Golgi targeting of Drosophila melanogaster β4GalNAcTB requires a DHHC protein family–related protein as a pilot

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Drosophila melanogaster β4GalNAcTB mutant flies revealed that this particular N-acetylgalactosaminyltransferase is predominant in the formation of laddINAc (GalNAcβ1,4GlcNAc)-modified glycolipids, but enzymatic activity could not be confirmed for the cloned enzyme. Using a heterologous expression cloning approach, we isolated β4GalNAcTB together with β4GalNAcTB pilot (GABPI), a multimembrane-spanning protein related to Asp-His-His-Cys (DHHC) proteins but lacking the DHHC consensus sequence. In the absence of GABPI, inactive β4GalNAcTB is trapped in the endoplasmic reticulum (ER). Coexpression of β4GalNAcTB and GABPI generates the active enzyme that is localized together with GABPI in the Golgi. GABPI associates with β4GalNAcTB and, when expressed with an ER retention signal, holds active β4GalNAcTB in the ER. Importantly, treatment of isolated membrane vesicles with Triton X-100 disturbs β4GalNAcTB activity. This phenomenon occurs with multimembrane-spanning glycosyltransferases but is normally not a property of glycosyltransferases with one membrane anchor. In summary, our data provide evidence that GABPI is required for ER export and activity of β4GalNAcTB.

Introduction

Glycosylation in the secretory pathway is a complex process in which hundreds of glycosyltransferases are involved (Taniguchi et al., 2002). Many glycosyltransferases appear in gene families specified mainly by the nature of the nucleotide sugar donor (Coutinho et al., 2003). Within a given family, individual glycosyltransferases differ regarding the recognized acceptor structures. To understand and modulate cellular glycosylation pathways, it is important to know how this substrate specificity is generated (de Graffenried and Bertozzi, 2004).

Important in this respect is the observation that acceptor specificity in many glycosyltransferases is not restricted to recognition of one or a few specifically linked monosaccharides. Some protein-specific glycosyltransferases obtain additional selectivity by recognizing specific peptide motifs in the acceptor. A classic example is the N-acetylgalactosaminyltransferase (GalNAcT), which modifies glycoprotein hormones with high selectivity (Smith and Baenziger, 1988).

Some glycosyltransferases require other proteins that are not part of the acceptor structure for their specific activity. β1,4-galactosyltransferase (β4GalT) acts on terminally positioned N-acetylgalactosamine (GlcNAc) residues conjugated to proteins or lipids. Its specificity changes if it builds a complex with α-lactalbumin. In the complex, free glucose is used as an acceptor, and lactose is formed (Brew et al., 1968). In the case of core 1 β3-galactosyltransferase (C1β3GalT), a molecular chaperone called Cosmc, with specificity for this single client, is required for folding and transportation to the Golgi (Ju and Cummings, 2002, 2005; Ju et al., 2008). Also, for O-mannosylation, two proteins, POMT1 and POMT2, are required (Manya et al., 2004). However, in this case, a two-protein enzymatic complex is proposed. The same is true in heparin sulfate biosynthesis in...
which two different exostosins are required for efficient biosynthesis (McCormick et al., 2000).

For several glycosyltransferases involved in glycolipid biosynthesis, data indicate that factors other than the enzyme and the acceptor substrate play a role. This is the case for β4GalT-V and -VI, which are homologues of the β4GalT mentioned in the previous paragraph and of Drosophila melanogaster β4GalNAcTB (the subject of this study). Under in vitro conditions, β4GalT-V and -VI transfer galactose (Gal) into β1-4 linkage to terminally expressed GlcNAc residues on glycoproteins (van Die et al., 1999; Guo et al., 2001). However, their involvement in the biosynthesis of lactosyl ceramide (Cer) by Gal transfer onto glucosyl Cer has been demonstrated as well (Nomura et al., 1998; Sato et al., 2000; Kolmakova and Chatterjee, 2005). At least in the case of galactosyltransferase V, this latter activity depends on the enzyme’s anchorage in the membrane (van Die et al., 1999; Sato et al., 2000). Other enzymes involved in glycolipid biosynthesis have been shown to exhibit very low activity (de Vries et al., 1995; Zhub et al., 1998; Togayachi et al., 2001) or no activity (Steffensen et al., 2000; Schwientek et al., 2002) activity if expressed as soluble proteins. In general, very little is known about how lipid acceptors are recognized by glycosyltransferases. However, it has been suggested that a membrane-bound activator protein is required to present glycolipid acceptors to the modifying glycosyltransferases (Ramakrishnan et al., 2002). This hypothesis is substantiated by analogy to the lysosomal sphingolipid degradation machinery in which the sphingolipid activator protein presents the glycolipid substrates to glycosidases (Kolter and Sandhoff, 2005).

In this study, we describe a novel mechanism of glycosyltransferase maturation and functionalization for the glycolipid-specific β4GalNAcTB from Drosophila. This enzyme, which has been described as an inactive homologue of β4GalNAcTA in a previous study (Haines and Irvine, 2005), is a member of the invertebrate branch of the β4GalT family involved in the biosynthesis of the lacdiNac (GalNAcβ1,4GlcNAc) epitope (Kawar et al., 2002; Vadaie et al., 2002; Haines and Irvine, 2005; Stolz et al., 2008). Because β4GalNAcT had not been cloned when this study was started, we searched for the corresponding activity using expression cloning (Bakker et al., 1997, 2005; Münster et al., 1998). In a heterologous approach, a cDNA library from Drosophila was used for expression in CHO cells, whereas formation of the lacdiNac epitope was monitored with a specific monoclonal antibody (van Remoortere et al., 2000). As will be demonstrated in this study, the expression of two cDNA clones was required to install the functionally active enzyme.

