FEBS Letters 450 (1999) 52–56 FEBS 21927

The acceptor substrate specificity of human β4-galactosyltransferase V indicates its potential function in *O*-glycosylation

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Received 22 March 1999

Abstract In order to assess the function of the different human UDP-Gal:GlcNAc β4-galactosyltransferases, the cDNAs of two of them, β4-GalT I and β4-GalT V, were expressed in the baculovirus/insect cell expression system. The soluble recombinant enzymes produced were purified from the medium and used to determine their in vitro substrate specificities. The specific activity of the recombinant β4-GalT V was more than 15 times lower than that of β4-GalT I, using GlcNAcβ-S-*p*NP as an acceptor. Whereas β4-GalT I efficiently acts on all substrates having a terminal β-linked GlcNAc, β4-GalT V appeared to be far more restricted in acceptor usage. β4-GalT V acts with high preference on acceptors that contain the GlcNAcβ1→6GalNAc structural element, as found in *O*-linked core 2-, 4- and 6-based glycans, but not on substrates related to *N*-linked or blood group I-active oligosaccharides. These results suggest that β4-GalT V may function in the synthesis of lacNAc units on *O*-linked chains, particularly in tissues which do not express β4-GalT I, such as brain.

Key words: Baculovirus; Insect cell; Glycosyltransferase; β4-Galactosyltransferase gene family

1. Introduction

UDP-Gal:GlcNAcβ-R β4-galactosyltransferase (β4-GalT) is an enzyme that functions in the biosynthesis of complex-type glycans by catalyzing the transfer of a galactose from UDP-Gal to an acceptor substrate carrying a terminal β-linked GlcNAc, resulting in the formation of a Galβ1→4GlcNAc (lacNAc) unit. In the lactating mammary gland, β4-GalT interacts with α-lactalbumin to form the lactose-synthase complex, which catalyzes the transfer of a galactose to free glucose, yielding lactose [1–3]. Targeted inactivation of the mouse β4-GalT I gene resulted in growth retardation and early death of the mice, indicating that this enzyme has an important function in embryonic development and further growth of the animals [4,5]. The β4-GalT I-deficient mice were unable to produce lactose; they showed, however, a low residual GalT activity.

Recently, many sequences related to the β4-GalT gene appeared in (EST) databases, indicating that the β4-GalT gene is part of a gene family, also including the related *Lymnaea stagnalis* β4-GlcNAcT gene that was cloned previously [6–9]. During the past years two chicken and three human homologs were cloned, and all appeared to be true β4-GalTs [8,10–12], which poses questions about their individual functions. In addition, a glucosylceramide-specific rat β4-GalT homolog has been cloned [13], revealing that the β4-GalT gene family contains enzymes with a wide variety of functions, including an enzyme with a different UDP-sugar specificity [6] as well as an enzyme using an acceptor deviant from those having a terminal β-linked GlcNAc [13].

In a previous study [11] we described the cloning of a novel human β4-GalT (GenBank accession number AB004550) that we named β4-GalT II, but later was referred to as β4-GalT IV [14], and β4-GalT V [12,15]. We showed in Northern blots that β4-GalT V, as we name it now, is widely expressed in human tissues [11]. Also β4-GalT I, β4-GalT II and β4-GalT III are expressed in a variety of tissues [8,9]. This indicates that in many tissues, and possibly in many cells, multiple β4-GalTs are expressed resulting in a mixture of enzymes, which makes it difficult to assign observed β4-GalT activity to individual enzymes. By a detailed study of the in vitro acceptor substrate specificity of the different β4-GalTs we aimed to get more insight into the in vivo function of the individual enzymes. In this study we investigate the acceptor substrate specificity and kinetic parameters of β4-GalT V and compare them with those of the well known β4-GalT I.

