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RESEARCH ARTICLE

"Add-on" domains of *Drosophila* β1,4-*N*-acetylgalactosaminyltransferase B in the stem region and its pilot protein

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Abstract The glycolipid specific *Drosophila melanogaster* β1,4-*N*-acetylgalactosaminyltransferase B (β4GalNAcTB) depends on a zinc finger DHHC protein family member named GalNAcTB pilot (GABPI) for activity and translocation to the Golgi. The six-membrane spanning protein actually lacks the cysteine in the cytoplasmic DHHC motif, displaying DHHS instead. Here we show that the whole conserved region around the DHHS sequence, which is essential for palmitoylation in DHHC proteins, is not required for GABPI to interact with β4GalNAcTB. In contrast, the two luminal loops between transmembrane domain 3–4 and 5–6 contain conserved amino acids, which are crucial for activity. Besides the dependence on GABPI, β4GalNAcTB requires its exceptional short stem region for activity. A few hydrophobic amino acids positioned close to the transmembrane domain are essential for the interaction with GABPI. Along with its catalytic domain, β4GalNAcTB, thus, requires an area in its own stem region and two small luminal loops of GABPI as "add-on" domains. Moreover, some inactive GABPI mutants could be rescued by fusion with β4GalNAcTB, indicating their importance in direct GABPI-β4GalNAcTB interaction.

Keywords DHHC · GABPI · Glycolipid · Glycosyltransferase

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Introduction

Little is known about what motivates Golgi localized glycosyltransferases to act on glycolipid acceptors. Several glycosyltransferases have been observed to require membrane anchoring to act on glycolipids [1, 30, 34]. We have recently reported that this is also the case for *Drosophila melanogaster* β1,4-*N*-acetylgalactosaminyltransferase B (β4GalNAcTB), which, in addition, requires a second protein named GalNAcTB pilot (GABPI) for activity. Without GABPI, β4GalNAcTB remains in the endoplasmic reticulum (ER) in an inactive state and upon co-expression resides as a complex with GABPI in the Golgi [11]. β4GalNAcTB acts exclusively on glycolipids, whereas a second, homologous *Drosophila* enzyme, β4GalNAcTA, also shows activity on glycoprotein acceptors and works independently from GABPI or its transmembrane domain [9, 11, 28]. Although both enzymes are able to generate the lacdiNAc (GalNAcβ1,4GlcNAc) glycotope on glycolipids, β4GalNAcTB does this, in the presence of GABPI, more efficiently than β4GalNAcTA. Also, mutant flies have revealed that β4GalNAcTB is the predominant enzyme for glycolipid biosynthesis [35]. Both enzymes show the typical type II membrane topology for Golgi glycosyltransferases, with a short N-terminal cytoplasmic domain and a stem region between transmembrane and C-terminal catalytic domain [36]. The most obvious difference between the two GalNAc-transferases is the

length of the stem region. β4GalNAcTB has an extraordinary short stem region and is inactive when it is exchanged for the stem of β4GalNAcTA.

GABPI is a member of the DHHC protein family. This family consists of multi-transmembrane proteins that present a variant of the zinc finger domain [22] in a cytoplasmic loop. It is defined by the eponymous Asp-His-His-Cys (DHHC) motif, embedded in a cysteine-rich domain. Characterized DHHC protein family members have been shown to be palmitoyltransferases, acting on cysteine residues in the cytoplasmic part of proteins (S-palmitoylation) [15]. Mutations in the DHHC amino acid sequence, especially replacement of the cysteine residue, abolish protein acyltransferase activity [6, 8, 16, 17, 27, 32], but DHHC proteins can have functions independent of the DHHC domain [4]. The DHHC sequence and the cysteine-rich domain are conserved throughout eukaryotes and multiple members of the DHHC protein family exist in each species [3, 18]. Although clearly a member of the DHHC protein family, GABPI shows a unique alteration of the eponymous DHHC motif to DHHS. It is striking that the DHHC motif, on the one hand, is highly conserved (all 23 human and all other 16 *Drosophila* family members show the DHHC sequence), but is, on the other hand, in most of GABPI's insect orthologs mutated in various ways. Since the motif itself has been shown to be crucial for palmitoyltransferase function, such activity was rather unlikely for GABPI. The lack of cysteines in the cytoplasmic domain of β4GalNAcTB strengthened the view that GABPI does not act as a palmitoyltransferase for β4GalNAcTB [11]. GABPI can be considered an "add-on" protein for a glycosyltransferase. An "add-on" domain has been defined as a protein or part of a protein outside of the catalytic domain of a glycosyltransferase that is required for activity [23]. This can be on a separate protein, like for α-lactalbumin, which modifies the acceptor specificity of β4galactosyltransferase [26], or on the same protein, for example a lectin-binding domain in α-GalNAc transferases [7, 13]. To narrow down the regions of GABPI that are directly involved in interaction with β4GalNAcTB, we have now resolved the membrane topology of GABPI and have generated truncations and point mutations of GABPI and β4GalNAcTB. Different properties of mutants revealed distinct functions of "add on" domains in GABPI and the stem of β4GalNAcTB.

