Identification and characterization of a Drosophila melanogaster ortholog of human β1,4-galactosyltransferase VII

Nadia Vadaie, Rebecca S. Hulinsky1, and Donald L. Jarvis2

Department of Molecular Biology, University of Wyoming, P.O. Box 3944, Laramie, WY 82071-3944, USA

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Drosophila melanogaster is widely considered to be an attractive model organism for studying the functions of the carbohydrate moieties of glycoconjugates produced by higher eukaryotes. However, the pathways of glycoconjugate biosynthesis are not as well defined in insects as they are in higher eukaryotes. One way to address this problem is to identify genes in the Drosophila genome that might encode relevant functions, express them, and determine the functions of the gene products using direct biochemical assays. In this study, we used this approach to identify a putative Drosophila β1,4-galactosyltransferase gene and determine its enzymatic activity of its product. Biochemical assays demonstrated that this gene product could transfer galactose from UDP-galactose to a β-xylosyl acceptor, but not to other acceptors in vitro. The apparent Km values for the donor and acceptor substrates indicated that this gene product is a functional galactosyltransferase. Additional assays showed that the enzyme is activated by manganese, has a slightly acidic pH optimum, and is localized in the insect cell Golgi apparatus. These results show that Drosophila encodes an ortholog of human β1,4-galactosyltransferase-VII, also known as galactosyltransferase I, which participates in proteoglycan biosynthesis by transferring the first galactose to xylose in the linkage tetrasaccharide of glycosaminoglycan side chains.

Key words: Drosophila/galactosyltransferase/insect/proteoglycan

Introduction

The availability of many different mutants, powerful genetic methods, a complete genome sequence, and an expressed sequence tag (EST) database has stimulated interest in the use of Drosophila melanogaster to study the functions of various glycoconjugates produced by higher eukaryotes (Seppo and Tiemeyer, 2000). However, the pathways mediating glycoconjugate biosynthesis in insects are not as well defined as those in higher eukaryotes. Furthermore, it has been effectively argued that the value of Drosophila as a model organism for these studies will be limited by differences in the glycomes of insects and mammals (Altmann et al., 2001). Therefore, it is important to clearly establish the nature of the machinery available for glycoconjugate biosynthesis in Drosophila and other insect systems. One way to do this is to identify genes in the Drosophila genome and/or EST databases that might be involved in glycoconjugate biosynthesis, express them, and determine the functions of the gene products using direct biochemical assays. In this study, we used this approach to identify a putative Drosophila β1,4-galactosyltransferase (β4GalT) gene and to show that it encodes an enzyme that probably functions in proteoglycan biosynthesis.

It is established that humans encode at least seven β4GalTs (Almeida et al., 1997, 1999; Lo et al., 1998; Amado et al., 1999; Okajima et al., 1999). The members of this human gene family are designated β4GalT-I to -VII, and each encodes an enzyme that transfers galactose from UDP-galactose in β1,4 linkage to an acceptor substrate. The in vitro acceptor specificities of the human β4GalT’s have been defined, and the results indicate that these enzymes participate in glycoconjugate biosynthesis in vivo (Almeida et al., 1997, 1999; Nomura et al., 1998; Sato et al., 1998; Schwientek et al., 1998; Okajima et al., 1999; Van Die et al., 1999). However, the acceptor substrates recognized by different family members are not identical, because these enzymes can use N-acetylgalcosamine, glucose, or xylose as acceptors. More specifically, the in vitro data indicate that β4GalT-I–VII are probably involved in glycoprotein and/or glycolipid biosynthesis, whereas β4GalT-VII, also known as galactosyltransferase I, is involved in proteoglycan biosynthesis (Almeida et al., 1999; Okajima et al., 1999). The in vitro activity of β4GalT-VII appears to reflect its true in vivo function because mammalian cell lines (Esco et al., 1987), C. elegans (Herman and Horvitz, 1999; Bulik et al., 2000), and humans (Quentin et al., 1999; Almeida et al., 1999) with mutant β4GalT-VII genes also have defects in glycosaminoglycan biosynthesis.