### Results

#### Expression cloning of a Drosophila β4GalNAcT

Other than in many invertebrates, the lacdiNac element has been identified on only a few glycoconjugates in mammals (Sato et al., 2003). We ascertained that CHO cells are negative for lacdiNac. Considering that in mammalian cells terminal GlcNAc residues are recognized by several galactosyltransferases, we additionally hypothesized that signals in the complementation cloning approach could be improved by the use of CHO Lec8 cells. They lack the Golgi UDP-Gal transporter and, consequently, show drastically reduced incorporation of Gal in glycans (Deutscher and Hirschberg, 1986). A cDNA library was constructed from Drosophila, subdivided into pools, and, in an established sibling selection procedure (Bakker et al., 1997), used to search for clones that rendered cells positive for lacdiNac. Cell surface lacdiNac expression was monitored with antibody 259-2A1, which was originally raised from Schistosoma mansoni-infected mice (van Remoortere et al., 2000). In this procedure, it became obvious that two cDNA clones were required for the expression of the lacdiNac epitope. Although clone one was a member of the β1,4GalT family (van Die et al., 1997), the second clone (flybase CG17257) encoded a type III membrane protein that was related to a gene family referred to as Asp-His-His-Cys (DHHC) proteins (Mitchell et al., 2006). This protein was termed β4GalNAcTB pilot (GABPI) to describe its crucial role in generating a functionally active β4GalNAcTB as shown in the following experiments. The identified β4GalNAcT was identical to the inactive β4GalNAcTB recently cloned by Haines and Irvine (2005) in a homology-based approach. In agreement with this study, we found no in vivo activity for full-length β4GalNAcTB expressed in mammalian cells. It is remarkable that expression cloning identified β4GalNAcTB and not the homologous β4GalNAcTA, which was shown in an earlier study (Haines and Irvine, 2005) to be an active enzyme. To resolve the controversial finding, β4GalNAcTA was cloned by PCR and expressed in comparison with β4GalNAcTB–GABPI in CHO and HEK293 cells. These experiments demonstrated that the cell surface lacdiNac expression detected with antibody 259-2A1 as a result of β4GalNAcTA was much lower than the lacdiNac formation after the combined expression of β4GalNAcTB and GABPI. Thus, the data demonstrate the existence of two functionally active β4GalNAcTs (β4GalNAcTA and β4GalNAcTB) in Drosophila of which β4GalNAcTB needs the cooperation of GABPI. Because CHO cells demonstrated a low tolerance to expression of the Drosophila N-acetylglactosamine (GalNAc) transferases and HEK293 cells turned out to be a more suitable expression system, subsequent experiments were performed exclusively in HEK293 cells.

**β4GalNAcTB specifically modifies glycolipids**

Despite elaborated analyses of Drosophila glycoproteins (North et al., 2006), the lacdiNac structure has so far only been found as a modification of glycolipids (Seppo et al., 2000). With both cloned enzymes at hand, we evaluated the question of acceptor specificity. HEK293 cells were transfected with β4GalNAcTA, β4GalNAcTB, or the combination β4GalNAcTB–GABPI and analyzed for the presence of lipid- and protein-bound lacdiNac using TLC followed by immunooxay (Fig. 1 A) and Western blotting (Fig. 1 B), respectively. In both systems, Drosophila S2 cells, which are naturally positive for the antibody epitope, and HEK293 cells transfected with Caenorhabditis elegans β4GalNAcT (Kawar et al., 2002) were used as controls. Although expression of the *C. elegans* enzyme confirmed the availability of β4GalNAcT acceptors on proteins, the absence of specific signals in both HEK293 cells transfected with the β4GalNAcTs
and in S2 cells confirmed the earlier observations in flies. In contrast, immunostaining of the lipid extracts resulted in positive signals for S2 cells as well as for HEK293 cells transfected with the β4GalNAcTB–GABPI pair. Expression of β4GalNAcTB alone was not sufficient to produce a signal, whereas faint signals were reliably obtained with β4GalNAcTA. It is important to mention that lipid specificity is preserved, although the glycolipid acceptor structures are different in Drosophila and HEK293 cells.

**In vitro activity of β4GalNAcTB–GABPI is detergent sensitive**

The observations that β4GalNAcTB is inactive if it is separately expressed in a heterologous cell system or if it is tested as a recombinant soluble protein (Haines and Irvine, 2005) encouraged further analyses to examine at which step β4GalNAcTB and GABPI interact with each other in the biosynthesis. In the first experiment, it was established that a soluble secreted construct of β4GalNAcTB was still inactive when coexpressed with GABPI. Subsequently, we wondered whether the two proteins expressed in separate cells have the capacity to form an active enzyme. Microsomal fractions of HEK293 cells transfected with either β4GalNAcTB or GABPI were isolated, mildly treated with detergent (saponin 0.01%), and functionally tested in mixtures. The assay system used to follow β4GalNAcT activity was adapted from an established assay (Palcic et al., 1988). In this assay system, [3H]UDP-GalNAc is the donor and [3H]UDP-Gal (endogenous activity as an internal control) as donor substrates. Each value represents the mean of three independent vesicle preparations with standard deviation. GalNAcT mix GABPI indicates that the proteins were expressed separately but mixed afterward for assays. (B and C) Microsomal fractions of HEK293 cells transfected with β4GalNAcTA (B) or the combination β4GalNAcTB–GABPI (C) were treated with Triton X-100 and saponin in various concentrations and assayed for GalNAcT activity as in A.
Depletion of GABPI in Drosophila S2 cells delocalizes GalNAcTB and reduces lacdiNAc-containing glycolipid formation

To additionally evaluate the influence of GABPI on β4GalNAcTB localization in the natural environment, RNAi experiments were performed. S2 cells were transiently transfected with identical treatment with detergent. In accordance with lacdiNAc formation in intact cells, β4GalNAcTB was not active when expressed alone, but it showed higher activity than β4GalNAcTA when expressed in combination with GABPI.