2. Materials and methods

2.1. Materials

The following persons kindly supplied us with compounds (numbers refer to the structures given in Table 1): **4** and **12**, Dr. O. Hindsgaul (University of Alberta, Edmonton, Alta, Canada); **13**, Dr. M. Haraldsson (University of Stockholm, Sweden); **20**, Dr. A. Veyrières (Université Paris-Sud, France); **21**, Dr. M. Messer (University of Sidney, Australia). Compounds **1** and **5** were purchased from Sigma Chemical Co. (St. Louis, MO, USA), **2**, **3**, **6-11** were obtained from Toronto Research Chemicals (Toronto, Ont., Canada) and **18** from Glycolipid Biochemicals (Birmingham, AL, USA). Oligosaccharides **14**, **16** and **17** were derived from α_1-acid glycoprotein by desialylation (acid treatment), digestion with PNGase F, fractionation of the resulting oligosaccharides and digestion with jack bean β-galactosidase. Glycopeptide as/ag-GP-F2 (**15**) was prepared from asialo-fibrinogen by pronase digestion as described previously [16] followed by enzymatic degalactosylation. Glycolipid **19** was derived from paragloboside, isolated from bovine erythrocyte ghosts [17], by enzymatic degalactosylation. UDP-[^{3}H]Gal (36 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). The sugar nucleotide donor was diluted with unlabeled UDP-Gal (Sigma) to the desired specific radioactivity. All other chemicals were obtained from commercial sources and were of the best quality available.

2.2. Plasmids and recombinant DNA techniques

Plasmids were propagated in the *Escherichia coli* K-12 strain XL-Blue (Stratagene). Synthetic oligonucleotides were obtained from Isogen Bioscience, Maarssen, The Netherlands. pVTBac-His was ob-

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PII: S0014-5793(99)00462-7

tained from Dr. D.H. Joziasse, and pVTBac-HisT, which shows a shift in reading frame in the multiple cloning site, from Dr. T. de Vries [18].

Isolation of plasmid DNA was carried out by a modification of the minilysate method, as described by [19], or with the QIAprep Spin Miniprep kit (Qiagen). Dideoxynucleotide chain terminating sequencing reactions [20], were performed on double stranded plasmid DNA, using the T7 sequencing kit (Pharmacia) and [α-^{35}S]dATP (Amersham). PCR was performed using Ampli-taq DNA polymerase (Perkin-Elmer) by 25 cycles (1 min 95°C, 1 min 63°C, 1 min 72°C). Before cloning, amplified fragments were purified according to the QIAquick PCR purification protocol (Qiagen).

2.3. Construction of pVTBacHis-β4GalT expression plasmids

Human β4-GalT I cDNA [21] was partially digested with *Pst*I and *Eco*RI and a 1.2 kb *Pst*I/*Eco*RI fragment, starting at base 176 of the coding region, was ligated in *Pst*I/*Eco*RI-digested pVTBac-His. A 5′ truncated β4-GalT V cDNA fragment was obtained by PCR of the complete cDNA [11] using the sense primer AAAGAGCTCCCAAGGCATTCTGATCCGG and the antisense primer ATTGAATTCAGTACTCGTTCACCTG. The PCR fragment was purified, digested with *Sac*I and *Eco*RI, and ligated into *Sac*I/*Eco*RI-digested pVTBac-HisT. The resulting expression plasmids were sequenced.

2.4. Maintenance and infection of insect cells

Trichoplusia ni ('BTI-TN-5B1-4') and *Spodoptera frugiperda* pupal ovary tissue (Sf9) insect cells were obtained from Invitrogen (San Diego, CA, USA) and grown in Graces Insect Medium (Invitrogen) supplemented with 10% fetal calf serum (Gibco-BRL), or High Five Serum-Free medium (Invitrogen), both containing 50 μg/ml gentamicin. Insect cells were grown as monolayers at 27°C. The Baculo-gold system (PharMingen) was used for construction of recombinant baculovirus and Sf9 cells were used to produce recombinant virus. To produce the recombinant enzymes, *T. ni* insect cells were seeded in High-Five serum-Free medium in T75 flasks. Baculovirus was added (multiplicity of infection of 6) when the cells showed 90–100% confluence, and after 3–5 days the medium was collected. The medium was centrifuged for 5 min at 1500 rpm in an Eppendorf centrifuge to remove detached cells.

2.5. Purification of the recombinant proteins and immunoblotting

Medium of recombinant baculovirus-infected *T. ni* insect cells was subjected to UDP-hexanolamine-Sepharose chromatography, essentially as described previously [22]. The protein concentration of the active eluted fractions was determined according to Peterson [23]. Proteins were separated by SDS-PAGE on a 10% polyacrylamide gel, using the Mini-Protean II system (Bio-Rad). For Western blotting [24] a monoclonal anti-polyhistidine antibody (Sigma Immunochemicals) was used as first antibody and goat anti-mouse peroxidase conjugate (TAGO Immunodiagnostic Reagents) as the second antibody.