It was recently reported that Drosophila melanogaster encodes three putative β4GalT genes (Altmann et al., 2001). However, no functional data are currently available to support the presumptive identification of these genes. In November 1999, we independently identified three putative β4GalT genes in the Drosophila genome. This report focuses on one of these genes, CG11780 (Genebank Accession No. AE003750), because bioinformatic analyses predicted that this gene is an insect ortholog of human β4GalT-VII and biochemical assays provided direct experimental evidence that the gene product is indeed a functional β4GalT-VII. These findings, together with the
recent identification of a *Drosophila* gene encoding a functional UDP-galactose transporter (Aumiller and Jarvis, 2002; Segawa et al., 2002), provide new information on the machinery available for glycosaminoglycan and proteoglycan biosynthesis in *D. melanogaster* and perhaps in other insects as well.

## Results

**Computer-assisted analysis of a putative *Drosophila* β4GalT-VII gene**

The initial intent of our bioinformatic analysis was to identify an insect β4GalT gene that might be involved in N-glycoprotein biosynthesis. For this reason, we used the human β4GalT-II sequence (Genbank Accession No. AF038660) as a representative β4GalT gene involved in N-glycoprotein biosynthesis in a BLAST-P analysis (Altschul et al., 1997) of the *Drosophila* databases. This search identified three different *D. melanogaster* genes, each of which had already been tentatively identified as β4GalT genes, based on sequence similarity alone (Flybase, 1999). We subsequently expressed each gene and performed preliminary β4GalT assays but obtained positive results only with CG11780 (data not shown). Therefore, we focused the remainder of this study on this single *Drosophila* gene and its product.

When the amino acid sequence encoded by CG11780 was used as the query for a second BLAST-P search, it was found to be most closely related to the seventh member of the human β4GalT family and to the β4GalT-VII encoded by the *C. elegans* sqv-3 gene (Table I). The putative *Drosophila* β4GalT was nearly 50% identical to these proteins, as compared to about 30% identical to the other human β4GalTs. In addition, the e values and bit scores associated with the human and *C. elegans* β4GalT-VII proteins were significantly better than those obtained with any of the other human β4GalTs. The phylogenetic tree analysis (Felsenstein, 1989) shown in Figure 1 also reveals that the putative *Drosophila* β4GalT protein is more closely related to the known β4GalT-VII proteins than to any of the other human β4GalT family members.

A CLUSTAL W multiple sequence alignment (Thompson et al., 1994) of the theoretical *Drosophila* protein, all seven human β4GalTs, and the *C. elegans* sqv-3 gene product is shown in Figure 2. Generally, all members of the β4GalT family have some highly conserved features, including predicted type II transmembrane topology, a metal binding site (DxD), four cysteine residues, and the major sequence motif WGWGDED (Bakker et al., 1994; reviewed by Amado et al., 1999). However, these features are not as well conserved in human β4GalT-VII, the *C. elegans* sqv-3 gene product, or the putative *Drosophila* β4Gal-T. Although the DVD motif is perfectly conserved in all the β4GalTs and the theoretical *Drosophila* protein, neither the WGWGDED motif nor all four cysteine residues are perfectly conserved in the known β4GalT-VIIs or the *Drosophila* protein (Figure 2). A close visual inspection of the amino acid sequence line-up revealed several other amino acids that are conserved in human β4GalT-I–VI but not in human β4GalT-VII, the *C. elegans* sqv-3 gene product, or the putative *Drosophila* β4GalT. Furthermore, in some of these positions, the amino acids in the β4GalT-VIIs and the putative *Drosophila* β4GalT are identical. Thus the results of our bioinformatics analyses strongly suggested that CG11780 is an insect ortholog of β4GalT-VII.

