



Review

The expanding roles of the Sd^a/Cad carbohydrate antigen and its cognate glycosyltransferase B4GALNT2



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ABSTRACT

Background: The histo-blood group antigens are carbohydrate structures present in tissues and body fluids, which contribute to the definition of the individual immunophenotype. One of these, the Sd^a antigen, is expressed on the surface of erythrocytes and in secretions of the vast majority of the Caucasians and other ethnic groups.

Scope of review: We describe the multiple and unsuspected aspects of the biology of the Sd^a antigen and its biosynthetic enzyme β 1,4-N-acetylgalactosaminyltransferase 2 (B4GALNT2) in various physiological and pathological settings.

Major conclusions: The immunodominant sugar of the Sd^a antigen is a β 1,4-linked N-acetylgalactosamine (GalNAc). Its cognate glycosyltransferase B4GALNT2 displays a restricted pattern of tissue expression, is regulated by unknown mechanisms - including promoter methylation, and encodes at least two different proteins, one of which with an unconventionally long cytoplasmic portion. In different settings, the Sd^a antigen plays multiple and unsuspected roles. 1) In colon cancer, its dramatic down-regulation plays a potential role in the overexpression of sialyl Lewis antigens, increasing metastasis formation. 2) It is involved in the lytic function of murine cytotoxic T lymphocytes. 3) It prevents the development of muscular dystrophy in various dystrophic murine models, when overexpressed in muscular fibers. 4) It regulates the circulating half-life of the von Willebrand factor (vWf), determining the onset of a bleeding disorder in a murine model.

General significance: The expression of the Sd^a antigen has a wide impact on the physiology and the pathology of different biological systems.

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1. Introduction

Many protein and lipid molecules embedded in the plasma membrane are decorated by sugar chains and are consequently referred to

as glycoproteins and glycolipids. A large proportion of the proteins dissolved in blood, body fluids and mucus are glycoproteins as well. The structure of the sugar chains of glycoproteins and glycolipids undergoes profound changes in a variety of pathological processes, including cancer [1], inflammatory diseases and aging [2].

Most of the blood group antigens described are carbohydrates. Formerly detected on the surface of erythrocytes, they were found to be widely distributed in other tissues [3] and consequently were named “histo-blood group” antigens in 1989 [4]. The most prominent features of histo-blood group antigens are: i) their polymorphic expression, in that only a percentage of the population is genetically determined to express a given antigen; ii) the existence of “natural” antibodies, in that individuals not expressing a given antigen frequently (but not necessarily) develop antibodies against that antigen even in the absence of previous immunization; iii) an individual (referred to as “non-secretor”) may express a given antigen on the erythrocytes and not in the secretions or *vice versa* [5].

The most important blood group system is the well-known ABO system. However, a variety of “minor” blood group systems, including

Abbreviations: aa, amino acids; AchR, acetylcholine receptor; B4GALNT1, β 1,4-N-acetylgalactosaminyltransferase 1; B4GALNT2, β 1,4-N-acetylgalactosaminyltransferase 2; bp, base pairs; CTL, cytotoxic T lymphocytes; DAPC, dystrophin-associated protein complex; DBA, *Dolichos biflorus* agglutinin; DMD, Duchenne muscular dystrophy; α -DG, α -dystroglycan; β -DG, β -dystroglycan; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; GdA, glycodeilin-A; GdC, glycodeilin-C; GdF, glycodeilin-F; GdS, glycodeilin-S; HPA, *Helix pomatia* agglutinin; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; N-CAM, neural cell adhesion molecule; NK, natural killer cell; nt, nucleotides; PHA, phytohaemagglutinin; Sia, sialic acid; sLe^a, sialyl Lewis a; sLe^x, sialyl Lewis x; THGP, Tamm-Horsfall glycoprotein; TLC, thin layer chromatography; VVA, *Vicia villosa* agglutinin; vWf, von Willebrand factor.

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Lewis, Kelly, and Duffy, contribute to determine the phenotype of blood and tissue cells. The histo-blood group Sd^a, qualified as “public” due to its high incidence, is one of these “non ABO” histo-blood group antigens. Since its discovery in 1967, its importance in a variety of physiological and pathological conditions has been increasingly recognized.