2.6. Galactosyltransferase assays and kinetic parameters

GalT assays were performed for 60–120 min at 37°C essentially as described in [25] in a 50 μl reaction mixture containing 5 μmol sodium cacodylate buffer pH 7.0, 25 nmol UDP-[^{3}H]Gal (1 Ci/mol), 1 μmol $MnCl_2$, 0.2 μmol ATP, 0.25 μl Triton X-100, 15 μl enzyme preparation and 50 nmol *p*-nitrophenyl-*N*-acetyl-1-thio-β-D-glucosaminide (GlcNAcβ-S-pNP) or acceptor substrate as indicated. Control assays lacking the acceptor substrate were carried out to correct for incorporation into endogenous acceptors. When mono- or oligosaccharides were used as acceptor, the labeled product was separated from unincorporated label by chromatography on a 1 ml column of Dowex 1-X8 (Cl^- form) [26]. When pNP substrates were used as the acceptor, the product was isolated using Sep-Pak C-18 cartridges (Waters) [27]. Kinetic parameters (K_m and V) were estimated from Eadie-Hofstee plots (acceptor substrate concentrations varied from 0.03 to 5 mM (100 mM for GlcNAc)).

3. Results

3.1. Expression of soluble β4-GalT I and β4-GalT V cDNA in insect cells

To obtain expression of human β4-GalT I and β4-GalT V in insect cells, 5′ truncated cDNA fragments encoding the putative stem region and catalytic domain of these β4-GalTs were inserted into baculovirus expression vectors downstream of the polyhedrin promoter, the cleavable mellitin signal sequence and a poly-histidine (His_6) tag. Expression of the recombinant baculoviruses in *T. ni* insect cells resulted in the secretion of soluble enzymes, carrying an amino-terminal poly-His_6 tag. No β4-GalT activity could be detected in the serum-free growth medium of insect cells infected with an unrelated recombinant baculovirus. The recombinant human β4-GalTs were purified from the medium. Western blotting with a His_6-specific monoclonal antibody revealed that similar amounts of protein were produced and purified for both recombinant enzymes (Fig. 1). The specific activity of the purified β4-GalT I (220 mU/mg protein), however, appeared to be more than 15 times higher than that of purified β4-GalT V (13.3 mU/mg protein).

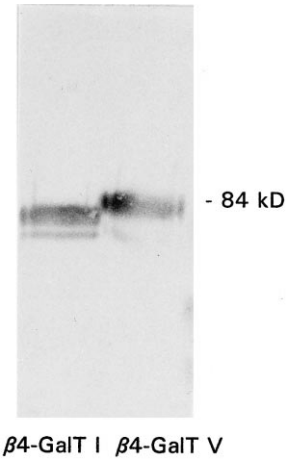

Fig. 1. Western blot analysis of β4-GalT I and β4-GalT V produced in High Five cells. The proteins of UDP-hexanolamine-Sepharose-purified medium of High-Five cells producing β4-GalT I or β4-GalT V (both 5.5 μg) were separated on SDS-PAGE. Proteins were transferred to nitrocellulose by Western blotting, and stained by an anti-polyHis antibody.

3.2. Acceptor specificity of the recombinant enzymes

The acceptor specificity of human β4-GalT V was compared with that of the well known human β4-GalT I (Table 1). Whereas all substrates terminating with a terminal β-linked GlcNAc were efficient acceptors for β4-GalT I, β4-GalT V appeared to be very restricted in its acceptor specificity. GlcNAcβ-S-pNP, and oligosaccharides representing *O*-linked structures (acceptor substrates **9–11**, Table 1) were good acceptor substrates for this enzyme. A hydrophobic aglycone generally improved the acceptor properties of the oligosaccharides. Optimal activity was found with acceptor substrates carrying a terminal GlcNAc β1→6 linked to an α-GalNAc residue (substrates **10** and **11**). Substrates representing *N*-glycans (**12–17**) were all poor acceptors, regardless of the presence of a hydrophobic aglycone (substrate **12**) or the GlcNAc linkage type. Also two acceptors related to blood group I-active structures (**20** and **21**) showed relatively low acceptor properties. Neither β4-GalT V nor β4-GalT I was active with the substrate glucosylceramide. In contrast to our previous results [11], the acceptor lacto-*N*-triaosylceramide (**19**) showed a low but distinct activity. Essentially no β4-GalT V activity

