to GalNAc-T4, GalNAc-T7 would only catalyze glycosylation of acceptor substrates with prior glycosylation.

2. Materials and methods

2.1. Identification and cloning of cDNA for GalNAc-T7

tBLASTn analysis of expressed sequence tags (EST) sequence information at The National Center for Biotechnology Information was used to identify a putative novel GalNAc-transferase isoform, designated GalNAc-T7. One EST clone (EST181335) contained part of an open reading frame with sequence similarity to GalNAc-transferases. 5′ sequence was obtained by 5′rapid amplification of cDNA ends (RACE) with a λgt10 cDNA library from the gastric carcinoma cell line MKN45 [3]. Two subsequent 5′RACE reactions with primers EBHC 1101 (5′-TAAGCGGCACAGTGCAAATGG-3′) and EBHC1106 (5′-GAGATCATGTCACTTGCCACC-3′) generated a total of 560 bp sequence 5′ of the EST clone. Further 5′-RACE experiments failed, and residual 5′ coding sequence (110 bp) was obtained by sequencing on genomic clone BACH-6dl-18083 (BAC-18083). This clone was isolated by use of the primer pair EBHC1106/1113 (5′-CGCAGCGGGCGGCCGCCCTGGAGAGAA-AGCCAAGC-3′) using Genome Systems BAC Human library screening services.

2.2. Expression of GalNAc-T7 in insect cells

An expression construct of a secreted form of GalNAc-T7, pAcGP67-GalNAc-T7-sol, was prepared by RT-PCR using primers EBHC1122 (5′- CGCAGCGGGCGGCCGCTGAGCAGGATGAG-GGAAGAC-3′) and EBHC1116 (5′- CGCAGCGGGCGGCCG-CTCTCTAAACACTATGGATGTTATTC-3′) with *Not*I overhangs and the product was cloned into the vector pAcGP67 (Pharmingen) and fully sequenced. RNA from MKN45 was used as template for RT-PCR. Control expression constructs included pAcGP67-GalNAc-T4-sol [5] and other GalNAc-transferases and irrelevant genes. Co-transfection of Sf9 cells with pAcGP67-GalNAc-T7-sol and BaculoGold DNA was performed as previously described [4].

2.3. Northern hybridization

Multiple tissue Northern (MTNIII) blot was obtained from Clontech. The soluble expression construct was used as the GalNAc-T7 probe. The probe was random-primed and labelled using [α-^{32}P]dCTP (Amersham) and Oligo labelling kit (Pharmacia). Blots were probed as described previously [4] and washed 5×at 42°C with 2×SSC, 0.1% SDS, once with 0.5×SSC, 0.1% SDS, and once at 55°C with 0.1×SSC, 0.1% SDS, in a mini-hybridization oven (HYBAID).

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Abbreviations: GalNAc, *N*-acetylgalactosamine; GalNAc-transferase, UDP-GalNAc:polypeptide αGalNAc-transferase (EC 2.4.1.41); EST, expressed sequence tags; RACE, rapid amplification of cDNA ends

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2.4. Polypeptide GalNAc-transferase assays