Experimental procedures

Generation of N- and C-terminally truncated proteins

N-terminal deletion constructs were amplified from Myc-GABPI [11]. Sense and antisense primers were flanked by XhoI and XbaI sites, respectively. PCR products were ligated into XhoI/XbaI sites of the expression vector pcDNA3 containing an N-terminal Myc-tag (MAQKLISE EDLNLRPLE) and a C-terminal HA-tag (SRYPYDV PDYASL).

Generation of proteins containing internal HA-tags and point mutations

Constructs encoding GABPI with internal HA-tags were generated by PCR. The construct Myc-GABPI-HA was used as template for the amplification. PCR products were ligated with each other via the AatII cleavage site within the HA-tag and cloned into the XhoI/XbaI sites of the expression vector pcDNA3 containing an N-terminal Myc-tag. Single point-mutations were generated by overlap extension PCR [10] on the original full-length constructs of *Drosophila* Myc-GABPI-HA and Flag- β4GalNAcTB.

Generation of GABPI- β4GalNAcTB fusion proteins

Fusion proteins of GABPI and β4GalNAcTB, and mutants thereof, were generated by XhoI/BamHI-restriction of GABPI and BamHI/XbaI-restriction of β4GalNAcTB. Products were ligated into XhoI/XbaI sites of the expression vector pcDNA3 containing an N-terminal Myc-tag.

Subcellular localization and topology studies by immunofluorescence

Subcellular localizations of recombinant Myc-GABPI-HA, truncated constructs, and Myc-GABPI containing internal HA-tags were carried out in HEK293 cells [5]. Therefore, cells were transfected in six-well plates using Metafectane (Biontex, Munich, Germany). After 2 days, the cells were resuspended and transferred into a 75-cm^2 flask. The cells were cultured for 3 weeks in the presence of G-418 (Calbiochem). For staining, cells were seeded onto glass coverslips, fixed in 4% PFA, and permeabilized for 30 min with 0.1% saponin in PBS containing 0.1% BSA or for 15 min at RT with digitonin solution (5 µg/ml digitonin, 0.3 M Sucrose, 0.1 M KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 10 mM HEPES, pH 6.9). After digitonin incubation, the cells were washed three times with PBS and incubated with blocking solution (0.1% BSA in PBS) for 30 min at RT. Samples were incubated with the respective primary antibodies (anti-HA tag 3F10, or anti-Myc tag 9E10, and rabbit anti-giantin (Covance) or anti-calnexin (Acris) as Golgi or ER marker) for 1.5 h at room temperature. After three washing steps (PBS, 0.1% BSA, 0.1% Tween 20), the cells were incubated with anti-mouse IgCy3 and anti-rabbit Ig Alexa 488 for 1 h at room temperature. After staining with the nuclear dye Hoechst 33258 (Hoechst Pharmaceuticals),

the slides were washed with water, mounted (DakoCytomation), and analyzed under a Carl Zeiss Axiovert 200M inverted microscope.

Immunocytochemistry staining

In vivo activity tests were carried out with transiently transfected HEK293 cells. Therefore, cells were transfected using Metafectane (Biontex, Munich, Germany). After 2 days, the cells grown in six-well plates were fixed with 1.5% glutaraldehyde, incubated with the anti-lacdiNAc monoclonal antibody 259-2A1 [37], followed by HRP-conjugated goat-anti-mouse (Jackson Immuno Research) and detected by tyramide signal amplification using biotin–tyramide [33], streptavidin-AP (Caltag), and Fast-Red (Sigma) as the chromogenic substrate.

Results

Topology of GABPI in the Golgi membrane

Topology analysis of GABPI using TopPred II [2] proposed a topology model of six transmembrane domains (TMDs), predicting the DHHS motif on the cytoplasmic face of the membrane. To investigate and confirm the membrane topology of GABPI, an epitope-insertion approach was used. Therefore, the full-length GABPI protein with an amino-terminal Myc and carboxy-terminal HA-tag was generated (Myc-GABPI-HA) and expressed in HEK293 cells. Additionally, based on the topology prediction, truncation constructs, in which GABPI was shortened from the N-terminal site between the TMDs, were produced (Fig. 1, 1–4).

Immunofluorescence was used to map the orientation of introduced tags. Transfected cells were treated with either the detergent saponin (Fig. 1, 1–4 A and 1–4 C) to permeabilize all cellular membranes [31] or digitonin (Fig. 1, 1–4 B and 1–4 D), which is only able to perforate the plasma membrane [12] and therefore only allows the detection of cytoplasmic tags. These experiments revealed that the C-terminus as well as the N-terminus was cytoplasmic (Fig. 1, 1 A–D). A truncation construct lacking the first 30 amino acids in front of the first predicted TMD (Myc-NΔ90GABPI-HA) remained detectable with saponin and digitonin (Fig. 1, 2 A–D), whereas the truncation of the first N-terminal TMD (Myc-NΔ117GABPI-HA) was exclusively visible in saponin-treated cells (Fig. 1, 3 A–D), indicating a lumenal orientation of the N-terminus of Δ117 in the Golgi. By further deletion of the second TMD, the Myc-tag of the construct (Myc-NΔ141GABPI-HA) proved to be cytoplasmically oriented (Fig. 1, 4 A and B).