In contrast to β4GalNAcTA, the β4GalNAcTB activity strongly depended on the detergent used. Only background levels were measured if membranes were treated with Triton X-100 (Fig. 2 B) or NP-40 (not depicted) at 0.5%, which is routinely used in glycosyltransferase assays. The milder detergent saponin increased activity over a wide concentration range (Fig. 2 B). The rather low activity measured in the absence of detergent was probably a result of limited transport of the substrates over the vesicle membranes. As saponin is known to perforate and not disrupt membranes (Schulz, 1990), these data suggest that the maintenance of protein complexes in intact membrane patches is required for β4GalNAcTB activity.

ER export of β4GalNAcTB requires GABPI

The data presented so far for the interaction between β4GalNAcTB and GABPI are highly reminiscent of the interactions between the human C1β3GalT generating the T antigen (core 1 O-glycan Galβ1-3GalNAcα1-Ser/Thr) and its client-specific molecular chaperone, Cosmc (Ju and Cummings, 2002). Cosmc supports functional folding of C1β3GalT in the ER but then dissociates and releases C1β3GalT (Ju et al., 2002b; Ju et al., 2008). Therefore, the following experiments addressed the subcellular localization of GABPI and β4GalNAcTs. Flag-β4GalNAcTs and Myc-GABPI were separately expressed in HEK293 cells and, after selection of stable clones, were detected by indirect immunofluorescence. Flag-β4GalNAcTA and Myc-GABPI colocalized with the Golgi marker α-mannosidase II (Fig. 3, A and C). Only the signal generated by Flag-β4GalNAcTB overlapped with the ER marker calnexin (Fig. 3 B). However, when GABPI was cotransfected (Fig. 3, D–F), the immunofluorescence images showed a clear shift of β4GalNAcTB to the Golgi. Moreover, as shown in Fig. 3 F, GABPI and β4GalNAcTB colocalized in this compartment. This experiment demonstrated that ER export of β4GalNAcTB needs piloting by GABPI, which by itself is an autonomous protein fully equipped with the information required for folding and transport to the Golgi.

As GABPI moves with β4GalNAcTB, the question was raised whether both proteins remain associated in the Golgi. To answer this question, GABPI was tagged with a C-terminal KKTN dilysine signal (Zerangue et al., 2001), which retains proteins in the ER. GABPI was indeed successfully localized in the ER using this approach (Fig. 3 G). More importantly, β4GalNAcTB was also retained in the ER in cells expressing KKTN-tagged GABPI (Fig. 3 H). In vitro enzymatic activity of β4GalNAcTB was about two times as high as the nonretained construct (Fig. 2 A, right bars), and cell surface lacdiNAc was also detectable in these cells. Although the latter probably required cycling to the Golgi of at least part of the enzyme, ER retention might have allowed a higher protein expression level that was enzymatically active in vitro. Together, these data demonstrated that the DHHC family-related protein has, in contrast to Cosmc, functions that go beyond those of a client-specific chaperone.

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with N-terminally tagged β4GalNAcTs, and localization of the enzymes was monitored. As shown in Fig. 4A, both β4GalNAcTs were colocalized in vesicular structures presumed to be the Golgi. β4GalNAcTB did not show any overlap with the ER-specific antibody anti-HDEL (Fig. 4B). Incubation of cells with double-stranded RNA (dsRNA; Clemens et al., 2000) corresponding to a central coding region of GABPI dissected the HA-β4GalNAcTB signal from Flag-β4GalNAcTA (Fig. 4C) and shifted the signal to structures that are part of the ER (Fig. 4D).

To answer whether the RNAi-induced redistribution of the enzyme is also followed by a change in activity, a second knockdown experiment was performed in which dsRNAs were designed to down-regulate β4GalNAcTA, β4GalNAcTB, or GABPI. S2 cells were cultured for 3 d in the absence or presence of dsRNA, after which the expression of lacdiNAc was displayed by immunocytochemistry and matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry (MS), as illustrated in Fig. 5. The intense staining of control cells with antibody 259-2A1 was in accordance with the detection of lacdiNAc-containing glycolipid structures by negative-ion mode MALDI-TOF-MS and β4GalNAcTA knockdown did not change the signal pattern in comparison with control cells, whereas depletion of either β4GalNAcTB or GABPI had comparably strong effects on lacdiNAc expression. In the negative-ion mode, reduction of glycolipids carrying lacdiNAc repeats was accompanied by an enrichment of GlcNAcβ3Galβ3GalNAcc,4GalNAcβ3GalNAcc,4(Pε-6)GlcNAcβ3Manβ3Galβ3Cer species with a molecular mass of 1,958.4 D, an acceptor structure for β4GalNAcT. The positive-ion mode analyses clearly demonstrated the accumulation of a second β4GalNAcT acceptor structure, GlcNAcβ3Manβ4Glcβ3Cer, having a molecular mass of 1,087.6 D. Changes in the glycolipid structures are very similar to changes observed in β4GalNAcTB knockout flies (Stolz et al., 2008) and thus are not further addressed in this paper. In addition, a new glycolipid species carrying lacdiNAc with a molecular mass of 1,796.3 D has been identified and characterized by MALDI-TOF-MS (Table S1 and Fig. S3). In summary, the results presented in Figs. 4 and 5 allow the conclusions that (a) β4GalNAcTB is the major lacdiNAc-synthesizing enzyme in S2 cells as it is in the fly, (b) GABPI enables Golgi targeting of β4GalNAcTB, and (c) β4GalNAcTB is essentially required to convey functionality.