Table 1
Acceptor specificity of recombinant β4-GalT I and β4-GalT V

Acceptor (1 mM theoretical acceptor sites)	Relative enzyme activity of β4-GalT I (%)	Relative enzyme activity of β4-GalT V (%)
Simple oligosaccharide acceptors		
1. GlcNAcβ-S-pNP	100	100
2. GlcNAcβ-O-pNP	87	18
3. GlcNAcα-O-pNP	17	< 1
4. GlcNAcβ-O-$(CH_2)_8$-$COOCH_3$	108	99
5. GlcNAc	43	3.2
6. SO_4^--6GlcNAcβ-O-pNP	11	< 1
7. Xylβ-O-pNP	< 1	1.4
8. GlcNAcβ1→4GlcNAc	85	3.7
Acceptors related to O-linked glycans		
9. GlcNAcβ1→3GalNAcα-O-pNP (core 3)	85	45
10. GlcNAcβ1→6GalNAcα-O-pNP (core 6)	117	134
11. GlcNAcβ1→6 Galβ1→3GalNAcα-O-pNP (core 2)	84	131
Acceptors related to N-linked glycans		
12. GlcNAcβ1→2Manα1→6 3Manβ-O-$(CH_2)_8$-$COOCH_3$ GlcNAcβ1→2Manα1→3	118	13
13. GlcNAcβ1→2Manα1→6 GlcNAcβ1→4Man GlcNAcβ1→2Manα1→3	52	2.0
14. GlcNAcβ1→2Manα1→6 3Manβ1→4GlcNAcβ1→4GlcNAc GlcNAcβ1→2Manα1→3	155	7.8
15. GlcNAcβ1→2Manα1→6 3Manβ1→4GlcNAcβ1→4GlcNAcβ1-peptide[a] GlcNAcβ1→2Manα1→3 (As/ag-GP-F2)	157	6.0
16. GlcNAcβ1→2Manα1→6 3Manβ1→4GlcNAcβ1→4GlcNAc GlcNAcβ1→2Manα1→3 GlcNAcβ1→4	164	12.5
17. GlcNAcβ1→6 GlcNAcβ1→2Manα1→6 3Manβ1→4GlcNAcβ1→4GlcNAc GlcNAcβ1→2Manα1→3 GlcNAcβ1→4	160	13.6
Glycolipid acceptors		
18. Glcβ-ceramide	< 1	< 1
19. GlcNAcβ1→3Galβ1→4Glcβ-ceramide	28	16.6
Acceptors related to blood group I-active structures		
20. GlcNAcβ1→6 3Gal GlcNAcβ1→3	176	10.9
21. GlcNAcβ1→6 3Galβ1→4Glc Galβ1→3	178	16.2

100% corresponds to an activity of 220 and 13.3 U/mg protein for the enzymes, respectively
[a]The peptide portion of the glycopeptide consists for >90% of Gly-Glu-Asn and Glu-Asn in a ratio of 3:2.

was found with monosaccharides other than GlcNAc, or an acceptor with a GlcNAc residue in α-anomeric configuration.

3.3. Interaction of β4-GalT V with α-lactalbumin

In the mammary gland of mammals, β4-GalT I interacts with α-lactalbumin (α-LA) to produce lactose. In Table 2 the effect of α-LA on the interaction of the soluble recombinant β4-GalT V and β4-GalT I is shown using different acceptor monosaccharides. Like β4-GalT I, human β4-GalT V is inhibited by α-LA in its action on GlcNAc. However, while the first enzyme is induced by α-LA to act on Glc to yield lactose, β4-GalT V is indifferent in this regard and acts with low activity on Glc regardless of whether or not α-LA is present. In previous studies we had observed that α-LA is able, although with low efficiency, to induce β4-GalT I to act on xylose, yielding the disaccharide Galβ1→4Xyl (D.H. van den Eijnden and R. Staffhorst, unpublished results). By contrast, β4-GalT V acts with very low efficiency on Xyl in the absence of α-LA, and not at all in the presence of this modifier protein.