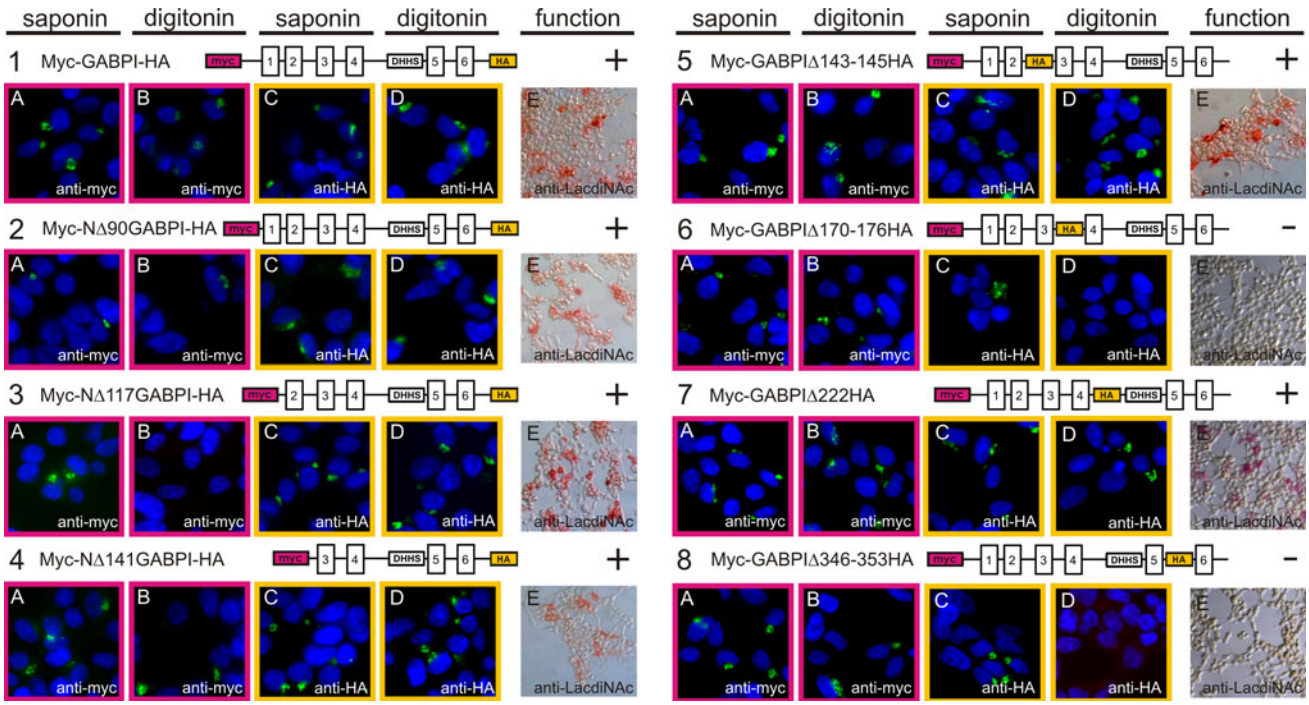

Fig. 1 The Myc-GABPI-HA truncation (**1–4**) and internal HA (**5–8**) constructs were expressed in HEK293 cells and stained with anti-Myc (*A* and *B*) or anti-HA antibodies (*C* and *D*) after either Saponin (*A* and *C*) or Digitonin treatment (*B* and *D*; tag is only visible, if positioned in the cytoplasm). The nuclei were stained by Hoechst 33258 (*blue*). Functionality of constructs, as revealed by cell surface staining of lacdiNAc after co-expression with β4GalNAcTB (*E*), is indicated by + and −

To investigate the orientation of predicted loop regions, GABPI constructs containing internal HA-tags and N-terminal Myc-tags were generated (Fig. 1, 5–8). As described above, the tags were detected immunologically in transfected HEK293 cells. Internal HA-tags in the predicted loops 3–4 and 5–6 were shown to be lumenally oriented (Fig. 1, 6 A–D and 8 A–D). In contrast, the HA-tags in predicted loop regions 2–3 and 4–5 close to the DHHS motif demonstrated cytoplasmic orientation of these parts (Fig. 1, 5 A–D and 7 A–D).

Taken together, all immunofluorescence experiments confirmed the predicted transmembrane topology model of GABPI. The protein consists of six transmembrane regions with three lumenal and two cytoplasmic loops. The amino- and carboxyterminal end, as well as the DHHS motif, is localized in the cytoplasm (see also Fig. 2).

GABPI requires four TMDs as minimal functional entity

To determine the minimal functional entity of GABPI, the generated deletion constructs (Fig. 1, 1–4) and additional N- and C-terminal deletion constructs were tested for their ability to activate β4GalNAcTB by immunocytochemical staining against the product of β4GalNAcTB, the GalNAcβ1,4-GlcNAc or lacdiNAc glycotope by antibody 259-2A1 [37] on the cell surface of transfected HEK293 cells. All deletion constructs containing not less than the last 4 TMDs remained fully functional, whereas further deletions from either side destroyed the ability to activate β4GalNAcTB. The four TMDs also need to be continuous. Expression of TMDs 3 + 4 and TMDs 5 + 6 separated in different proteins and co-expressed in HEK293 cells did not restore activity.