2. Structure, distribution and biosynthesis of the Sd^a/Cad antigen

2.1. Structure and distribution

In 1967, two groups independently reported the presence of a carbohydrate antigen, called Sd^a, on the erythrocytes and in secretions of the vast majority of individuals of Caucasian origin [6,7]. The Sd^a was found to be inherited as a dominant character suggesting the occurrence of a new blood group antigen. In 1970, a comprehensive study was published on the distribution of the Sd^a antigen in human and animal body tissues and fluids, using the inhibition of the agglutination of Sd^a⁺ erythrocytes by anti-Sd^a antibodies as a measure of Sd^a activity [8]. According to this study, summarized in Table 1, human saliva, milk, meconium and urine can express the Sd^a antigen with the highest concentration being detected in urine. More than half of the people whose red cells group as Sd^a[−], still secrete Sd^a substance in the urine or saliva [8,9], indicating that a different regulation of the biosynthesis of this antigen occurs in kidney, salivary glands and bone marrow. The percentage of individuals with an Sd^a negative phenotype in both erythrocytes and urine was 4%, while anti-Sd^a antibodies were present in about 50% of Sd^a[−] non-secretors. Among animals, the Sd^a antigen was detected in a variety of mammals, with the highest Sd^a activity detected in urine and kidney of guinea-pig, whereas no activity was detected in the five bird species tested [8]. In considering these data, it should be kept in mind that sometimes the number of individuals analyzed was very small. Consequently, a percentage of 100% Sd^a⁺ individuals determined in a group of 10 or less does not have the same accuracy as the percentage calculated on hundreds of individuals.

Table 1
Sd^a activity in human secretions^a.

Secretion	No. of individuals	% individuals with Sd ^a activity in the secretion ^b
Urine		
General population (above 5 years)	361	96
Individuals with Sd ^a ⁺ erythrocytes	22	100
Individuals with Sd ^a [−] erythrocytes	13	75
Individuals with Sd ^a [−] erythrocytes and anti Sd ^a in serum	9	0
Newborns	16	100
Children (1–9 years)	20	100
Pregnant women	16	100
Post partum	14	100
Milk	4	25
Feces		
Newborns	4	100
Children (3–12)	4	75
Adults (19–84 years)	12	8
Saliva		
Individuals with Sd ^a ⁺ erythrocytes	66	94
Individuals with Sd ^a [−] erythrocytes	14	43
Newborns	12	100

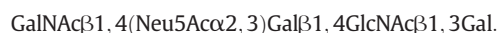
Data have been deduced from Ref [8].

^a The test used was based on the ability of the sera of some individuals to induce the erythrocyte agglutination of other ABO-compatible individuals. In the standard test, the erythrocytes of a single reference individual, considered to be Sd^a⁺, were incubated with the serum from another reference individual of Sd^a[−] phenotype and possessing anti-Sd^a antibodies, resulting in erythrocyte agglutination. The addition to this reaction of secretions from other individuals can inhibit agglutination, depending on the presence in the secretion of molecules carrying the Sd^a epitope.

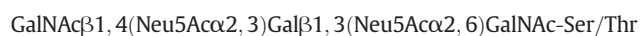
^b The percentage of Sd^a⁺ individuals determined among small groups is not statistically precise, owing to the small sample size and cannot be compared with the percentage calculated on larger samples.

Several lines of evidence indicated that the well-known urinary Tamm–Horsfall glycoprotein (THGP) is a major carrier of the Sd^a antigen in urine. In fact, the Sd^a activity of THGP from different donors strictly correlated with their Sd^a red blood cell phenotype [10], while THGP from Sd^a⁺ individuals was precipitated with anti-Sd^a sera [11]. In kidney, the Sd^a antigen and THGP showed the same tissue distribution in the distal convoluted tubules and collecting ducts [12]. However, at least one other protein carrier of the Sd^a antigen with mucin-like properties, different from THGP, is present in urine [13].

The structure of the Sd^a antigen was elucidated in the eighties. First, a close relationship was established between the *N*-acetylgalactosamine (GalNAc) content and Sd^a activity of THGP [10]; second, it was established that the immunodominant GalNAc residue was β1,4-linked to a galactose (Gal) residue [14]; third, a pentasaccharide with the ability to inhibit anti-Sd^a-induced agglutination of Sd^a⁺ erythrocytes was isolated by endoglycosidase treatment from the *N*-linked chains of THGP and found to possess the following structure [15]:



The Cad antigen was first described in 1968 as a dominant character in three members of a Mauritian family [16]. Agglutination of Cad erythrocytes by GalNAc-specific lectins (*Dolichos biflorus*, *Helix pomatia*) (Table 2) led to the conclusion that the immunodominant sugar of the Cad antigen was GalNAc [17]. The observation that Cad samples reacted strongly with anti-Sd^a antibodies [18] led to the conclusion that Cad was a very reactive form of Sd^a, and was called super Sid or Sd^a⁺⁺. Membrane glycoproteins of the erythrocytes of Cad individuals displayed reduced electrophoretic mobility [19]. The major Cad structure associated with glycophorin A was a type 3 O-linked carbohydrate chain with the following structure [19–21]:



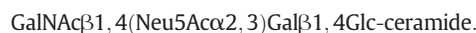
and was a powerful inhibitor of the anti-Sd^a agglutination of Sd^a⁺ erythrocytes. Interestingly, a comparative study of glycophorin A from Cad, Sd^a⁺ and Sd^a[−] individuals revealed the absence of the above-reported Cad-active O-linked pentasaccharide in glycophorin A from both Sd^a⁺ and Sd^a[−] individuals [22]. In addition, in erythrocytes from Cad individuals, the glycolipid sialylparagloboside was largely modified with a β1,4-linked GalNAc, giving rise to the structure [23]:



The percentage of sialylparagloboside molecules elongated by the GalNAc residues was about 70% in erythrocytes from Cad individuals and less than 20% in those from Sd^a⁺ individuals [24]. Thus, the three Sd^a/Cad-active structures reported above share the following minimal structure:



This trisaccharide is also found in the G_{M2} ganglioside:



However, an enzyme preparation from human kidney catalyzed the transfer of radiolabeled GalNAc to sialylparagloboside but not to ganglioside G_{M3} (the G_{M2} precursor), indicating that the GalNAc-transferase responsible for the biosynthesis of Sd^a/Cad antigens was not able to convert G_{M3} into G_{M2} [24].

In conclusion, Cad and Sd^a blood group antigens appear to share the antigenic determinant, which can be expressed by *N*- or O-linked chains of glycoproteins and by long sugar chain gangliosides (sialylparagloboside), but not by short sugar chain gangliosides (G_{M3}). Sd^a and Cad antigens are likely the product of the same biosynthetic enzyme. The difference between the two phenotypes may

Table 2
Binding specificity of the most widely used tools for the detection of the Sd^a/Cad antigen.

Antibody or lectin	Specificity	References
<i>Dolichos biflorus</i> lectin (DBA)	α-GalNAc	[16]
<i>Helix pomatia</i> lectin (HPA)	Cad antigen	[24]
<i>Vicia villosa</i> B4 lectin (VVA)	α-GalNAc	[17]
CT1 antibody	Siaα2,3(GalNAcβ1,4)Gal-R	[43]
CT2 antibody	G _{M2} containing Neu5Ac	[93,94]
KM 531 antibody	Siaα2,3(GalNAcβ1,4)Gal-R	[43]
KM694 antibody	Sd ^a antigen containing Neu5Gc	[93,94]
	Sd ^a /Cad>G _{M2}	[67]
	Sd ^a /Cad>G _{M2}	[68]

Antibodies CT1 and CT2 have both been reported to react with human THGP [43] whose sialic acid is Neu5Ac, because Neu5Gc is not synthesized by human tissues. However, in another study Neu5Gc was found to be mandatory for reactivity with CT2 [94]. This discrepancy can be explained by the different tests used.

result from a higher activity, or a slightly different substrate specificity of the biosynthetic enzyme in Cad individuals leading to the addition of the β1,4-linked GalNAc on a higher percentage of carrier molecules. Moreover, some acceptor molecules, which could be poor acceptors for the enzyme of Sd^{a+} individuals, could be efficiently glycosylated by the enzyme of Cad individuals, leading to structures not detectable in Sd^{a+} individuals, such as the O-linked structure derived from glycophorin A reported above. Thus, the difference between Cad and Sd^a appears to be both quantitative and qualitative.

2.2. Identification of the Sd^a synthase: β-1,4-N-acetylgalactosaminyltransferase 2 (B4GALNT2)

The very high Sd^a activity in kidney and urine of guinea-pigs [12] prompted F. Serafini-Cessi to investigate the presence of the Sd^a biosynthetic enzyme in guinea-pig kidney. This approach led to the identification of a GalNAc-transferase, now known as β-1,4-N-acetylgalactosaminyltransferase 2 [official symbol of the human gene, according to HUGO (<http://www.genenames.org/>): B4GALNT2. Previously used names: UDP-GalNAc:Neu5Acα2-3Galβ-R β1,4-N-acetylgalactosaminyltransferase 2; β1,4GalNAcT-II; GALGT2]. This glycosyltransferase catalyzes the transfer of GalNAc from UDP-GalNAc to the N-linked chains of THGP and fetuin and not to their asialylated counterparts. This finding suggested that in the sequential synthesis of the Sd^a antigen the addition of β1,4-linked GalNAc occurred after the addition of α2,3-linked sialic acid [25]. Despite the fact that only THGP from Sd^{a-} individuals lacked GalNAc residues, both THGP from Sd^{a+} and Sd^{a-} individuals were good acceptors of this GalNAc-transferase activity *in vitro* [25], suggesting that several free potential acceptor sites of GalNAc-transferase exist in the THGP of Sd^{a+} individuals. It was subsequently reported that the guinea-pig enzyme could catalyze the transfer of a GalNAc residue to a free α2,3-sialylated oligosaccharide, and not to its α2,6-sialylated isomer or to the G_{M3} ganglioside [26]. In addition, this GalNAc residue could be released only by β-hexosaminidase and not by α-hexosaminidase treatment, provided that the sialyl residue was preliminarily removed by chemical hydrolysis [26]. Altogether, these data indicated that the guinea-pig enzyme had properties consistent with those of an Sd^a synthase. Strikingly, the enzyme could not be extracted by conventional incubation with a non-ionic detergent, such as Triton X-100, and also required KCl [27]. The partially purified enzyme showed activity also towards O-linked derived structures, such as Neu5Acα2,3Galβ1,3GalNAcol. Interestingly, the enzyme was also highly expressed in colon and kidney medulla [28].