Standard reaction mixtures (50 μl final volume) contained 25 mM cacodylate (pH 7.4), 10 mM $MnCl_2$, 0.25% Triton X-100, 200 μM UDP-[^{14}C]GalNAc (2000 cpm/nmol)(Amersham), and 200–500 μM acceptor peptides. Products were quantified by scintillation counting after chromatography on Dowex-1, octadecyl silica cartridges (Baker-bond) or HPLC (PC3.2/3 or mRPC C2/C18 SC2.1/10 Pharmacia, Smart System). The panel of acceptor peptides tested included TAP25 (TAPPAHGVTSAPDTRPAPGSTAPPA; human MUC1 tandem repeat [11]), Muc2 (PTTTPISTTTMVTPTPTPTC; human intestinal mucin MUC2 [12]), Muc5AC (Ac-SAPTTSTTSAPT; human respiratory gland mucin MUC5AC [13]), Muc7 (Ac-CPPTPSATTPAPPSSSAPPETTAA, human salivary gland mucin MUC7 [14]), EA2 (DSTTPAPTTK; rat submandibular gland mucin [15]), VTHPGY (Ac-PFVTHPGYD; human fibronectin [16]), zonadhesin (PTERTTTPTKRTTTPTIR; human zonadhesin [17]), OSM fragment (LSESTTQLPGGGPGCA; ovine submaxillary mucin [18]), hCG-β (PRFQDSSSSKAPPPLPSPSRLPG; human chorionic gonadotropin β-subunit [19]), and PSGL-1b (Ac-QATEYEYLDYDFLPETEPPEM; N-terminus of human P-selectin ligand-1 [20]). GalNAc-glycopeptides of the peptides of TAP25, Muc2, Muc5AC, Muc7 and EA2 were produced using cold UDP-GalNAc and purified human recombinant GalNAc-T2 (Table 1). Different GalNAc-glycoforms of EA2 were produced by limiting the ratio of UDP-GalNAc to 2–5 mol per mol of acceptor peptide. Glycopeptides were purified on Supelclean LC-18 columns (1 ml, Supelco) and the number of GalNAc residues incorporated evaluated by matrix-assisted laser desorption/ionization mass spectrometry time-of-flight (MALDI-TOF). Spectra were acquired on a Voyager-DE mass spectrometer (Perseptive Biosystem) equipped with delay extraction. The enzyme sources used were semipurified as previously described by successive sequential ion-exchange chromatographies on Amberlite (IRA95, Sigma) or DEAE Sephacel (Amersham Pharmacia Biotech) and S-Sepharose Fast Flow (Amersham Pharmacia Biotech) [5,9]. Secreted GalNAc-T4 was obtained from a stably transfected CHO line (CHO/GalNAc-T4/21A) [5] grown in roller bottles in HAMS F12 supplemented with 10% fetal bovine serum. Secreted GalNAc-T7 was obtained from infected High Five cells grown in serum-free medium (Invitrogen) in upright roller bottles shaken 140 rpm in waterbaths at 27°C. The activities found with purified GalNAc-T4 expressed in CHO cells deviated slightly from those reported previously for GalNAc-T4 expressed in insect cells [5,8]. These differences may in part be due to higher levels of contaminating endogenous GalNAc-transferase activities in the medium of insect cells.

3. Results

3.1. Cloning of GalNAc-T7

GalNAc-T7 was identified by analysis of the EST database [8], and the human sequence obtained by a combination of PCR-based cDNA cloning and by genomic cloning. The composite sequence contained an open reading frame of 1974 bp, which predicted a type II transmembrane protein with a hydrophobic signal anchor sequence in residues 10–24 (Fig. 1). The 3′UTR is almost 2.2 kb and contains a consensus polyadenylation signal. Multiple human ESTs are derived from the 3′UTR, while only two 5′ESTs contain the coding sequence of GalNAc-T7. The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL data bank with accession number AJ002744. GalNAc-T7 had no consensus *N*-linked glycosylation sites. Multiple sequence alignment analysis of the seven human GalNAc-transferases cloned and expressed so far (Fig. 1), shows that GalNAc-T7 share common sequence motifs with GalNAc-transferases. GalNAc-T7 is distinct in sequence outside these shared motifs from other members of this gene family, indicating that this gene does not fit within any known subfamilies of other identified genes [8]. This is further supported by preliminary analysis of the genomic organization of the gene for GalNAc-T7, designated *GALNT7*, (E.P. Bennett, unpublished), which is different from other characterized GalNAc-transferases [8,21]. *GALNT7* was localized to chromosome 4q31.1 between D4S1566 and D4S1596 at 157.9-169.1 cM by 3′EST mapping data (stSG31267) as reported under Hs.62817 in Unigene. The 3′UTR sequence identified by sequencing on genomic clone BACH-6dl-18083 was identical to the sequence of this Unigene cluster.

Northern analysis revealed high expression of a 4.2 kb transcript in most of the human organs analyzed suggesting a quite ubiquitous expression pattern (Fig. 2). The 3′ and 5′ESTs derived from GalNAc-T7 originated from a large variety of different organs including pancreas, uterus, retina, kidney, small intestine, omentum, stomach and CNS. Murine ESTs derived from the putative GalNAc-T7 orthologue showed a similar broad pattern of expression. There were additional 5′ESTs covering the coding sequence.