Lumenal loop regions are insect-conserved and essential for GABPI-β4GalNAcTB interaction

Having determined the membrane orientation and minimal active domain of GABPI, we continued to mutate specific domains. Functional tests of the internal HA-tagged constructs demonstrated that insertions of HA-tags between TMDs 3 and 4, as well as between TMDs 5 and 6 resulted in proteins that were not able to activate β4GalNAcTB any longer. In contrast, the insertion of an HA-tag in the DHHS loop region did not affect functionality. A sequence alignment of these luminal loop regions of GABPI and related insect DHHC proteins showed high similarity. In contrast, the human ortholog zDHHC23 is significantly different, especially in loop 5–6, which is shorter than GABPI's. For detailed functional analyses addressing the loop regions of GABPI, insect-conserved amino acids, which differ from the human ortholog (Fig. 2), were selected for site-directed mutagenesis and constructs were

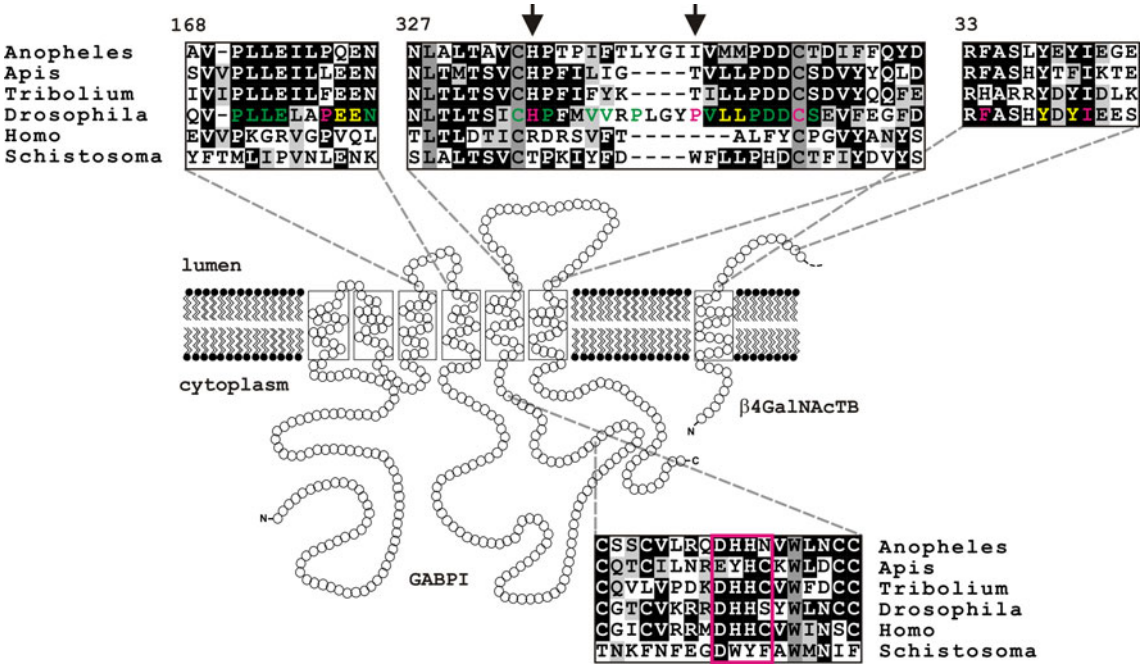

Fig. 2 Alignment of GABPI orthologs and human zDHHC23 (loop regions 3/4 and 5/6 as well as the DHHC motif) and homologs of β4GalNAcTB (stem region near the TMD). For the *Drosophila* proteins, the effect of mutation is indicated by various colors: *green* no effect, *magenta* abolished activity, *yellow* abolished activity only, if neighboring amino acid is also mutated. The *arrows* mark the amino acids, whose effect on activity can be rescued by fusion of GABPI and β4GalNAcTB, *numbers* above alignments refer to *Drosophila* GABPI or β4GalNAcTB

Table 1 Impact of point mutations in conserved GABPI loop regions 3–4 and 5–6

Construct	Activity	Subcellular localization	β4GalNAcTB-piloting
GABPI loop 3–4 mutations			
P170A	+	Golgi	+
LL171/172SS	+	Golgi	+
LL171/172AA	+	Golgi	+
E173A	+	Golgi	+
L174S	+	Golgi	+
P176A	−	ER	−
E177A	+	Golgi	+
E178A	+	Golgi	+
EE177/178AA	−	Golgi	−
EE177/178 fusion	−	*Golgi*	+
N179A	+	Golgi	+
GABPI loop 5–6 mutations			
C334A	+	Golgi	+
H335A	−	Golgi	−
H335A fusion	+	*Golgi*	+
P336A	+	Golgi	+
V339S	+	Golgi	+
V340S	+	Golgi	+
VV339/340SS	+	Golgi	+
P342A	+	Golgi	+
P346A	−	Golgi	−
P346A fusion	+	*Golgi*	+
V347A	+	Golgi	+
L348S	+	Golgi	+
L349S	+	Golgi	+
LL348/349SS	−	Golgi	−
LL348/349AA	−	Golgi	−
LL348/349AA fusion	−	*Golgi*	+
P350A	+	Golgi	+
DD351/352AA	+	Golgi	+
C353A	−	Golgi	−
C353A fusion	−	*Golgi*	+
S354A	+	Golgi	+