**Endogenous β4GalT activities in an established *Drosophila* cell line**

The *in silico* identification of a putative *Drosophila* β4GalT-VII gene prompted us to examine extracts of S2 cells, which are derived from *D. melanogaster* embryos (Schneider, 1972), for the presence of various endogenous β4GalT activities. Standard biochemical assays were used to measure the transfer of galactose from UDP-galactose to various acceptor substrates in vitro, as described in Materials and methods. Control assays were performed with boiled extracts to establish background levels for each assay, and additional controls were performed with no acceptor to establish the potential contribution of endogenous acceptor substrates in the crude lysates. The results showed that S2 cells contained substantial amounts of galactosyl-transferase activity toward p-nitrophenyl β-D-xylopyranoside (pNP-xylose; Figure 3). They also showed that these cells had low levels of activity toward pNP N-acetyl-β-
D-galactosaminide (GalNAc) or glucocerebroside. These results were consistent with the idea that D. melanogaster encodes and expresses a β4GalT-VII ortholog.

Expression and biochemical analysis of the putative Drosophila β4GalT-VII

Subsequent biochemical assays were designed to determine the function of the protein encoded by the putative Drosophila β4GalT-VII gene. The open reading frame was subcloned into an expression plasmid under the transcriptional control of the promoter from a baculovirus ie1 gene and the resulting plasmid was used to express the putative Drosophila β4GalT-VII in Sf9 cells. We used Sf9 cells as the hosts for the remainder of our biochemical assays because they have no detectable endogenous β4GalT activity toward pNP-xylose (data not shown; also see Figures 4–5). Sf9 cells were transfected with the immediate early expression plasmid encoding Drosophila β4GalT-VII or the empty expression plasmid as a negative control. Extracts were prepared from the transfected Sf9 cells, positive control extracts were prepared from untransfected S2 cells, and normalized aliquots of each were assayed for the ability to transfer galactose from UDP-galactose to pNP-xylose. The results of these assays showed clearly that Sf9 cells transfected with the putative Drosophila β4GalT-VII gene, but not the empty vector control, had significant levels of galactosyltransferase activity toward pNP-xylose (Figure 4). It is note-
worthy that both the induced (Sf9) and endogenous (S2) \(\beta 4\)GalT activities were nearly 10-fold higher than the level of endogenous (S2) activity observed in the previous experiment (compare Figures 3–4). Higher levels of activity were observed in this latter experiment because the MnCl\(_2\) concentration was increased to 40 mM to match the optimal concentration reported for human \(\beta 4\)GalT-VII (Almeida et al., 1999). Additional assays were performed to determine the apparent \(K_m\) values of the putative \textit{Drosophila} \(\beta 4\)GalT-VII for its donor and acceptor substrates. The results showed that the apparent \(K_m\) values were approximately 50 \(\mu\)M for UDP-galactose and 1.2 mM for pNP-xylose (data not shown). The apparent \(K_m\) values previously reported for human \(\beta 4\)GalT-VII were 170 \(\mu\)M for UDP-galactose (Quentin et al., 1990) and 3.4 mM for pNP-xylose (Okajima et al., 1999). In addition, partially purified human \(\beta 4\)GalT-VII was reported to have a \(K_m\) for UDP-galactose of about 50 \(\mu\)M (Almeida et al., 1999). These data extended the results of the bioinformatic analyses and provided the first direct biochemical evidence that the \textit{Drosophila} CG11780 gene encodes an insect ortholog of \(\beta 4\)GalT-VII. Henceforth, we will refer to this gene and its product as Dm\(\beta 4\)GalT-VII.