3.2. Kinetic properties of GalNAc-T7

A secreted construct of GalNAc-T7 was expressed in insect cells and no activity was demonstrated in initial assays with a large panel of peptide substrates as described in the Section 2 (not shown). As shown in Table 1, GalNAc-T7 had catalytic activity with selected GalNAc-glycosylated peptides derived from mucin tandem repeats. Catalytic activity with $GalNAc_{1-2}$Muc2 and $GalNAc_{1-2}$EA2 is 10-fold greater than the non-glycosylated peptide substrate. GalNAc-T7 showed no activity with the GalNAc glycosylated Muc1 substrate. The activity of GalNAc-T7 with glycopeptides was different from GalNAc-T4.

Previously, we found that GalNAc-T4 had unique activity with two sites in the tandem repeat of MUC1 if the three other sites in the repeat were GalNAc glycosylated using the glycopeptide, $GalNAc_4$TAP24 (TAPPAHGVTSAPDTRPAPGSTAPP, GalNAc attachment sites underlined) [5]. The

Table 1
Substrate specificities[a] of semi-purified recombinant GalNAc-T4 and -T7

	TAP24		MUC2		MUC5AC		MUC7		EA2	
	−[b]	+[c]	−	+	−	+	−	+	−	+
GalNAc-T4	0.4	1.8	3.33	16.1	0.7	5.3	2.9	2.0	7.4	1.0
GalNAc-T7	0	0	0	10.3	0.1	0.3	0	0.4	0.3	4.2

$GalNAc_4$TAP24 is terminal glycosylated with GalNAc-T2 (4 mol GalNAc/mol peptide). Other GalNAc-glycopeptides are glycosylated with GalNAc-T2 using limitation in donor substrate (2 mol UDP-GalNAc/mol peptide) as described under Section 2. Mass spectrometric analysis of the used glycopeptides indicates that 1–2 mol was incorporated (not shown).

[a]milliunits/ml, 1 unit of enzyme is defined as the amount of enzyme that transfer 1 μmol GalNAc in 1 min using the standard reaction mixture.

[b]−, non-glycosylated peptide substrates.

[c] +, GalNAc glycosylated peptide substrates, glycosylated using human GalNAc-T2.