Activity was tested by cell surface staining with the mAb 259-2A1. ER or Golgi localization of GABPI and piloting of β4GalNAcTB was scored using antibodies against respective Myc and Flag tags. The fusion constructs of GABPI and β4GalNAcTB are italised

tested for functionality by cell surface staining, using the anti lacdiNAc antibody, and for their ability to pilot β4GalNAcTB to the Golgi, as detected by immunofluorescence (Table 1). Additionally, localization studies of GABPI itself were performed to distinguish properly Golgi localized from misfolded ER localized proteins and therefore malfunctioning proteins. All point-mutated constructs, with the exception of the P176A mutation, were still able to reach the Golgi (Table 1). Several of the mutated amino acids in the luminal loops 3–4 and 5–6 of GABPI comprise β4GalNAcTB activation. In contrast, mutations of any amino acid of the DHHS motif, as well as depletion of the whole cysteine-rich domain did not affect functionality. This confirmed that this sequence, unlike for other DHHC family members, has no relevance for this specific GABPI function [11]. All inactive mutants, as revealed by cell surface staining for the produced lacdiNAc epitope, where also not able to pilot β4GalNAcTB to the Golgi (Table 1).

Some defective GABPI mutants can be rescued by direct fusion to β4GalNAcTB

When GABPI is directly C-terminally fused to β4GalNAcTB, it forms an active complex, which remains, like the separately expressed proteins, sensitive to high detergent concentrations in in vitro assays (data not shown). The fusion protein reaches the Golgi apparatus (Fig. 3A) and is able to carry out lacdiNAc synthesis. However, when this protein was co-expressed with a separate β4GalNAcTB construct, it was no longer able to pilot it to the Golgi (Fig. 3B). Thus, GABPI can interact with the fused β4GalNAcTB, but is then occupied and cannot bind a second transferase.

Defective GABPI constructs were fused to β4GalNAcTB as well to estimate if the mutant's defect could be overcome by forcing the two proteins together. The functionality of the H335A and P346A mutants could be rescued by fusion to β4GalNAcTB, whereas other defective mutants remained inactive after fusion (Fig. 4). This indicated that histidine 335 and proline 346 are essential for GABPI-β4GalNAcTB binding but not required for activity if the two proteins are forced together. The other GABPI mutants, which remained inactive as fusion protein, were still able to reach the Golgi, indicating that delivery of β4GalNAcTB to the Golgi by itself does not lead to an active enzyme but that GABPI is in addition required for activity.

A hydrophobic area in the stem of β4GalNAcTB is crucial for GABPI interaction

Previous experiments on β4GalNAcTB had revealed the importance of the luminal stem region for GABPI interaction. Hybrid proteins of β4GalNAcTA, the GABPI independent enzyme, and β4GalNAcTB were autonomously active as long as they carried the catalytic domain of β4GalNAcTA. As soon as they possessed the catalytic domain of β4GalNAcTB, however, they only showed activity if co-expressed with GABPI. In addition, β4GalNAcTB required its own stem region, but not its own