The properties of the human kidney enzyme were very similar, but not identical, to those of the guinea-pig enzyme, in that only the latter was able to transfer GalNAc to glycophorin [29]. Moreover, the human

enzyme was able to act on a long sugar chain ganglioside, such as sialylparagloboside, although not on G_{M3} [24,29]. A β1,4GalNAc-transferase activity with similar, if not identical, enzymatic properties was identified in human plasma [30], urine [31] and large intestine, in which it shows a proximal to distal gradient of expression [32]. Although microarray analysis has suggested that a transcript similar to B4GALNT2 may be expressed in intestinal cells of developing zebrafish [33], no information on this enzyme activity in lower vertebrates is available.

2.3. The Sd^a antigen and B4galnt2 in mouse lymphocytes: the CT antibodies

It has been known since the seventies that populations of mouse cytotoxic T (CT) lymphocytes expressed on specific glycoproteins a carbohydrate antigen which could be recognized by the GalNAc-specific lectin from *Vicia villosa* (Table 2) [34,35]. This antigen allowed CTL to be discriminated and isolated from other lymphocyte populations, including those with cytotoxic activity but not belonging to the T cell lineage (such as NK cells). In 1984, it was reported that the VV6 clone of the murine CTL cell line B6.1.SF.1 resistant to *V. villosa* lectin lacked β1,4-linked GalNAc residues at the non-reducing ends of conventional O-linked structures [36]. Enzymatic studies showed that the VV6 clone lacked GalNAc-transferase activity [37] which, in wild type cells, displayed very similar properties to those of the GalNAc-transferase described one year earlier in guinea-pig kidney [25]. Among murine T cell populations, the level of GalNAc-transferase activity was generally higher in those with cytotoxic function [38]. The monoclonal antibodies CT1 and CT2 (Table 2) were obtained from a screen for antibodies reacting with a murine CTL clone and were found to recognize carbohydrate antigens on CTL [39] and to block specific target cell lysis [40]. The causal relationship between lytic activity and CT antigen expression was confirmed by the finding that the induction of lytic ability by IL-2 in a mouse hybridoma cell line paralleled the expression of CT antigens [41]. Subsequent analysis showed that CT1 and CT2 antibodies recognized similar, but not identical, sialic acid-containing epitopes [42]. Both antibodies bound to Tamm-Horsfall glycoprotein of Sd^{a+} but not Sd^{a-} individuals in a solid phase assay and to erythrocytes of Cad individuals [43]. Among lymphocytes associated with mouse small intestine, the expression of the CT antigens was associated with those expressing the Lyt-2 antigen (CD8), not only with those with constitutive cytotoxic activity [44]. Interestingly, in the small intestine the CT1 antibody stained the epithelial cells of the lower crypt (which contain the intestinal stem cells), while CT2 stained the entire villi [44]. During mouse embryonic development, the CT1 antigen was expressed by a relevant percentage of thymocytes, namely a subset of immature double negative (CD4⁻/CD8⁻) thymocytes [45]. During thymocyte differentiation in adult mice, the CT1 antigen was expressed only by early T-lineage-committed cells: the signal leading to down-regulation of B4GALNT2 and consequently to switch-off of the CT1 antigen appears to be mediated through the pre-TCR complex [46]. Priming murine CTL precursors with different antigens gave rise to polarized CTL populations: one produced a cytokine spectrum similar to that of T helper 1 cells involved in anti-microbial immunity (IFN-γ and TNF-α, but not IL-4, IL-5, and IL-10), while the second produced a cytokine spectrum (IL-4, IL-5, IL-10 but little or no IFN-γ and TNF-α) similar to that of T helper 2 cells involved in anti-parasite defence. Interestingly, only CTL of the first type were found to react with CT1 [47], suggesting that this antigen distinguishes functionally different CTL populations.

In human peripheral blood lymphocytes, CT1 and CT2 antibodies marked only a small percentage of double positive CD4⁺ + CD8⁺ cells. This percentage was higher in patients with systemic lupus erythematosus but not in those with other autoimmune diseases [48]. Stimulation with phytohaemagglutinin (PHA) or anti-CD3 antibodies increased the percentage of CD4⁺ + CD8⁺ lymphocytes reactive with CT1 or CT2 antibodies [48]. The latter data suggest that in humans, the expression

of CT antigens is not restricted to lymphocytes of the CTL lineage and is affected by lymphocyte stimulation.