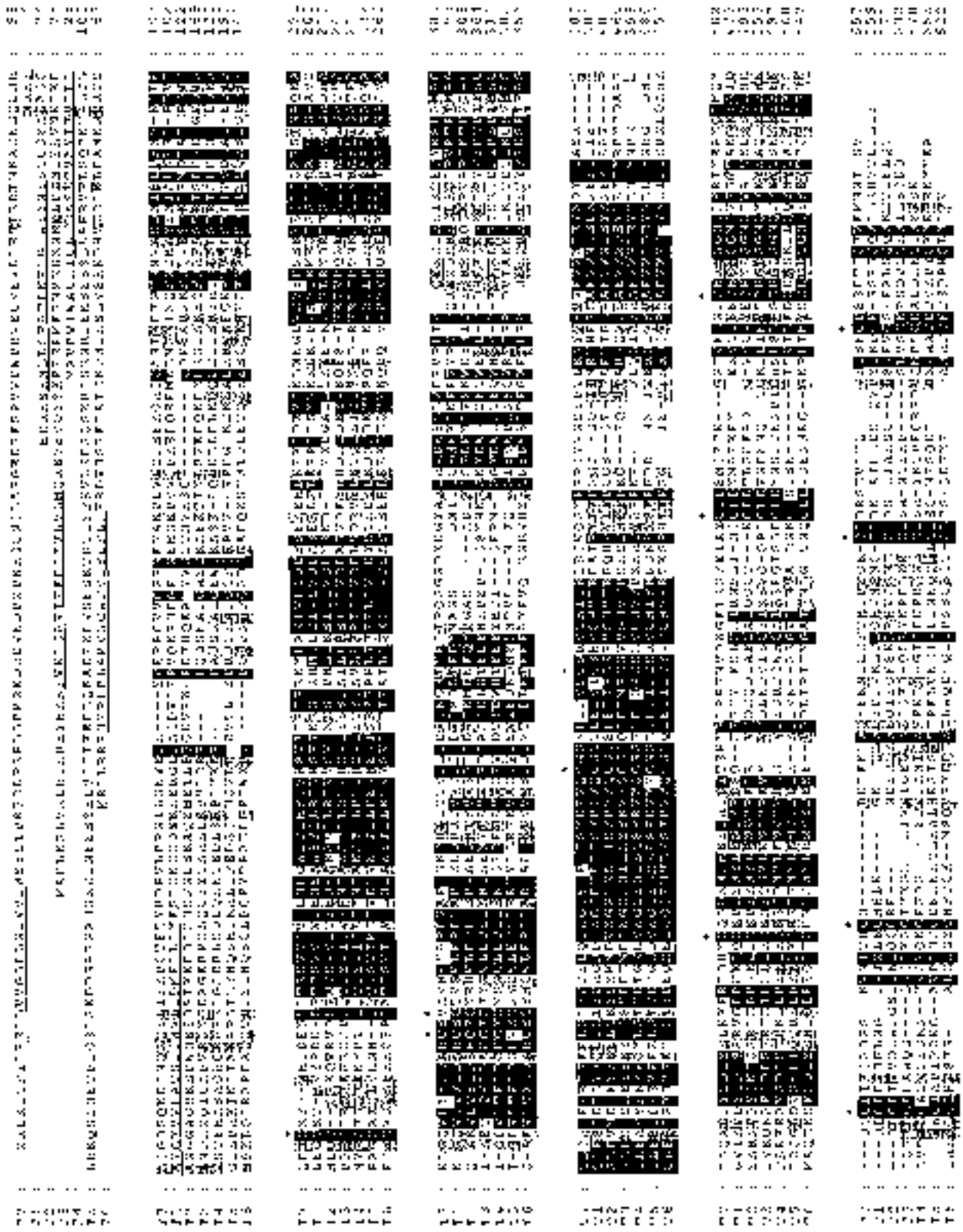

Fig. 1. Structure of human GalNAc-T7. Multiple sequence alignment (ClustalW, DNASTAR) of seven human GalNAc-transferases. The human sequence of GalNAc-T5 shown represents the orthologue of the rat gene reported by Hagen et al. [7] and only 653 amino acids of the catalytic domain are shown.

two sites, S in -VTSA- and T in -PDTR-, are not utilized by other characterized GalNAc-transferases, whereas GalNAc-T1, -T2 and -T3 can transfer to the three sites, T in -VTSA- and ST in -GSTA- [9]. GalNAc-T4 also utilize several non-glycosylated peptide substrates, including those derived from the tandem repeats of MUC2, MUC7 and a rat submandibular mucin (peptide from latter designated EA2) [5]. Here, we found that catalytic activity of GalNAc-T4 with the Muc2 peptide was enhanced five-fold by prior incorporation of 1–2 mol of GalNAc by GalNAc-T2 (Table 1). Similarly, an

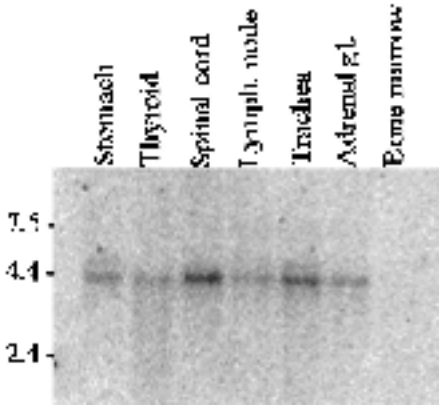

Fig. 2. Northern blot analysis of human tissues. Multiple human Northern blot MTNIII (Clontech) was probed with ^{32}P-labeled GalNAc-T7.

eight-fold enhancement was observed for the Muc5AC peptide with the GalNAc-glycoform. In contrast, GalNAc-glycosylation had no effect on glycosylation of the Muc7 peptide substrate and reduced activity with EA2 substrates seven-fold. The latter finding is interesting as the EA2 substrate is one of the best non-glycosylated peptide substrates identified for both human and mouse GalNAc-T4 [5,6].