We also performed assays designed to determine if Dm\(\beta 4\)GalT-VII had any in vitro activity toward several other acceptor substrates. Sf9 cells were transfected with the immediate early expression plasmid encoding Dm\(\beta 4\)GalT-VII or the empty vector, as a negative control. Extracts were prepared and normalized amounts used for galactosyltransferase assays with various acceptor substrates. Assays were performed using boiled lysates or no acceptor to establish background for each assay, as described. The results of these experiments demonstrated that the expression plasmid encoding Dm\(\beta 4\)GalT-VII only induced high levels of in vitro galactosyltransferase activity toward pNP-xylose (Figure 5A). By expanding the ordinates 10-fold, one can see evidence of minor activity toward pNP-GlcNAC (Figure 5C). However, the same result was obtained when Sf9 cells were transfected with the empty vector, suggesting that this activity was induced by transfection, independently of Dm\(\beta 4\)GalT-VII expression. No significant activities were observed with pNP-GalNAc (Figure 5B) or glucocerebroside (Figure 5D) as the acceptor substrates. The ability of this \textit{Drosophila} gene to induce high levels of activity toward \(\beta\)-xylosyl but no other acceptor substrate tested strongly supports the identification of the gene product as a \(\beta 4\)GalT-VII.

Although it had been reported that 40 mM MnCl\(_2\) provides optimal human \(\beta 4\)GalT-VII activity (Almeida et al., 1999), as mentioned, it was of interest to examine the influence of various metals on the \textit{Drosophila} enzyme. Galactosyltransferase assays were performed in the absence or presence of various metals using normalized extracts of Sf9 cells transfected with the immediate early expression plasmid encoding Dm\(\beta 4\)GalT-VII (Figure 6). The results showed that in vitro transfer of galactose to pNP-xylose was strongly activated by manganese, slightly activated by cobalt, and very slightly activated by magnesium and nickel. No activity was observed in the absence of metals or in the presence of calcium or zinc. The influence of various concentrations of manganese and cobalt is shown in Figure 7. The optimal concentration of MnCl\(_2\) was 20 mM, which is half the optimal concentration for the human enzyme (Almeida et al., 1999). The insect enzyme was also activated to a similar extent by either 20 or 40 mM CoCl\(_2\), but the highest activity observed in the presence of cobalt was still about 20 times less than that observed in the presence of manganese.

Finally, considering that insect cells thrive in slightly acidic growth medium, we determined the optimal pH for Dm\(\beta 4\)GalT-VII activity in vitro. Sf9 cells were transfected with the immediate early expression plasmid encoding SfDm\(\beta 4\)GalT-VII, the cells were extracted, and normalized aliquots of the extracts were assayed for galactosyltransferase activity toward pNP-xylose at various pH values. The results showed that the optimal pH for this insect cell enzyme was about 6.5 (Figure 8). Interestingly, the \textit{Lymnaea stagnalis} \(\beta 4\)N-acetylgalcosaminyltransferase, which is another invertebrate member of the \(\beta 4\)GalT family, has the same pH optimum (Bakker et al., 1997).
Intracellular distribution of Drosophila β4GalT-VII

Human β4GalT-VII contributes to the biosynthesis of proteoglycans by transferring galactose to a xylose residue in the linkage tetrasaccharide region of the glycosaminoglycan side chain (Almeida et al., 1999), probably in the Golgi apparatus (Sugumaran and Silbert, 1991). Thus, we examined the intracellular distribution of Dmβ4GalT-VII to obtain another clue to its in vivo function. An expression plasmid encoding a nearly full-length version of Dmβ4GalT-VII with a C-terminal green fluorescent protein (GFP) tag was used to transiently express the fusion protein in S2 cells, as described in Materials and methods. Confocal fluorescence microscopy of living cells revealed that the fusion protein was distributed in a punctate pattern throughout the cytoplasm (Figure 9A). This pattern coincided almost perfectly with the pattern obtained by staining the same cells with BODIPY®TR ceramide (Figure 9C, merged with GFP pattern in Figure 9B), a Golgi-specific dye. No green fluorescence was observed when S2 cells were transfected with the empty expression plasmid (data not shown). Based on these data, we concluded that the majority of the Dmβ4GalT-VII protein is localized in the Golgi apparatus of this Drosophila cell line. It is possible, however, that the addition of the GFP tag to the nearly full-length Dmβ4GalT-VII protein could have altered its intracellular distribution.