2.4. Molecular cloning of *B4GALNT2*

Taking advantage of the availability of the Sd^a-reactive CT1 and CT2 antibodies, the first mouse *B4galnt2* cDNA was successfully cloned in 1994 using a mammalian transient expression cloning approach [49]. The cloned cDNA predicted a 510 amino acid transmembrane protein with a type 2 topology, characteristic of other Golgi-glycosyltransferases, with a single potential *N*-glycosylation site. In a murine CTL cell line, a single 4.4 kb transcript was detected by Northern blot analysis. Like the previously characterized B4GALNT2 from various origins, the cloned recombinant enzyme displayed an absolute requirement for an α 2,3-linked sialic acid in the acceptor. The first partial human *B4GALNT2* cDNA was reported two years later [50], while the first human full-length clones were independently obtained from the human colon cancer cell line Caco-2 by two groups in 2003 [51,52]. The human *B4GALNT2* gene maps on 17q21.33 and encompasses 11 coding exons. Northern blot [52] and PCR analyses [51] indicated the existence of multiple transcripts diverging in their 5'- and 3'-UTR, some of which were very long (about 9000 bp), and the occurrence of at least two alternative first exons (Fig. 1). Five *B4GALNT2* transcripts were mainly expressed in colon and to a lower extent in ileum, stomach and kidney [52]. The long exon 1 (exon 1L) is 253 nt long, while the short exon 1 (exon 1S) is 38 nt long, leading to two transcript variants differing at their 5' end (GenBank accession number NM_153446 and NM_001159387). Both exon 1L and 1S contain a translational start site. Consequently, the human *B4GALNT2* gene can give rise to at least two different transmembrane peptides, divergent in their amino-terminal portion (Fig. 1): a 566 aa long form with a very long cytoplasmic tail and a 506 aa short form. Recombinant expression of either protein isoform in the colon cancer cell line LS174T revealed that the shorter form was associated with higher enzyme activity than the long form [53]. Beside these two experimentally detected transcript forms, GenBank predicts a third transcript variant (GenBank accession

number NM_001159388) with a third, 51 nt long, first exon (called 1 Middle, 1M in Fig. 1) which has never been experimentally reported. Exon 1M lacks any ATG translational start codon, while two in frame ATG potential translational start codons are located in exon 2, around the end of the transmembrane domain. Consequently, the predicted peptide would lack the transmembrane region, generating a putatively soluble form of the enzyme (Fig. 1). It has been generally thought that the enzymatically active soluble forms of B4GALNT2 described in plasma [30], urine [31] and conditioned medium of differentiated Caco-2 cells [54] derive from the proteolytic cleavage of the membrane-bound form(s), as occurs for other glycosyltransferases, including the well-known sialyltransferase ST6GAL1 [55]. However, the experimental detection of a transcript containing exon 1M would support the existence of another mechanism, based on alternative first exon use and/or different promoter usage.

The long protein isoform of B4GALNT2 is characterized by the unusual length of its cytoplasmic tail (67 aa), which is one of the longest among glycosyltransferases and further suggests its involvement in a very specific function. This is reminiscent of B4GALT1, another *trans*-Golgi glycosyltransferase, which also exists in short and long forms diverging in their N-terminal portion. Both B4GALT1 protein isoforms are Golgi-resident enzymes, while the long protein isoform may also be localized to the cell membrane, acting as a receptor for extracellular matrix components [56,57]. B4GALT1 provides an interesting example of a mechanism used by mammals to recruit a gene which serves a dual role: *N*-glycan biosynthesis in most tissues and lactose synthesis specifically in lactating mammary glands [58,59].

A soluble recombinant enzyme displayed good activity towards α 2,3-sialylated type 1 (Gal β 1,3GlcNAc), type 2 (Gal β 1,4GlcNAc) and core 1 O-linked (Gal β 1,3GalNAc) acceptors [52]. The structure of the carbohydrate chains shown to be carriers of the Sd^a/Cad antigen or to be acceptors for the B4GALNT2 enzymatic activity is displayed in Fig. 2. B4GALNT1 (G_{M2} synthase) catalyzes the addition of GalNAc in β 1,4-linkage to the Gal residue of ganglioside G_{M3}, giving rise to G_{M2} [60]. This reaction is similar to that catalyzed by B4GALNT2, except for the presence of α 2,3-linked sialic acid in the acceptor, which is not