3.4. Kinetic properties of the recombinant β4-GalTs

To substantiate the differences in the observed acceptor substrate specificity of human β4-GalT I and β4-GalT V, the K_m and V values with four different substrates were estimated for both enzymes (Table 3). The affinity of β4-GalT I and β4-GalT V for GlcNAcβ-S-pNP and the *O*-linked core 2 structure (**11**) appeared to be of the same order, whereas the V values for β4-GalT V were much lower than those for β4-GalT I. This resulted in kinetic efficiencies for β4-GalT V that are 30–40 times lower than those for β4-GalT I. The kinetic efficiency with which β4-GalT V acts on the *N*-linked glycopeptide (**15**), however, appears to be much lower than that found with GlcNAcβ-S-pNP and the core 2 structure, due to both a lower affinity and lower V. The low kinetic efficiency of β4-GalT V found with GlcNAc is mainly due to the low affinity of the enzyme for this substrate.

4. Discussion

Acceptor specificity studies with the soluble forms of β4-GalT V and β4-GalT I have shown that β4-GalT V displays a much more restricted acceptor specificity than β4-GalT I. In accord with previous studies, β4-GalT I acts on virtually any substrate that carries a β-GlcNAc residue at its non-reducing terminus at a relatively high intrinsic rate. By contrast, β4-GalT V clearly prefers substrates that contain the GlcNAcβ1→6GalNAcα structural element suggesting that it might function in the formation of lacNAc units at the β6-

Table 2
Activity of β4-GalT I and β4-GalT V on GlcNAc, Glc and Xyl in the presence (+) or absence (−) of 10 mg α-lactalbumin (α-LA) per ml

Acceptor (5 mM)	Relative activity (%) of β4-GalT I		Relative activity (%) of β4-GalT V	
	−α-LA	+αLA	−α-LA	+α-LA
GlcNAc	100	5	100	15
Glc	0.1	72	9	7
Xyl	<0.1	6	10	<1

100% corresponds to an activity of 313 and 2.8 mU/mg protein for the enzymes, respectively.

Table 3
Apparent kinetic parameters of recombinant human β4-GalT I and β4-GalT V for several acceptor substrates

Acceptor	β4-GalT I			β4-GalT V		
	K_m (mM)	V (mU.mg^{-1} protein)	kinetic efficiency ($V.K_m^{-1}$)	K_m (mM)	V (mU.mg^{-1} protein)	kinetic efficiency ($V.K_m^{-1}$)
5. GlcNAc	5.2	886	170	71	25.7	0.36
1. GlcNAcβ-S-pNP	0.58	526	907	0.96	21.8	22.7
11. GlcNAcβ1 ↘6 Galβ1→3GalNAcα-O-pNP	0.50	428	856	1.3	33.2	25.5
15. As/ag-GP-F2	3.4	1544	454	17.5	6.6	0.38

In the assays the concentrations of the acceptor substrates were varied over a range of 0.03–5.0 mM (1–100 mM for GlcNAc with β4-GalT V) using otherwise standard incubation conditions.

linked GlcNAc of core 2-, 4-, or 6-based *O*-linked glycans. A role of β4-GalT V in the formation of lacNAc units on *N*-glycans is less likely as this enzyme acts with low efficiency on all substrates that form part of *N*-linked glycans. Also acceptors that are related to blood group I-active substances (containing the GlcNAcβ1→6Gal element) are relatively poor substrates. Recently a GlcNAc-6-*O*-sulfate-specific β4-GalT activity was demonstrated in human colorectal mucosa [28]. This activity, however, cannot be assigned to β4-GalT V as this enzyme is not active with this substrate. It thus appears that β4-GalT V is also not involved in the synthesis of 6-sulfated structures such as 6-sulfo-sialyl Lewis^x which has been identified as a ligand for L-selectin [29].