Discussion

Although there is significant interest in using D. melanogaster to study the functional significance of glycosylation pathways in higher eukaryotes (Seppo and Tiemeyer, 2000), the value of the fruitfly as a model organism for these studies is debatable (Altmann et al., 2001). One basis for this debate is the view that insect cells are unable to produce N-glycoproteins containing complex glycans. Historically, it has appeared that the most highly processed N-glycans produced by insect cells are highly trimmed paucimannose (Man3GlcNAc2) structures. But more recent structural studies have provided evidence that some lepidopteran insect cell lines can produce at least minor subpopulations of hybrid or complex N-glycans containing terminal β-linked galactose residues (Ogonah et al., 1996; Hsu et al., 1996).
et al., 1997; Rudd et al., 2000). Furthermore, these cell lines contain low but detectable levels of β4GalT-I activity (Van Die et al., 1996) and it was recently shown that D. melanogaster encodes and expresses a functional UDP-galactose transporter (Aumiller and Jarvis, 2002; Segawa et al., 2002). Thus, insect cells appear to have more extensive glycan processing machinery than has been generally appreciated.

One way to obtain further information about insect cell glycan processing machinery is to use bioinformatics to search the Drosophila databases for genes encoding relevant functions. The emerging view that insect cells can produce galactosylated N-glycoproteins prompted us to use this approach to search for D. melanogaster genes that appear to be involved in glycosaminoglycan biosynthesis. These genes include those that appear to encode a Drosophila ortholog of human β4GalT-VII.

An in vitro galactosyltransferase assay was used to examine endogenous β4GalT activities in a Drosophila cell line and to determine the actual function of the CG11780 gene product. The results showed that Drosophila S2 cells have an enzymatic activity that can transfer galactose from UDP-galactose to β-xyllosyl but not to other acceptors in vitro. They also showed that this activity can be induced by transfecting Sf9 cells with an immediate early expression plasmid encoding the Drosophila CG11780 gene product. This enzyme had apparent $K_m$ values of about 50 μM and 1.2 mM for UDP-galactose and pNP-xyllose, respectively; was strongly stimulated by MnCl$_2$; and had an optimal pH of about 6.5. Finally, a GFP-tagged version of the gene product was localized to the Golgi apparatus of insect cells. The combined evidence obtained from the in silico, in vitro, and in vivo experiments reported in this manuscript indicates that the CG11780 gene does indeed encode a Drosophila cognate of human β4GalT-VII. We designated this gene and its product Dmβ4GalT-VII to conform to the nomenclature introduced by Lo and co-workers (1998) and concluded that it probably contributes to Drosophila proteoglycan biosynthesis by transferring galactose to the xylose residue in the linkage tetrasaccharide of glycosaminoglycan side chains.

The value of Drosophila as a model system for studying the role of glycosaminoglycans in proteoglycan function is well established. First, it is clear that this animal produces glycosaminoglycans and proteoglycans (Seppo and Tiemeyer, 2000b; Toyoda et al., 2000b; Selleck, 2001). Second, it is clear that mutant phenotypes can arise from mutations in fly genes that appear to be involved in glycosaminoglycan biosynthesis. These genes include sugarless, sulfateless, and tout velu, which appear to encode Drosophila cognates of UDP-glucose dehydrogenase, N-deacetylase N-sulfotransferase, and heparan sulfate copolymerase, respectively (reviewed by Seppo and Tiemeyer, 2000; Toyoda et al., 2000a,b). However, to our knowledge, this report is the first to identify a Drosophila galactosyltransferase that is likely to be involved in glycosaminoglycan biosynthesis. According to Flybase (1999), this gene is located on chromosome 3R and has been cytologically mapped to 96B13-14. There are no recorded mutant alleles, but there is a deficiency (DF[3R]96B)

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Fig. 7. Influence of various concentrations of manganese and cobalt on Dmβ4GalT-VII activity. Sf9 cells were transfected with an immediate early expression plasmid encoding Dmβ4GalT and extracted, and normalized amounts of the extracts were assayed for galactosyltransferase activity towards pNP-xyllose in the presence or absence of various concentrations of MnCl$_2$ or CoCl$_2$, as described in Materials and methods. The graph shows the average specific activities measured in triplicate assays with SDs indicated by the error bars (note that some SDs were too small to show up as error bars on the graph).