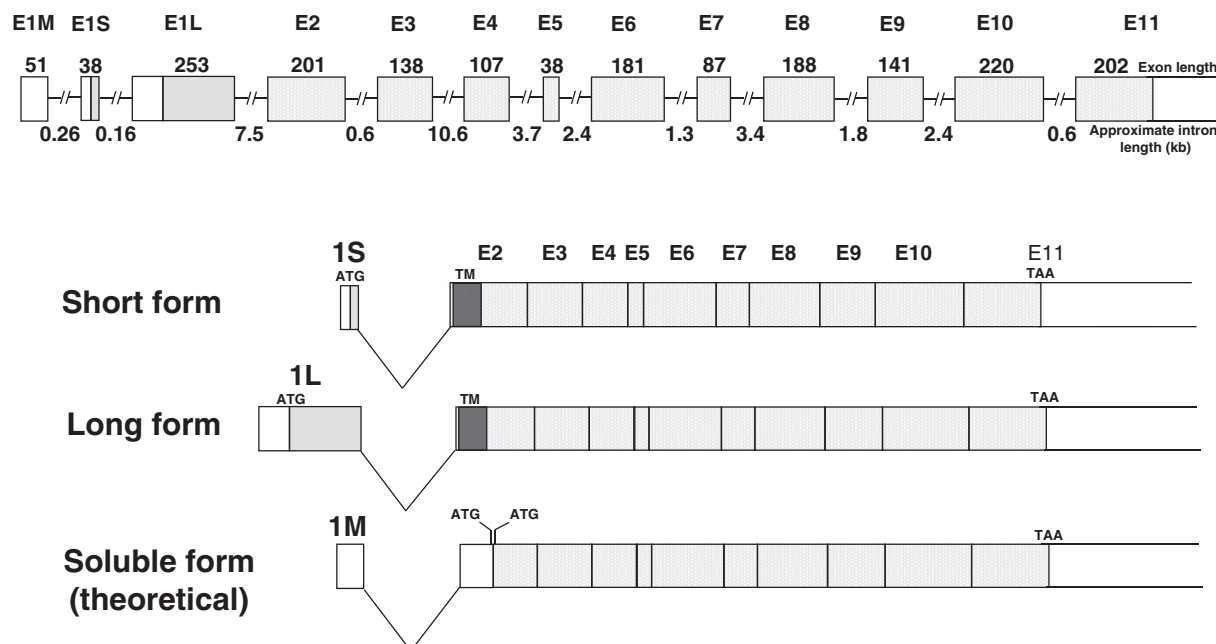


Fig. 1. Organization of the human *B4GALNT2* gene and its transcripts. The gene is comprised of 11 exons with at least three alternative first exons: exons 1S, 1L and 1M. Exon length (in bp) is reported above the exons. Numbers below the introns indicate the approximate intron length expressed in kb. The coding regions are depicted in gray. The length of exon 11 refers to the coding portion only. The short and long transcript forms are originated by the alternative presence of exon 1S or 1L. Both contain a translational start codon and give rise to two transmembrane proteins diverging in their amino-terminal portion. The predicted transmembrane domain is depicted in dark gray. The “soluble form” is predicted by GenBank, but has never been confirmed experimentally so far. It is originated by exon 1M which lacks a translational start codon. Two potential ATG starting codons of this putative transcript are inside exon 2, around the end of the transmembrane encoding sequence. Consequently, the protein encoded by this transcript should be a soluble form of B4GALNT2.

strictly required by B4GALNT1 [61,62] and the ceramide portion in close proximity to the reaction site, which is incompatible with the activity of B4GALNT2. A comparison of the predicted amino acid sequences of B4GALNT1 and B4GALNT2 reveals extensive homology in the C-terminal region encompassing the catalytic domain, suggesting a common phylogenetic origin of the two genes.

3. The Sd^a antigen in physio-pathological conditions

3.1. Onco-developmental regulation

The dependence of B4GALNT2 on the mechanisms regulating ontogenic development and cell differentiation was formerly suggested by the observation that the guinea-pig kidney enzyme showed a fivefold increase after birth [28]. A developmental regulation of B4GALNT2 was also observed in rat colon, in which the enzyme was practically absent at birth, increased slowly in the first days of life and then rapidly after weaning [63]. Consistently, the Sd^a antigen was not detected in human fetal colonic mucins [64]. However, as mentioned in Table 1, the feces, urine and saliva of human newborns have been reported to express high Sd^a activity [8]. The origin of this discrepancy remains to be established.

A higher B4GALNT2 activity has been observed in the poorly differentiated cells of the colonic crypt [63], in agreement with the observation that CT1 antibody selectively stained the cells at the basis of the intestinal crypt [44]. Conversely, Caco-2 cells, one of the few *in vitro* models of intestinal differentiation, showed an increased B4GALNT2 expression concomitantly with a differentiated phenotype [65].

In human colonic cancer, B4GALNT2 activity exhibits a dramatic down-regulation, compared with the normal surrounding mucosa [32,53], due to down-regulated expression of the mRNA [50]. Interestingly, a small percentage of individuals expressing little or no activity in normal colon was found by both European [32,53] and Japanese [50] studies, suggesting that these individuals were probably Sd^a-. Sd^a antigen expression was reduced in colon cancer, compared with normal mucosa paralleling that of the enzyme expression [66].