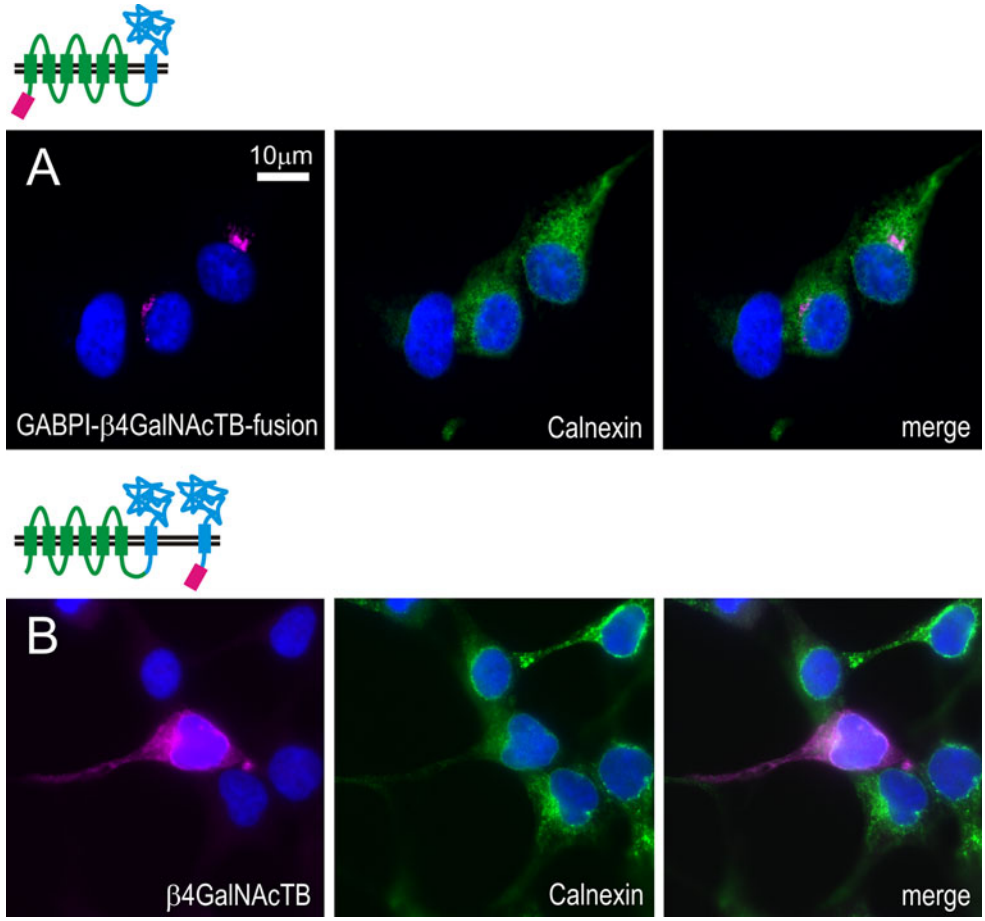

Fig. 3 The fusion protein GABPI-β4GalNAcTB reaches the Golgi (**a**), whereas β4GalNAcTB co-expressed with GABPI-β4GalNAcTB is still retained in the ER (**b**). The proteins were expressed in HEK293 cells and visualized by indirect immunostaining (*magenta*) of Myc (**a**) or Flag (**b**) tags. The ER was stained with anti-Calnexin (*green*). The nuclei were stained by Hoechst 33258 (*blue*)

cytoplasmic and transmembrane domain to be active [11]. A sequence alignment of the stem region with related insect β4GalNAcTs showed high similarity in the area of the stem region close to the transmembrane domain (Fig. 2). Initial indication that this area is important was obtained by generating an additional hybrid of N-terminal β4GalNAcTA and C-terminal β4GalNAcTB. Whereas a hybrid at the border between transmembrane and stem domain showed to be active, the activity was lost when the switch was made ten amino acids further in the stem region. Interestingly, the conserved amino acids in the β4GalNAcTBs are predominantly of hydrophobic character. Mutation of four amino acids at once (F34, Y38, Y40, and I41) to alanine as well as to serine destroyed the activity of the enzyme. All these amino acids were also addressed in single point-mutations to serine. Here, the single mutations F34S and I41S destroyed the activity (Fig. 5, C1 and D1), whereas the single mutations Y38S and Y40S did not. In a double mutation of both tyrosines, the activity again vanished. Single point-mutations F34A and I41A, however, left the enzyme intact (Fig. 5, C3 and D3). Mutation of both tyrosines to the structural more similar phenylalanine also did not harm the activity. After all, the inactive β4GalNAcTB mutants were consequently fused to GABPI, but, whereas activity of some of the GABPI mutants could be restored by generation of a fusion, none of the β4GalNAcTB mutants could be rescued this way (Fig. 5, C2 and D2). However, when the subcellular localization was determined, a striking difference was observed. All inactive β4GalNAcTB mutants were retained in the ER when co-expressed with GABPI, but in a hybrid the F34/Y38/Y40/I41S mutant was localized in the ER, whereas the quadruple mutant to alanine was reaching the Golgi (Fig. 5, A and B). Once more, like for the hybrids of inactive GABPIs, a mutant which had an intact catalytic domain was localized to the Golgi, but showed no activity.

Discussion

Since it was clear that GABPI's influence on β4GalNAcTB could not be explained by a palmitoylation and functional

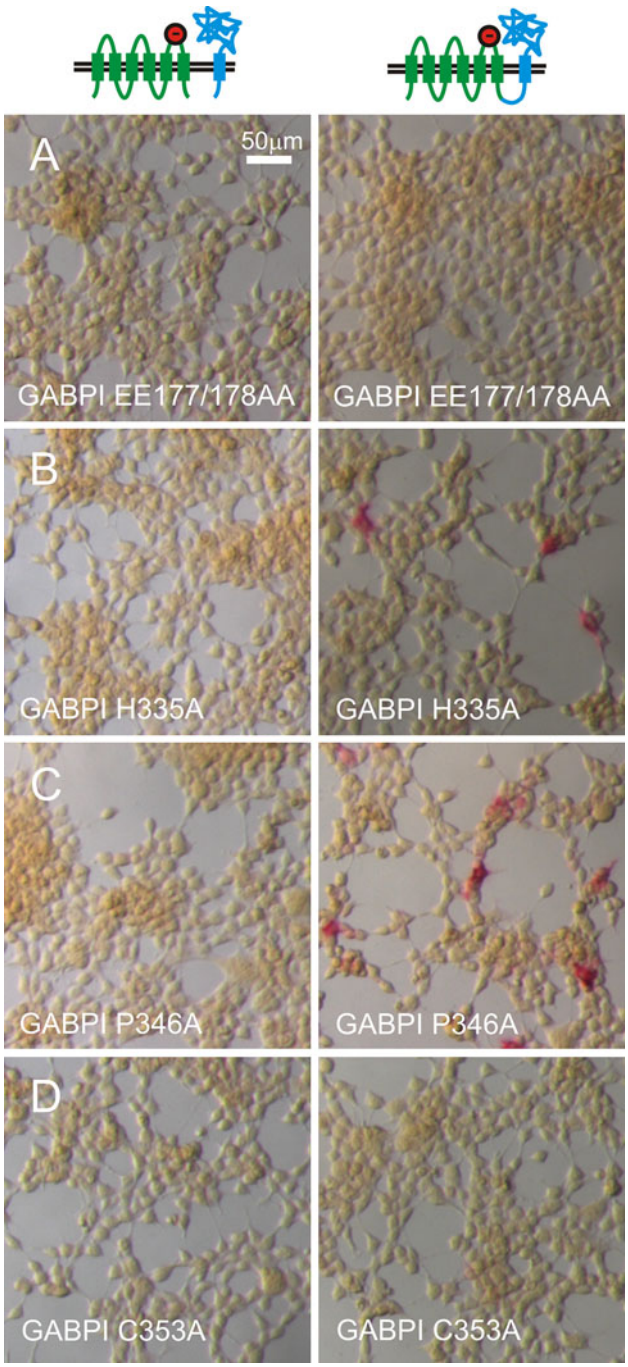

Fig. 4 Rescue of inactive GABPI mutants by generation of hybrid GABPI-β4GalNAcTB. *Left column* GABPI mutants, co-expressed with β4GalNAcTB; *right column* GABPI-β4GalNAcTB fusion proteins. In vivo activity was determined by cell surface staining of the lacdiNAc epitope after transient expression in HEK293 cells