The primary structure of β4-GalT V shows more resemblance to that of the glucosylceramide-specific β4-GalT [13] than to any other β4-GalT cloned so far. Yet the soluble β4-GalT V tested in this study showed no activity with glucosylceramide. By contrast the full-length enzyme produced in insect Sf9 cells [14] showed a low but distinct activity with this glycolipid substrate (T. Sato and K. Furukawa, unpublished). Similarly, truncated, soluble forms of α3/4-fucosyltransferases show a diminished capability to act on glycolipids compared to the full-length enzymes [30]. These results suggest that the membrane spanning domain of glycosyltransferases somehow is important for activity with glycolipids, perhaps by enabling a close and properly oriented association between enzyme and substrate in the membrane. On the other hand the soluble form of β4-GalT V shows some activity with lacto-*N*-triaosylceramide (Lc_3Cer), while the chimeric β4-GalT V-protein A form of the enzyme was found to be inactive with this substrate [11]. These data indicate that glycosyltransferase activities with glycolipid substrates may be highly sensitive to the form (soluble, fusion protein, full-length) of the enzyme. In addition to β4-GalT V and β4-GalT I (this study), the soluble forms of β4-GalT III [8] and β4-GalT IV [12] also are capable of acting on Lc_3Cer, leaving the question unanswered whether the synthesis of paragloboside (nLc_4Cer) in vivo is catalyzed by one or more β4-GalTs.

In contrast to β4-GalT I, β4-GalT V is not stimulated by α-LA to act more efficiently on Glc [11,14] but shows a low intrinsic activity with this sugar. Its activity with GlcNAc, however, is strongly inhibited at an α-LA concentration of 10 mg/ml. Furthermore, β4-GalT I and β4-GalT V differ also in their relative activities with Xyl (the ancestor pentose of Glc) in the absence and presence of α-LA, but the activities of the other β4-GalTs with this acceptor have not been reported. It may be expected that the β4-GalT functioning in the synthesis of the GlcAβ1→3Galβ1→3Galβ1→4Xylβ-*O*-Ser core of proteoglycans (such as chondroitin sulfate and heparin sulfate) [31] is also a member of the β4-galactosyltransferase family and that this enzyme displays a high specificity for xylosides. The properties of β4-GalT V do not meet this criterion and therefore this enzyme appears not to have this function.

Although the *O*-linked core 2 and the core 6 structures are the most efficient substrates for β4-GalT V, the kinetic efficiency with which this enzyme acts on the core 2 structure is about 30-fold lower than that shown by β4-GalT I. This implies that it is not likely that β4-GalT V is the only β4-GalT involved in the galactosylation of the core 2 oligosaccharide, in particular because β4-GalT I is prominently present in most tissues [9,11]. However, in brain β4-GalT I transcripts are hardly detectable while β4-GalT V, β4-GalT III and β4-GalT VI are well expressed in this tissue [8,9,11,12]. This brain-specific expression pattern of β4-GalTs might explain the under-galactosylation of the *N*-linked glycans of certain brain glycoproteins [32,33] as it is consistent with the poor activity β4-GalT V shows with acceptors that are related to

these glycans. In the absence of β4-GalT I, β4-GalT V might indeed have a specific function in the galactosylation of *O*-linked structures.

While our work was in progress a study appeared favoring a specific role of β4-GalT IV rather than of β4-GalT I or β4-GalT V in the synthesis of polylactosamine chains on *O*-linked core 2 structures [15]. Although we did not test the acceptor specificity of β4-GalT IV our results on β4-GalT I and β4-GalT V clearly contradict the conclusion drawn by these authors. Also we did not encounter the severe substrate inhibition of β4-GalT I by the core 2 substrate which underlines the function of the latter enzyme in the galactosylation of both *N*- and *O*-linked glycans.

In this study, we compared the kinetic properties and the in vitro acceptor specificity of two human β4-GalTs. These data are important to better understand their role in the synthesis of lacNAc-based glycans. The in vivo function of these enzymes, however, will also depend on potential competition with other β4-galactosyltransferases and β4-*N*-acetylgalactosaminyl transferases [34], subcellular localization, and protein-directed effects.

Acknowledgements: We thank Drs. O. Hindsgaul, A. Veyrières, M. Haraldsson for their kind gifts of oligosaccharides, and Drs. M. Fukuda, D.H. Joziasse and T. de Vries for providing plasmids.

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