Fig. 8. pH optimum of Drosophila β4GalT-VII. Sf9 cells were transfected with an immediate early expression plasmid encoding Dmβ4GalT and extracted, and normalized amounts of the extracts were assayed for galactosyltransferase activity toward pNP-xyllose at various pH values, as described in Materials and methods. The graph shows the average specific activities measured in triplicate assays with SDs indicated by the error bars (note that some SDs were too small to show up as error bars on the graph).
in this region (96A2:96C2) that has a recessive lethal phenotype. Thus, future studies of Drosophila mutants could provide further information on the in vivo role of the Dmβ4GalT-VII gene identified in this study and on the biological significance of proteoglycan production in this organism.

Materials and methods

Cells and cell culture

Sf9 cells (Vaughn et al., 1977) were routinely maintained as a shake-flask culture in TNM-FH medium containing 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 1% (w/v) pluronic F68, as described previously (Jarvis et al., 1996). This medium was designated complete TNM-FH. S2 cells (Schneider, 1972) were routinely maintained as a shake-flask culture in Schneider’s Drosophila Medium (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (HyClone) and 1% (w/v) pluronic F68.

Plasmid constructs

The immediate early expression plasmid pAcP(-)IEdGalT3 was used to express the full-length Dmβ4GalT-VII gene under the control of a baculovirus ie1 promoter. This plasmid was constructed by subcloning the 1.13 kb HincII–BamHI fragment from the Drosophila EST clone designated CK02622 (GenBank accession number AA142310; Research Genetics, Huntsville, AL; Rubin et al., 2000) into the SmaI and BglII sites of pAcP(-)IETV6 (Jarvis et al., 1996). The expression plasmid pAc5.1DmGalT-VII-GFP was used to express a nearly full-length, C-terminally GFP-tagged version of the Dmβ4GalT-VII protein under the transcriptional control of a Drosophila actin promoter. The 1.05 kb KpnI–BspEI fragment, which encodes all but the last 12 amino acids of the Dmβ4GalT-VII protein, was excised from CK02622, and the BspEI end was repaired with T4 DNA polymerase I. The resulting fragment was gel-purified and subcloned into the XmaI site of pEGFP-N1 (Clonetech Laboratories, Palo Alto, CA), which also had been repaired with DNA polymerase I.

The resulting plasmid was isolated, amplified, purified, and used to excise the 1.82 kb KpnI/Xbal fragment encoding the Dmβ4GalT-VII-GST fusion protein, which was gel-purified and subcloned into the corresponding sites of pAc5.1/A (Invitrogen). To produce each of these constructs, Escherichia coli transformants were isolated and screened using standard alkaline lysis plasmid isolation and restriction mapping procedures (Birnboim and Doly, 1979; Sambrook et al., 1989). The final plasmid preparations used for transient expression in insect cells were isolated from 200 ml transformed E. coli cultures by alkaline lysis (Birnboim and Doly, 1979) and isopycnic ultracentrifugation on CsCl-ethidium bromide density gradients (Sambrook et al., 1989).