**Pull down of the complex formed between β4GalNAcTB and GABPI**

Because the data shown so far consistently argue for the existence of β4GalNAcTB and GABPI as a complex in the Golgi, we examined this contact in pull-down experiments. HEK293 cells transiently transfected with cDNA constructs encoding Myc-GABPI–HA and Flag-β4GalNAcTA and -B were lysed with buffer containing 1% NP-40. The anti-HA antibody 12CA5 coupled to Sepharose beads was used to precipitate Myc-GABPI–HA. To control the expression of recombinant proteins, total cell lysates were analyzed by Western blotting in parallel to precipitated proteins (Fig. 6). Both proteins were well expressed, as shown in the total cell lysates, whereas only Flag-β4GalNAcTB and not Flag-β4GalNAcTA was precipitated via Myc-GABPI–HA. This provided additional evidence for a tight interaction between β4GalNAcTB and GABPI.

**The DHHC family-related protein GABPI is not an acyltransferase**

Characterized DHHC protein family members are palmitoyltransferases in which the cysteine residue in the conserved DHHC motif is essential for activity (Lobo et al., 2002; Roth et al., 2002; Valdez-Taubas and Pelham, 2005). GABPI, in contrast to all mammalian and the other *Drosophila* members in the family, has exchanged this motif from DHHC to DHHS. This was already an argument against its function as acyltransferase. To validate this assumption, a series of mutants was constructed with which a potential involvement of the DHHS motif could be tested. The ability of GABPI to install a functional β4GalNAcTB was not abolished by reconstruction of the DHHC motif, by replacement of the serine by alanine, or by successive replacement to AAAA (unpublished data). In addition, the critical cysteine residue (C29) that may serve as acyl residue acceptor in β4GalNAcTB was mutated. Again, no effect on GABPI–β4GalNAcTB Golgi localization and activity was found (unpublished data).

**The stem region of β4GalNAcTB is needed for activation by GABPI**

Because the β4GalNAcTs isolated from *Drosophila* are highly homologous proteins, it was of relevance to identify primary sequence elements responsible for the strict GABPI dependency...
of β4GalNAcTB. The aligned primary sequences indicated the stem region to be the domain of highest variability. Consequently, hybrids were made by domain swapping, as shown in Fig. 7. The chimera in which cytoplasmic and transmembrane domains of β4GalNAcTA were added to stem and catalytic regions of β4GalNAcTB (hybrid A-B-B) remained GABPI dependent for Golgi localization (Fig. 7, A and B) and activity (not depicted). However, additional replacement of the stem region destroyed activation by GABPI. The resulting protein was inactive and retained in the ER (Fig. 7, C and D). Because the stem region in β4GalNAcTA is considerably longer than in β4GalNAcTB, additional constructs were prepared in which the size was trimmed from the N and C termini to the exact length of the β4GalNAcTB stem region. All constructs remained inactive (unpublished data), allowing the conclusion that information contained in the stem region of β4GalNAcTB is essential for its function. In contrast, β4GalNAcTA remained Golgi localized and active independently of GABPI when fused to the cytoplasmic and stem region of β4GalNAcTB (construct B-B-A; Fig. 7 E). This is in agreement with the fact that the catalytic domain of β4GalNAcTA can be produced as soluble enzyme and, therefore, is an independent active entity.

Discussion

Using a heterologous expression cloning approach, we isolated β4GalNAcTB as the major enzyme responsible for the biosynthesis of 4acdiNac structures in Drosophila. In this study, this enzyme was demonstrated to depend on the cooperation of a multimembrane-spanning protein related to the DHHC protein family. To point out the complexity of its involvement in forming a functionally active β4GalNAcTB, it was called GABPI. GABPI was cloned simultaneously to β4GalNAcTB in a classical expression cloning approach. This demonstrates the power of this technique, which exclusively screens for activity. The fact that the second β4GalNAc transferase in Drosophila,
β4GalNAcTA (Haines and Irvine, 2005), was not detected in the expression cloning approach as a result of the much lower activity of this enzyme, which we confirmed in vitro and in cellular test systems as well as on the systemic level (Stolz et al., 2008). Although β4GalNAcTA has been shown to act on protein acceptors in vitro (Sasaki et al., 2007), the low activity of β4GalNAcTA in HEK293 cells observed in this study does not allow a conclusion on the nature of the acceptor. Obviously, β4GalNAcTB is strictly lipid specific. This specificity is remarkable because glycan structures added to lipid anchors are different between mammals and flies. This suggests that selectivity is at least partly established through the lipid anchors. GABPI might be involved in the lipid specificity, but β4GalNAcTB also requires GABPI for in vitro activity with the synthetic acceptor substrate GlcNAc-pNP. In particular, these types of small hydrophobic aglycon-linked monosaccharide acceptors usually overcome the restricted specificity of glycosyltransferases; even the glycoprotein hormone-specific β4GalNAcT is reactive with such acceptors (Smith and Baenziger, 1988).