domains of GABPI should rather be positioned on the luminal side of the Golgi membrane, we started resolving the topology of GABPI. The only described topology of a DHHC protein (Akr-1) shows a six-transmembrane domain protein which presents its eponymous motif to the cytoplasm [21]. Although an involvement of GABPI's DHHS-motif in the activation was unlikely, since introduced alterations did not interfere with β4GalNAcTB interaction, a change of orientation could not be excluded without further analysis. However, the orientation of GABPI could clearly be shown to be analogous to Akr-1 and therefore shows no difference to other DHHC proteins. After showing that only transmembrane domains 3–6 were required for interaction with β4GalNAcTB, there remained only two small loops of 12 and 35 amino acids that were exposed to the lumen. We now have shown that these two luminal loops indeed play an essential role in the interaction between β4GalNAcTB and GABPI. Insertion of HA-tags in these regions abolished activity and several point mutations in the loops resulted in an inactive enzyme as well.

Although all eukaryotic species contain numerous DHHC proteins, clear orthologs of GABPI can be identified in most other metazoans. This is zDHHC23 in mammals, for which palmitoylation activity has not yet been established, but which displays an unaltered DHHC motif. This is different in orthologs from insects. Most of them, like *Drosophila* GABPI, have lost the DHHC sequence, but in different ways. Unlike in vertebrates, the two luminal loops, however, are highly conserved (Fig. 2). As all these insects also exhibit an ortholog of β4GalNAcTB, it is most likely that the same function is provided by insect orthologs of GABPI, however, not by orthologs from mammals, which lack β4GalNAcTB. The longer loop between TMD 5 and 6, which shows more conservation than loop 3–4, displays a preserved structure with two cysteines, several prolines and two stretches of hydrophobic amino acids. Exchange of individual amino acids revealed the loop's necessity, but also some redundancy. Beside the dependency on GABPI, we had already observed that β4GalNAcTB required its own stem region to be active [11]. We have shown that an insect-conserved area close to the transmembrane domain contains several essential amino acids. We have mutated three conserved aromatic amino acids and an isoleucine, and could show that they were all relevant for the function of β4GalNAcTB. We could furthermore illustrate that the hydrophobic nature of the amino acids was fundamental. An interesting observation was, however, made when the inactive β4GalNAcTB was fused to GABPI. The quadruple alanine mutant allowed the enzyme to reach the Golgi, whereas the serine mutant remained in the ER. All mutants of GABPI, on the other hand, targeted β4GalNAcTB to the Golgi in the fusion constructs, independently of the generation of an active complex. We thus have obtained three types of mutants. GABPI mutants (H335A and P346A) that could be fully rescued by generation of a GABPI-β4GalNAcTB fusion, GABPI and β4GalNAcTB mutants that remained inactive as fusion constructs, but reached the