Transfection and extraction of Sf9 cells for biochemical assays

Sf9 cells were transfected with the purified immediate early expression plasmids described or the parental vectors, as negative controls, using a modified calcium phosphate method. Briefly, 2 x 10⁶ cells were seeded into 25-cm² tissue culture flasks (Corning, Corning, NY), washed with Grace’s medium, and transfected with 10 µg pAcIE1 plus 10 µg of the relevant expression plasmid or parental vector, as described previously (Jarvis et al., 1996). The cells were incubated for 2 h at 28°C, washed twice with complete TNM-FH, and fed with 5 ml of the same medium. The cells were then incubated for another 22 h at 28°C, removed from the culture flasks, washed with ice-cold buffer (25 mM Tris–HCl, pH 7.4; 140 mM NaCl), and extracted with an appropriate galactosyltransferase buffer. The buffers used for each type of assay performed in this study were chosen from the literature to provide optimal activity in each type of β4GalT assay. These buffers were: 25 mM sodium cacodylate, pH 7.0, containing 0.25% Triton-X-100 for pNP-xylene assays (Almeida et al., 1999); 141 mM sodium cacodylate, pH 7.0, containing 0.7% Triton-X-100 and 10 mM ATP for pNP-GlcNAc and pNP-GalNAc assays (Bakker et al., 1994); and 200 mM sodium cacodylate, pH 7.2, containing 0.375% Triton-X-100 for glucocerebroside assays (Nomura et al., 1998). The cells were extracted for 10 min on ice, then the extracts were clarified by centrifugation for 10 min at 4°C at
top speed in a microcentrifuge (Hermle Model Z180M). The supernatants were harvested and used for the assays.

Galactosyltransferase assays

Total protein concentrations in the cell extracts were determined using a commercial bicinchoninic assay kit (Pierce, Rockford, IL). Triplicate samples of each extract containing 50 μg total protein were then assayed in a total reaction volume of 0.1 ml of the appropriate buffer, as described. Each assay also contained various concentrations of various metals (specified in the figure legends), 0.3 μCi UDP-galactose, [galactose-1-3H(N)] (Perkin-Elmer Life Sciences, Boston, MA; 9.1 Ci/mmole), and 1.0 mM of the appropriate acceptor substrate (Sigma, St. Louis, MO; also specified in the figure legends). The reactions were incubated for 1 h at 37°C, then terminated by adding 0.5 ml ice-cold 50 mM ethylenediamine tetra-acetic acid, pH 8.0. The spent reactions were applied to Sep-Pak C18 cartridges (Millipore, Bedford, MA), unincorporated radioactivity was eliminated by washing with methanol. The eluants were added to 5 ml Scintisafe Plus 50% scintillation cocktail (Beckman Coulter Instruments, Palo Alto, CA). All data were presented as the average pmol of galactose transferred per mg of total protein per h, with standard deviations calculated from the individual measurements.

The galactosyltransferase assay described was modified slightly to determine the apparent K_m values for UDP-galactose and pNP-xylene. In each case, duplicate reactions were performed using 50 μg total protein from Dmβ4GalT-VII-transfected S9 cell extracts prepared in pNP-xylene assay buffer supplemented with 20 mM MnCl_2. To determine the apparent K_m for the donor substrate, reactions were performed in the presence of 2.0 mM pNP-xylene, nonradioactive UDP-galactose concentrations ranging from 10–500 μM, and a constant ratio of UDP-[galactose-1-3H(N)] with a specific activity of 9.1 Ci/mmole. To determine the apparent K_m for the acceptor substrate, reactions were performed in the presence of 50 μM nonradioactive UDP-galactose, 0.3 μCi of UDP-[galactose-1-3H(N)], and pNP-xylene concentrations ranging from 0.1–5.0 mM.

Living cell fluorescence experiments

S2 cells were seeded into 25-cm² tissue culture flasks (Corning) at a density of 3 × 10^6 cells, the cells were incubated overnight at 28°C, then they were transfected with 20 μg of either pAc5.1DmGalT-VII-GFP or pAc5.1/A using a standard calcium phosphate method described for use with the Drosophila Expression System (Invitrogen). The transfected cells were incubated with the DNA precipitate for 24 h at 28°C, washed, fed, and incubated for another 40 h at 28°C. The growth medium was then removed and the cells were stained for 1 h with fresh growth medium containing 350 nM BODIPY®TR ceramide (Molecular Probes, Eugene, OR). After staining, the cells were rinsed with fresh medium and examined under a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany).

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Abbreviations

β4GalT, β4-galactosyltransferase; EST, expressed sequence tag; GalNAc, N-acetyl-β-D-galactosaminide; GlcNAc, N-acetyl-β-D-glucosaminide; GFP, green fluorescent protein; pNP-xylene, p-nitrophenyl β-D-xylanoylase.

References