Trials to assemble an active enzyme by combining vesicle preparations containing β4GalNAcTB and GABPI separately failed. This was also the case for the O-mannosyltransferases (Manya et al., 2004). This shows that GABPI and β4GalNAcTB do not act in a sequential reaction mechanism. Combined with the experiments in which it was shown that β4GalNAcTB remains in the ER in an inactive state if expressed alone and can only reach the Golgi in the presence of GABPI, it can be concluded that interaction between β4GalNAcTB and GABPI most likely starts in the ER and requires coexpression of the two proteins. Most importantly, GABPI is an autonomous protein equipped with all of the information needed for Golgi destination. In this respect, GABPI seems to be different from Cosmc, the client-specific molecular chaperone required to activate C1β3GalT. A soluble, active form of recombinant C1β3GalT can be produced (Ju et al., 2002a), although Cosmc is not associated with this enzyme (Ju and Cummings, 2002). Purified rat liver C1β3GalT was also devoid of Cosmc (Ju et al., 2002b). According to the classical definition of a chaperone, Cosmc releases an active C1β3GalT (Ju et al., 2002b, 2008). In contrast, GABPI moves with β4GalNAcTB to the Golgi and retains β4GalNAcTB in the ER if it is retained itself. This and the fact that the proteins can be coimmunoprecipitated argue for a stable complex of both.

An insertion of the complex in an intact membrane patch is indispensable for functionality. Proof of this is provided by the fact that β4GalNAcTB activity tested with GlcNAc-pNP was almost completely abolished after addition of Triton X-100 or NP-40. Although these are rather mild detergents that normally do not dissociate protein complexes, their presence interferes with membrane integrity. This in turn may cause deformation of associated complexes. In contrast, saponin, which only perforates membranes, most likely increased activity by allowing the substrates to enter the vesicles without disturbing the proper embedding of the enzyme in the membrane. Detergent sensitivity is a property of many mannosyltransferases in the ER (Schützbach, 1997), including the protein O-mannosyltransferase complex (POMT1 and POMT2), which is inactivated by Triton X-100 (Manya et al., 2004), and egghead, the mannosyltransferase acting two steps upstream of β4GalNAcTB in Drosophila glycolipid biosynthesis (Wandall et al., 2003). These enzymes are multitransmembrane-spanning proteins. Glycosyltransferases of the Golgi containing one transmembrane domain are usually not sensitive to detergents. As β4GalNAcTB is a typical member of the Golgi type II transmembrane glycosyltransferases, the observed detergent sensitivity is expected to be conveyed by disturbance of GABPI or the GABPI-β4GalNAcTB complex.

In line with the experiments in HEK293 cells, dsRNA-induced knockdown of GABPI in Drosophila S2 cells separated β4GalNAcTB from β4GalNAcTA, depleted cell surface expression of the lacdiNAc epitope, and provoked an accumulation of the β4GalNAcT glycolipid acceptor structures. These effects observed at the cellular level were exactly phenocopied in a Drosophila mutant with an inactivated β4GalNAcT gene (Stolz et al., 2008).

The knowledge that all functionally characterized DHHC family proteins are palmitoyltransferases prompted experiments designed to determine whether GABPI could function as an
mologous which GABPI activates the name-giving DHHC motif is exchanged by serine in GABPI, ( Mitchell et al., 2006 ) as well as the only cysteine residue that may serve as acyl acceptors in activity in GABPI. All residues critical for a potential acyltransferase Figure 7. Protein domains of β4GalNAcTB involved in the interaction with GABPI. Hybrids of Flag-β4GalNAcTA (red) and -B (blue) were cloned by domain swapping of the cytoplasmic (Cyt) and transmembrane domain (TM), stem region, and catalytic domain (Cat) as illustrated and expressed in HEK293 with and without GABPI. The intracellular localization was analyzed by indirect immunofluorescence using α-mannosidase II and calnexin as markers for Golgi and ER, respectively.

acyltransferase. All residues critical for a potential acyltransferase activity in GABPI ( Mitchell et al., 2006 ) as well as the only cysteine residue that may serve as acyl acceptors in β4GalNAcTB were point mutated. None influenced the functionality of GABPI or activity of β4GalNAcTB. The functionally crucial cysteine in the name-giving DHHC motif is exchanged by serine in GABPI, which argues against its function as a palmitoyltransferase.

In experiments aimed at understanding how β4GalNAcTB and GABPI interact, we demonstrated that the selectivity with which GABPI activates β4GalNAcTB and not the highly homologous β4GalNAcTA is attributed to a structural element in the stem region. However, this area cannot be the solely responsible element. Additional sequences in the catalytic domain must be involved in determining GABPI dependency.