Using the monoclonal antibody KM531 (Table 2), specific for both G_{M2} and Sd^a antigen, G_{M2} was not expressed by samples of normal gastric mucosa or of peptic ulcer but was expressed by gastric carcinoma specimens [67]. However, in the same samples of normal gastric mucosa KM531 detected other glycolipids with a lower TLC mobility, mostly

sialylparagloboside with a β 1,4-linked GalNAc residue (see above). These long sugar chain gangliosides were not detected in the few gastric cancer specimens examined [67]. Owing to the known ability of B4GALNT2 to glycosylate sialylparagloboside [24] and not G_{M3}, it was likely that in gastric cancer B4GALNT2 was down-regulated while B4GALNT1 (G_{M2} synthase) was up-regulated. Indeed, the B4GALNT2 gene was found to be expressed in gastric mucosa and down-regulated in gastric cancer because of a down-regulation of its mRNA [50]. Later on, the same group [68] showed that in normal human stomach, B4GALNT2 activity and Sd^a active glycolipids (detected with another anti Sd^a-specific monoclonal antibody: KM 694) were both present in a cell fraction enriched with chief cells (the stomach cells releasing pepsinogen).

Sialyl Lewis x (sLe^x) and sialyl Lewis a (sLe^a) antigens are well-known fucosylated carbohydrate structures (Fig. 3A) which act as ligands for the cell adhesion molecules of the selectin family and play a fundamental role in leukocyte extravasation. The frequent overexpression of these antigens in cancer cells contributes to metastasis formation because it allows the interaction of the circulating cancer cells released by the tumor with selectins expressed on endothelial cells. In colon cancer, the overexpression of sLe^{x/a} is not due to the overexpression of their cognate fucosyltransferases [69]. An intriguing possibility is that the level of B4GALNT2 plays a role in regulating sLe^{x/a} expression (Fig. 3). The structural similarity between the Sd^a and sLe^{x/a} antigens suggests that their biosynthesis might be mutually exclusive. In fact, both derive from the substitution of an α 2,3-sialylated Gal β 1,3/4GlcNAc chain: the first with a GalNAc residue β 1,4 linked to galactose, the second with a fucose residue α 1,3/4-linked to GlcNAc (Fig. 3A). *In vivo*, it was shown that mucins from normal descending colon highly expressed the Sd^a epitope and poorly expressed sLe^x-containing structures, whereas no carbohydrate chains carrying both the Sd^a and the sLe^x determinants were detected [70]. *In vitro*, two groups independently demonstrated that the forced expression of B4GALNT2 in cancer cell lines of gastrointestinal origin resulted in a down-regulation of sialyl Lewis antigens. One group expressed the long protein isoform of B4GALNT2 in gastric cancer KATO III cells and in colon cancer HT29 cells, observing the loss of sLe^x expression in both cell lines and a striking reduction of their metastatic ability [68]. The other study showed that both the long and the short protein isoforms of B4GALNT2 were able to induce the expression of the Sd^a antigen and the inhibition of sLe^x in LS174T cells [53]. In all these *in vitro* cellular models the Sd^a or

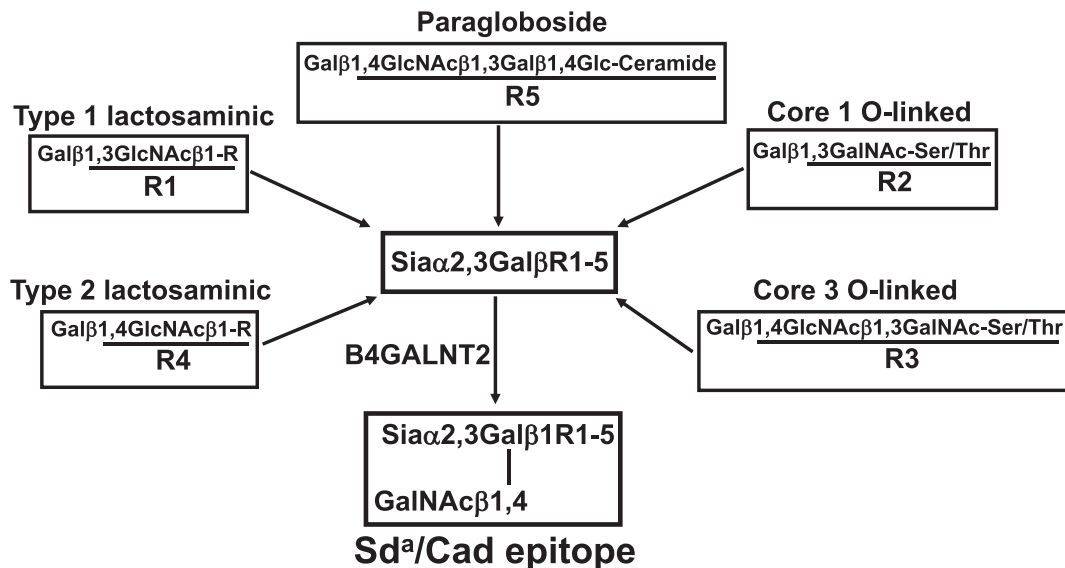


Fig. 2. Structure of the major oligosaccharide chains carrying the Sd^a/Cad antigen. The terminal galactose residue of different types of carbohydrate chains, including type 1 and type 2 lactosaminic chains, (both present in the distal regions of N- and O-linked chains), core 1 and core 3 O-linked chains, as well as long sugar chain glycolipids, such as paragloboside (indicated as R1–R5 structures), can undergo α 2,3-sialylation. The subsequent addition of a β 1,4-linked GalNAc, mediated by B4GALNT2, leads to formation of the Sd^a/Cad antigen.