GalNAc-T7 showed a different pattern of activity with these glycopeptides; it was inactive with the MUC1 derived glycopeptide ($GalNAc_4$TAP24), and weakly active with Muc5AC and Muc7 glycoforms (Table 1). Similar to GalNAc-T4, there was high activity with GalNAc-Muc2; however, while GalNAc-T7 showed high activity with the EA2 glycopeptide (13-fold enhancement), the activity of GalNAc-T4 with this substrate was dramatically reduced. These results demonstrate that the glycopeptide specificities of GalNAc-T4 and -T7 differ and that the enzymes show distinct acceptor peptide sequence specificities. Detailed analysis of the sites of

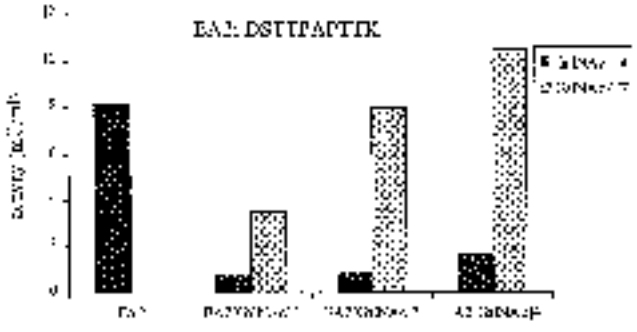

Fig. 3. GalNAc-T4 and -T7 activities with EA2 peptide and GalNAc glycosylated EA2 peptides. Designations: EA2, non-glycosylated peptide; EA2[$GalNAc]_1$, EA2 glycosylated with GalNAc-T2 to yield a glycoform with 1 mol GalNAc incorporated (mass spectral analysis indicated that the majority of the substrate contained 1 mol GalNAc with minor amounts of glycoforms with 2 and 3 mol of GalNAc incorporated); EA2[$GalNAc]_3$, EA2 glycosylated with GalNAc-T2 to yield a glycoform with 3 mol GalNAc incorporated (mass spectral analysis indicated that the majority of the substrate contained 3 mol GalNAc with minor amounts of glycoforms with 2 and 4 mol of GalNAc incorporated); EA2[$GalNAc]_4$, EA2 glycosylated with GalNAc-T2 to yield a glycoform with 5 mol GalNAc incorporated (mass spectral analysis indicated that the substrate contained 3 and 4 mol GalNAc glycoforms).

GalNAc attachment to the GalNAc-peptide substrates (glycosylated by GalNAc-T2) and products produced (by GalNAc-T7) is required for further understanding of the substrate specificity of GalNAc-T7. However, it was clear from the mass spectrometric analysis of the substrates (apart from TAP24 [5]) that these were not glycosylated in full at positions used by GalNAc-T2; hence, clear assignment of site(s) used subsequently by GalNAc-T7 are difficult to define.

The activities of GalNAc-T4 and -T7 with the EA2 substrate were studied in more detail (Fig. 3). GalNAc-EA2 glycopeptides with different densities of glycosylation were prepared by in vitro glycosylation with GalNAc-T2. The activity of GalNAc-T4 with the EA2 peptide is eliminated after incorporation of 1 mol GalNAc into the peptide. In contrast, the activity with GalNAc-T7 is initiated with the 1 mol glycoform of EA2 and further enhanced with higher density glycoforms including the 3–4 mole glycoform. Analysis of the products of terminal reactions with GalNAc-T7 of the three GalNAc-EA2 substrates (shown in Fig. 3) by mass spectrometry, showed that GalNAc-T7 only incorporated 1 mol of GalNAc regardless of the degree of prior glycosylation (not shown). In particular, GalNAc-T7 increased the ratio of the 4 mol GalNAc glycoform from the 3–4 mol substrate, and no evidence of incorporation of a 5th mol of GalNAc was found. This suggests that GalNAc-T7 utilizes one site in the EA2 peptide and that this is a site poorly glycosylated by GalNAc-T2.