The exact function of GABPI in priming activity of β4GalNAcTB in the Golgi is difficult to address. However, several glycosyltransferases acting exclusively in the glycolipid bio-synthetic pathways need membrane anchorage and cannot be expressed as soluble recombinant proteins ( Amado et al., 1998; Steffensen et al., 2000; Schwientek et al., 2002 ). One of these enzymes is brainiac ( Schwientek et al., 2002 ), a β3GlcNAc transferase acting right upstream of β4GalNAcTB in glycolipid biosynthesis of Drosophila. The factors determining membrane dependency of brainiac are not yet identified. Because we found the product of brainiac accumulated in S2 cells treated with RNAi against GABPI ( Fig. 5 ), an involvement of GABPI for brainiac function in vivo is not likely. However, because mammalian lipid-modifying enzymes have been suggested to form multienzyme complexes ( Giraudo and Maccioni, 2003 ), GABPI, being an essential part of β4GalNAcTB, might be an anchor position in the pathway without being essential for the activity of all enzymes. A striking parallel exists between β4GalNAcTB and β4GalT-V and -VI described in the Introduction. These mammalian galactosyltransferases are members of the same gene family and are essentially dependent on membrane contact for transfer of Gal onto glucosyl Cer ( van Die et al., 1999; Sato et al., 2000 ). As soluble enzymes, β4GalT-V and -VI recognize terminal GlcNAc residues instead of glucose. Therefore, it can be speculated that these enzymes require a cofactor similar to GABPI, which mediates glycolipid acceptor recognition. Orthologues of GABPI are found in arthropod and vertebrate species but not in nematodes, indicating that GABPI homologues might play a role in higher eukaryotes as well. In summary, it can be concluded that the identification of GABPI reveals a novel mechanism to generate specificity in the complex glycosylation pathway.

Materials and methods

Expression cloning
A cDNA library from Drosophila larval poly(A)+ RNA ( Clontech Laboratories, Inc. ) was constructed in pCMV-Script using the pCMV-Script XR cDNA library construction kit ( Agilent Technologies ). The library was divided into pools of 10,000 independent clones and used for expression cloning after the sibling selection strategy described previously ( Bakker et al., 1997, 2005 ). The CHO cell line Lec8 ( Deutscher and Hirschberg, 1986 ) grown in α-MEM supplemented with 10% FCS ( both obtained from Biochrom AG ) was used as the host. Pools or clones of the cDNA library were transfected into Lec8 cells using Metafectane ( Biontex ). After 2 d, cells grown in 6-well plates were fixed with 1.5% glutaraldehyde, incubated with the anti-lactadilNac monoclonal antibody 259-2A1 ( van Romenoorte et al., 2000 ) followed by HRP-conjugated goat anti-mouse antibody ( Jackson Immuno-Research Laboratories ), and detected by tyramide signal amplification using biotin-tyramide ( Speel et al., 2006 ), streptavidin-AP ( Invitrogen ), and Fast-Red ( Sigma-Aldrich ) as chromogenic substrate.

Plasmid constructs
All tagged mammalian expression constructs were made in pcDNA3 ( Invitrogen ). Myc-GABPI ( flybase gene number CG17257 ) contains an N-terminal Myc tag ( MAQKIKSEEDINLPFE { antibody-bound sequence underlined } ) and Myc-GABPI-HA, an additional C-terminal HA tag ( SRYPDVDYPDASYL ). Flag-β4GalNAcTB ( CG14517 ), Flag-β4GalNAcTA ( CG8536 ), and C. elegans GalNAcT ( Kawar et al., 2002 ) contain N-terminal Flag tags ( MDYKDDDDKQGS ). The Myc-GABPI-KTN construct was cloned by PCR using Myc-GABPI as a template. For expression in Drosophila S2 cells, Flag-β4GalHA ( A and HA ) and Flag-β4GalNAcTB ( N-terminal HA tag; MYPYDVPDYAGS ) were cloned in pB/V5-His ( Invitrogen ). Hybrids of β4GalNAcTA and β4GalNAcTB are identified by a three-letter code, whereby the first letter indicates the cytoplasmic plus transmembrane region, the second letter indicates the stem region, and the third letter indicates the catalytic domain ( e.g., A-B-B ). Borders between the three regions are after amino acids 29 and 133 in β4GalNAcTA and after 33 and 65 in β4GalNAcTB. Flag- or Myc-tagged constructs were used for all experiments unless indicated.
Transiently transfected HEK293 cells were grown in DME/HAM's F-12 supplemented with 10% FCS (both obtained from Biochrom AG). Cells transiently transfected as described in the Expression cloning section for CHO cells were washed with PBS and collected by centrifugation (5 min at 1,500 g). The cell pellets from three 175-cm² plates (9 x 10⁷ cells) were resuspended in 7 ml of lysis buffer (10 mM Hepes-Tris, pH 7.4, 0.8 M sorbitol, and 1 mM EDTA) containing an EDTA-free protease inhibitor mixture (Roche). After 10 strokes in a Dounce homogenizer, the lysate was centrifuged (10 min at 1,500 g). The supernatant was collected, and the pellet was subjected to a second homogenization/centrifugation round. The ER/Golgi-rich fraction was obtained by centrifugation of the combined supernatants at 100,000 g for 1 h. Pelleted vesicles were resuspended in 500 μl of assay buffer (0.1 M MOPS, pH 7.5) and 20% glycerol kept at -80°C. Protein concentrations were determined using a bicinchoninic acid kit (Thermo Fisher Scientific).