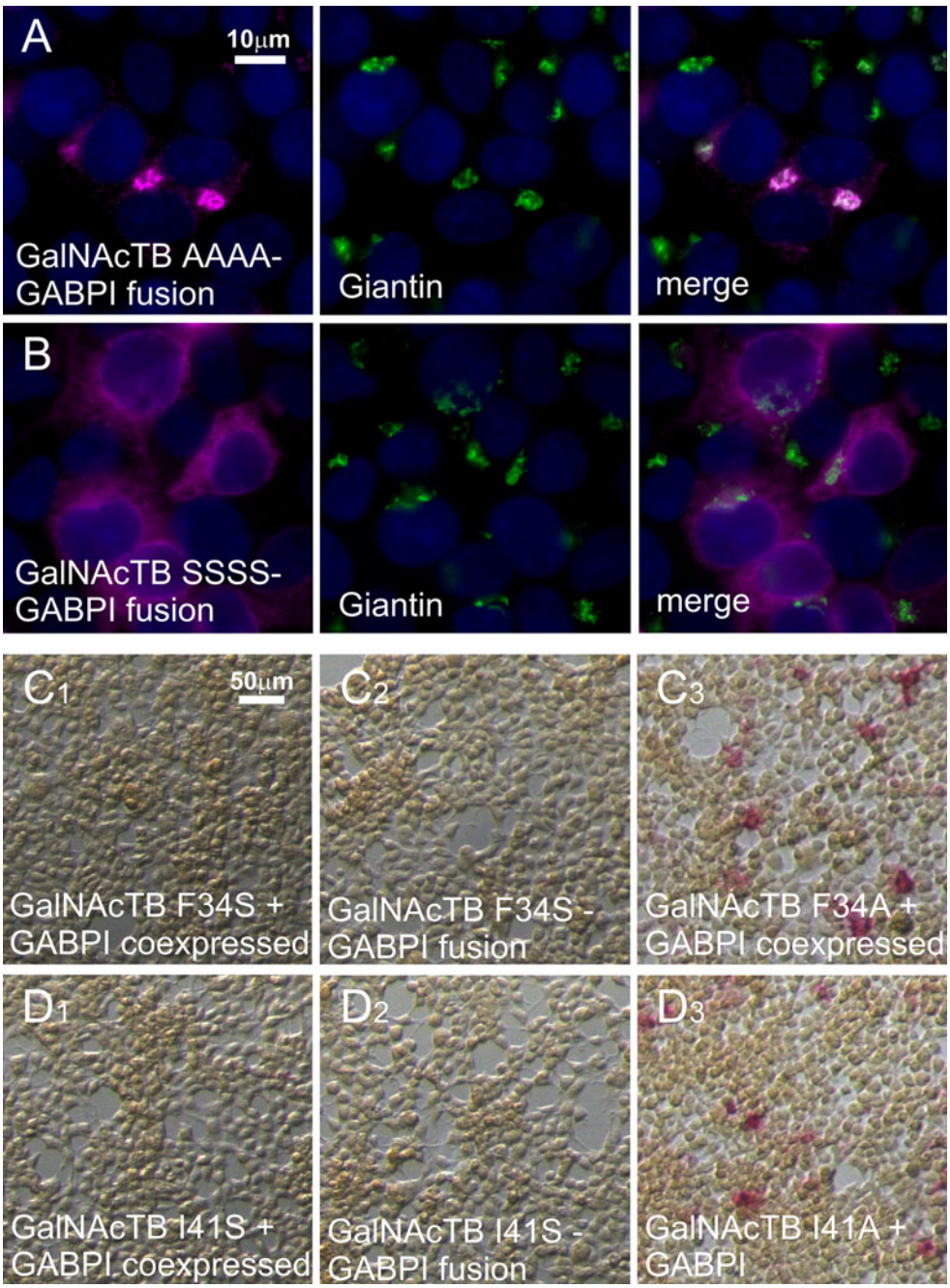

Fig. 5 The GABPI-β4GalNAcTB fusion protein displaying quadruple alanine mutations of β4GalNAcTB F34, Y38, Y40, and I41 reach the Golgi (**a**), whereas the likewise serine mutant is kept in the ER (**b**). The subcellular localization was analyzed in HEK293 cells via the N-terminal Myc-tag (*magenta*). The Golgi apparatus was stained with anti-Giantin (*green*). The nuclei were stained by Hoechst 33258 (*blue*). In vivo activity of point mutations of β4GalNAcTB F34 and I41 mutants. F34S and I41S mutants show no activity (**c1** and **d1**), activity can not be restored by fusion to GABPI (**c2** and **d2**), and F34A and I41A remain active (**c3** and **d3**)

Golgi, and the inactive β4GalNAcTB mutant that remained in the ER as fusion protein. The rescue of mutants by fusion indicates a direct involvement in GABPI-β4GalNAcTB interaction, but not in enzymatic activity. Amino acids addressed in not-rescued mutants might have a role in catalysis or glycolipid recognition. The hydrophobic stem region of β4GalNAcTB must have a function in both.

We have now narrowed down domains on GABPI and in the stem of β4GalNAcTB that are responsible for the joined activity of the two proteins. We have identified them as "add-on" domains, a term coined by Qasba and Ramakrishnan [23] to describe important domains for glycosyltransferase activity outside of the catalytic domain. It was also predicted that additional factors were required

for glycolipid-specific glycosyltransferases [24, 25] Still, the exact way GABPI is involved in activating β4GalNAcTB remains elusive.

Can the situation in *Drosophila* be transferred to other animals? The observation in mammalian glycolipid-specific glycosyltransferases never went further than that, at least for some enzymes, membrane anchorage was required for activity [1, 34] and that one enzyme (β3GalT4) shows a very hydrophobic, putative stem region that differs significantly from homologous enzymes not acting on glycolipids [1]. If a factor like GABPI is required for membrane-linked activity is still not known. There are, however, two mammalian homologs of β4GalNAcTB that can transfer galactose to glucosylceramide [14, 19, 20]. These enzymes are β4GalT5 and -6, and at least β4GalT5 can do this only when membrane bound [29]. Interestingly, β4GalT5 and -6 show a conserved YLF sequence in the stem at the same distance to the membrane as the YDYI sequence in *Drosophila* β4GalNAcTB. Such concentration of large hydrophobic amino acids is not found in β4GalT1 until 4. Similar mechanisms could thus play a role in glycolipid biosynthesis in other species than insects. The ortholog of GABPI in mammals, zDHHC23, is not a likely candidate to play a role in glycolipid biosynthesis. GABPI's ortholog in *Schistosoma*, however, lacks almost the complete DHHC-motif and the surrounding cysteine-rich domain (Fig. 2). It nevertheless shows the characteristic conserved luminal loops in a predicted four TMD topology and was not found until we searched for homologous proteins, specifically considering loop 5–6. Complex glycolipids are made by *Schistosoma* in which potential homologs of β4GalNAcTB are involved and thus a similar mechanism as in *Drosophila* could generate specificity for glycolipids in Trematodes.

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