4. Discussion

The initiation step of mucin-type *O*-glycosylation is a complex and regulated process carried out by multiple polypeptide GalNAc-transferase isoforms [1]. The kinetic properties of GalNAc-transferase isoforms are different and isoforms clearly have different functions in terms of sites of attachment in proteins [22]. Differential expression of GalNAc-transferase isoforms allows for cell specific glycosylation patterns. Analyses of the functions of recombinant transferases in in vitro assays show that apart from qualitative differences in acceptor substrate specificities, isoforms may also have common acceptor sites. The latter is frequently found with peptide substrates derived from tandem repeat sequences of mucins, which contain high density of potential Ser and Thr acceptor sites often in clusters. Detailed analysis of the order in which different GalNAc-transferase isoforms incorporate GalNAc residues reveal striking differences [9,10,23] and suggests that the enzymes work in a coordinate fashion transferring to different sites. The finding that the action of one GalNAc-transferase, GalNAc-T4, with unique acceptor sites in the MUC1 tandem repeat, is dependent on prior actions of other GalNAc-transferases [5], further support that GalNAc-transferases function in a coordinate process. The present data extend these observations and presents a novel human GalNAc-transferase isoform, GalNAc-T7, that apparently show exclusive acceptor substrate specificity for glycopeptides. In contrast to GalNAc-T4, the only substrates identified for GalNAc-T7 were partially GalNAc glycosylated. Thus, the kinetic properties of GalNAc-transferase isoforms with respect to absolute and preferential specificities for sequence contexts of acceptor sites as well as specificities for acceptor sequences flanked by GalNAc glycosylated sites, allow for a high degree of regulation in the process of initiation of *O*-glycosylation. Differential topography of GalNAc-transfer-

ase isoforms in the secretory pathway may also influence the functions of GalNAc-transferase isoforms; however, analysis of three isoforms by immunocytolocalization of tagged expression constructs suggests that these are found throughout the Golgi stacks, albeit with some differences in the profile [24].

It is clear that the primary sequence context of acceptor substrates for *O*-glycosylation is the major determining factor for the substrate specificities of GalNAc-transferases [22]. GalNAc glycosylation of selected sites in a multiple acceptor site peptide sequence may induce conformational changes favoring the substrate specificities of GalNAc-transferase isoforms. Several studies have demonstrated relative effects, negative as well as positive, of adjacent and distant *O*-glycans on the kinetics of subsequent in vitro *O*-glycosylation [23,25]. In the case of GalNAc-T7 (and GalNAc-T4 with the MUC1 derived substrates), the effect of preceding *O*-glycosylation is an ultimate prerequisite for function. One hypothesis predicts that these enzymes recognize a GalNAc residue in the context of its attachment to the polypeptide backbone and that this recognition event activates enzymatic function in an allosteric manner. In this respect, Hazes [26] and Imberty et al. [27] have identified a ricin lectin-like motif in the C-terminal region of GalNAc-transferases, but functionality for this domain has not been demonstrated. Hagen et al. [28] have recently shown that the catalytic activity of GalNAc-T1 with an unsubstituted peptide substrate is not significantly affected by detrimental mutations in the lectin domain. It remains open whether this domain plays a role in the observed glycopeptide specificities of GalNAc-transferases like GalNAc-T4 and -T7. Additional GalNAc-transferases with glycopeptide specificities functioning as follow-up enzymes may exist. The putative rat GalNAc-T7 orthologue recently cloned was shown to exhibit glycopeptide substrate specificity using the MUC5AC derived peptide substrate glycosylated with GalNAc-T1 [29].

Interestingly, a comparative analysis of GalNAc-T4 and -T7 activities with partially GalNAc-glycosylated peptides, derived from mucin tandem repeats, showed that these have different peptide substrate specificities. GalNAc-T7 did not show activity with the MUC1 derived glycopeptide, whereas both enzymes showed high activity with the glycopeptide derived from MUC2. Several non-glycosylated peptide substrates have been identified for GalNAc-T4 including a sequence derived from rat submandibular mucin (EA2) [5,6]. The number and position of acceptor site(s) has not been characterized but the activities of GalNAc-T4 and -T7 with glycoforms of EA2 (Fig. 3) clearly demonstrate differences in activities. More detailed structural information is required on the substrates used and products formed in those reactions.

In conclusion, the results indicate that several GalNAc-transferases function in an independent fashion after the action of other GalNAc-transferases. These 'follow-up' enzymes have unique acceptor substrate specificities, and they may play a significant role in the glycosylation of tandem repeats of mucins, which are characterized by high density clustering of serine and threonine acceptor sites.

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