**In vitro [4GalNAc] assays**

Standard enzyme assays were performed with 20 μl of the ER/Golgi preparations in 50 μl of assay buffer (0.1 MOPS, pH 7.5, 20 mM MnCl₂, 10 mM ATP, 100 mM GalNAc, 0.1% BSA, and 0.01% saponin). Therefore, 20-μl aliquots of the ER/Golgi vesicle preparation were supplemented to obtain the appropriate buffer composition and 0.5 mM of the radio-labeled nucleotide sugars UDP-[³⁵S]Gal (specific activity of 25 Bq/nmol; GE Healthcare) or UDP-[³H]GalNAc (specific activity of 36 Bq/nmol; PerkinElmer) diluted with cold nucleotide sugars [Sigma-Aldrich]. Reactions were started by adding the acceptor substrate Glc[Nac-pNp (Sigma-Aldrich) at 1 mM and were incubated for 2 h at 28°C. Control samples were incubated in the absence of Glc[Nac-pNp and subtracted from the reaction. Reactions were quenched by adding 1 ml of a 1:10 dilution of ice-cold water, and products were isolated on columns (Sep Pak Plus C₁₈ Waters Corporation) as described previously (Palic et al., 1988). The elutes were dried and counted in 2 ml of scintillation cocktail (Luma Safe Plus; Lumac LSC). Incorporated radioactivity was measured in a counter (LS 6500; Beckman Coulter).

Analyses of glycosphingolipids and proteins from transfected HEK293 cells

Transiently transfected HEK293 cells were washed with PBS, scraped off the plate, and collected by centrifugation (10 min at 1,500 g). Drosophila S2 cells were harvested by centrifugation and extracted in the same way. The cell pellets (10⁷ cells) were resuspended in 300 μl of water and sonicated for 5 min in a bath sonicator. 2-propanol and hexane were added to obtain a solvent ratio of 55:25:20 (2-propanol/hexane/water) and the mixture was sonicated again for 5 min. Samples were centrifuged for 10 min at 1,500 g, and supernatants were dried under nitrogen. The extracts were resuspended in chloroform/methanol/water [3:47:48] and desalted by reverse-phase chromatography (Sep Pak Plus C₁₈ columns; Williams and McCluer, 1980). The eluted glycosphingolipids were dried under nitrogen, and one fourth of each sample was spotted onto a TLC plate (Merck, Darmstadt, Germany). Seeblie and Thompson developed in running solvent composed of chloroform/methanol/0.25% aqueous KCl [3:4:1]. For immunostaining, the silica plate was fixed in 0.1% polyisobutylmethacrylate (Sigma-Aldrich) in acetone. The plate was blocked overnight with 1% BSA in TBS at 4°C followed by incubation with primary antibody (mouse anti-LacNAc 259-2A1) for 2 h at room temperature and with secondary antibody goat anti-mouse IRDye 800 (LI-COR Biosciences) for 30 min. After washing, the plate was analyzed on an infrared imaging system (Odyssey; LI-COR Biosciences).

Protein samples for Western blotting were isolated from the same cells by dissolving 10⁷ cells in 750 μl of lysis buffer (2 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, and 1% NP-40 supplemented with a protease inhibitor mixture). After centrifugation (300 g for 3 min) and washing twice with 50 mM Tris-HCl, pH 8.0, and 1% NP-40, the proteins were separated in SDS-PAGE, transferred to nitrocellulose membranes (Biochrom AG). For RNAi knockdown experiments (Clemens et al., 2000), 10⁶ cells were plated per 6 wells in serum-free medium, and RNAi treatment was repeated with a concentration of 18.5 nM dsRNA. 2 d after transfection, cells were transferred to 2 ml of Schneider's medium containing FCS and 4 μg/ml of the target dsRNA. For RNAi knockdown experiments (Clemens et al., 2000), 10⁶ cells were plated per 6 wells in serum-free medium, and RNAi treatment was repeated with a concentration of 18.5 nM dsRNA. 2 d after transfection, cells were transferred to concanavalin A–coated coverslips for 1 h, fixed in 4% PFA, and further processed as described in the previous section for HEK293 cells except that 0.1% saponin was kept in all incubation and washing solutions. Mouse anti-Flag M5 (Sigma-Aldrich) in combination with rabbit anti-HA (Sigma-Aldrich) was used to visualize the targeted protein. In addition, whereas the ER was stained with mouse anti-HDEL (Santa Cruz Biotechnology, Inc.). Secondary antibodies used were goat anti-mouse–Alexa Fluor 488, and goat anti-rabbit–Cy3. Fluorescent images were made using a microscope (Axiovert 200M) while using the aforementioned zwitterionic glycosphingolipid extracts prepared as described in Analyses of glycosphingolipids and proteins from transfected HEK293 cells for s2 cells before and after dsRNA treatment were analyzed by MALDI-TOF-MS in a TOF/TOF mass spectrometer (Ultraflex II; Bruker Daltonics) as described previously (Wurher and Deelder, 2005; Stolz et al., 2008).

**Immunoprecipitation**

Transiently transfected HEK293 cells were lysed for 30 min at 4°C using 750 μl of lysis buffer (2 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, and 1% NP-40 supplemented with protease inhibitor mixture). After centrifugation for 30 min at 12,000 g, anti-HA antibody 12CA5 coupled to Sepharose A beads was added to supernatants and incubated for 3 h at 4°C on a rotating wheel. Immunocomplexes were pelleted by centrifugation (12,000 g for 3 min) and washed twice with 50 mM Tris-HCl, pH 8.0, and 1% NP-40, twice with 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 1% NP-40, and once with the first washing buffer. Immunoprecipitated proteins were separated in SDS-PAGE, blotted onto polyvinylidene difluoride membranes (Waters Corporation), and stained with mouse anti-Flag M5 or rat anti-HA antibody.

**Online supplemental material**

Table S1 shows newly registered zwitterionic glycosphingolipid species. Fig. S1 shows negative-mode MALDI-TOF-MS of S2 cell dsRNA.
Fig. S2 shows MALDI-TOF/TOF-MS fragmentation analysis of two zwitterionic glycolipid species containing lactoNAC tandem repeats. Fig. S3 shows MALDI-TOF/TOF-MS analysis of two zwitterionic glycolipid species. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801071/DC1.

We would like to thank Drs. Françoise Router and Birgit Lütig for critical reading of the manuscript. Supporting financial resources for this study were obtained from the Hannover Medical School bonus system Leistungsorientierte Mittel and Regenerative Biology to Reconstructive Therapy, a Cluster of Excellence financed by the Deutsche Forschungsgemeinschaft.

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Glycobiology shows MALDI-TOF/TOF-MS analysis of two zwitterionic glycolipid species containing lactoNAC tandem repeats. Fig. S3 shows MALDI-TOF/TOF-MS analysis of two zwitterionic glycolipid species. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801071/DC1.

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Figure S1. **Negative-mode MALDI-TOF-MS of S2 cell glycosphingolipids.** Glycosphingolipid extracts of nondepleted S2 cells were analyzed by negative-ion mode MALDI-TOF-MS in the reflectron mode using 2,5-dihydroxybenzoic acid as matrix for sample preparation. A list of newly registered masses and assigned glycosphingolipid structures compared with known GSL structures in *Drosophila* is given in Table S1. b, glycosphingolipid with a Cer composition of C14:1 tetradecasphingenine and C20:0 arachidic acid; a and c, glycosphingolipid with Cer masses that are 28 D (two methylene groups, \(\text{C}_2\text{H}_4\)) lower (a) or higher (c) than for b. Blue circles, glucose; green circles, mannose; yellow circles, galactose; yellow squares, GalNAc; blue squares, GlcNAc; white square, N-acetylhexosamine; PE, phosphoethanolamine; m/z, mass/charge.
Figure S2. MALDI-TOF/TOF-MS fragmentation analysis of two zwitterionic glycolipid species containing LacdiNAc tandem repeats. (A and B) Zwitterionic glycosphingolipid species with a hexasaccharide glycan moiety (mass/charge = 1,796) with one phosphoethanolamine (PE) modification (A) and the hexasaccharide glycan moiety (mass/charge [m/z] = 1,919) containing two phosphoethanolamine residues (B) were predicted to contain two hexose and four N-acetylhexosamine residues. Fragmentation patterns by MALDI-TOF/TOF-MS (in deprotonated form using 2,5-dihydroxybenzoic acid as matrix) confirmed the presence of a tetra N-acetylhexosamine sequence with one or two phosphoethanolamine modifications, which can only be interpreted as a repeat of two LacdiNAc units. Blue circles, glucose; green circles, mannose; blue squares, GlcNAc.
Figure S3. MALDI-TOF/TOF-MS analysis of two zwitterionic glycolipid species. (A and B) Zwitterionic glycosphingolipid species with five N-acetylhexosamine residues (mass/charge [m/z] = 2,027; A) and six N-acetylhexosamine sugars (mass/charge = 2,487; B) were analyzed by MALDI-TOF/TOF-MS in a deprotonated form using 2,5-dihydroxybenzoic acid as a matrix. Fragmentation patterns confirmed the presence of an additional terminal N-acetylhexosamine residue compared with known structures present in Drosophila, probably a GalNAc residue in α-1,4 linkage in analogy to other Drosophila zwitterionic glycosphingolipid structures. Blue circles, glucose; green circles, mannose; yellow circles, galactose; yellow squares, GalNAc; blue squares, GlcNAc; white square, N-acetylhexosamine; PE, phosphoethanolamine.
**Table S1. Newly registered zwitterionic glycosphingolipid species**

<table>
<thead>
<tr>
<th>Zwitterionic GSL</th>
<th>Proposed structure</th>
<th>Registered mass</th>
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<tbody>
<tr>
<td>Nz6*</td>
<td>GalNAcβ,4(PE-6)GlcNAcβ,3GalNAcβ,4GlcNAcβ,3Manβ,4GlcβCer, GalNAcβ,4GlcNAcβ,3GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer</td>
<td>1,796.3/1,824.4*</td>
</tr>
<tr>
<td>Nz,6*</td>
<td>GalNAcβ,4(PE-6)GlcNAcβ,3GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer</td>
<td>1,919.4</td>
</tr>
<tr>
<td>Nz7*</td>
<td>(HexNAc1-)GalNAcβ,4GlcNAcβ,3GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer</td>
<td>1,999.5/2,027.5*</td>
</tr>
<tr>
<td>Nz,7*</td>
<td>(HexNAc1-)GalNAcβ,4(PE-6)GlcNAcβ,3GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer</td>
<td>2,122.5</td>
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<tr>
<td>Nz9*</td>
<td>(HexNAc1-)GalNAcβ,4(PE-6)GlcNAcβ,3Galβ,3GalNAcα,4GalNAcβ,4PE6 GlcNAcβ,3Manβ,4GlcβCer</td>
<td>2,364.7</td>
</tr>
<tr>
<td>Nz,9*</td>
<td>(HexNAc1-)GalNAcβ,4(PE-6)GlcNAcβ,3Galβ,3GalNAcα,4GolNAcβ,4(PE6) GlcNAcβ,3Manβ,4GlcβCer</td>
<td>2,487.7</td>
</tr>
</tbody>
</table>

GSL, glycosphingolipid. Zwitterionic glycosphingolipids were registered as [M–H]²⁻ species and are alternative structures to those described by Seppo et al. (2000). *Glycosphingolipid with a Cer species with a 28-D higher mass, which reflects two additional methylene groups in the Cer moiety.*

**References**