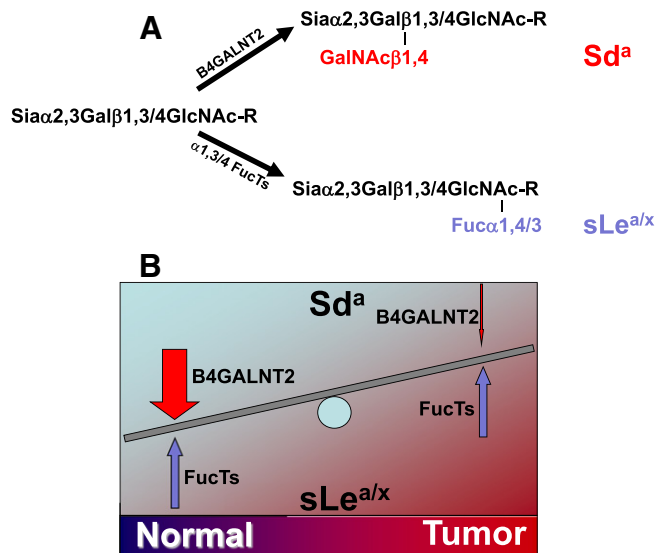


Fig. 3. Competition between the Sd^a and $sLe^{a/x}$ antigens. A: The Sd^a and $sLe^{a/x}$ antigens derive from the substitution of a $\alpha 2,3$ -sialylated type 1 or type 2 chain. The addition of $\beta 1,4$ -linked GalNAc to Gal leads to the expression of the Sd^a antigen, while that of $\alpha 1,4/3$ -linked fucose to GlcNAc leads to the expression of $sLe^{a/x}$ antigens. The expression of the two types of antigen is mutually exclusive because the Sd^a antigen is not a substrate of $\alpha 1,4/3$ -fucosyltransferases and *vice versa*. B: In colon cancer, the marked increase in sLe^x expression is not supported by increased fucosyltransferase expression (arrows of the same width), while the reduced $B4GALNT2$ activity present in cancer tissues (narrow arrow) is likely responsible for the shift of the Sd^a/sLe^x equilibrium towards sLe^x .

sLe^x antigens appeared to be alternatively carried by the same molecules. A model in which the regulation of sLe^x expression in colonic tissues is controlled by the level of competing $B4GALNT2$ is depicted in Fig. 3B.

3.2. Expression in intestinal infections

In rat small intestine, infection with the parasite *Nippostrongylus brasiliensis* causes different alterations of mucin glycosylation, including a decrease of the Neu5Gc/Neu5Ac ratio and the appearance of O-linked oligosaccharides terminating with the Sd^a epitope [71]. The maximum expression of Sd^a carrying structures increased from day 8 postinfection to peak at the time of parasite expulsion. This change could be due to activation of $B4GALNT2$ in small intestine of infected rats, but no evidence of such activation has been provided so far. In mice small intestine, parasite infection induced the synthesis of an O-linked oligosaccharide with a putative terminal GalNAc but devoid of sialic acid. Owing to the known strict specificity of $B4GALNT2$ for sialylated acceptors, its involvement in the biosynthesis of such a structure can be ruled out [72].

The gut microbiota comprises trillions of microbes living in the intestine and forming a complex ecological community that influences normal physiology and susceptibility to disease. A recent study showed that *B4galnt2* KO mice displayed significant alterations of the gut microbiota compared with normal mice [73]. This finding suggests that *B4galnt2* exerts a role at the interface between commensal microbiota and host immune system. However, unlike other glycosyltransferases such as fucosyltransferase 2, whose expression requires bacterial colonization of the gut, *B4galnt2* also seems to be expressed in germ-free mice [73].

4. Epigenetic regulation of *B4GALNT2*

Exons 1L, 1S and 1M of the human *B4GALNT2* gene are embedded in genomic sequences with the features of a CpG island, suggesting that DNA methylation could play a role in regulating *B4GALNT2* gene expression, in particular in the down-regulation observed in gastrointestinal

cancers. This point was formerly investigated in a paper showing that the two previously described transcripts driving the expression of either the long or short protein isoforms of *B4GALNT2* were detectable by RT-PCR (but only after 40 amplification cycles) in some gastrointestinal cell lines and not in others [74]. In the cell lines not expressing the *B4GALNT2* transcripts, the promoter region was found to be methylated and *B4GALNT2* gene expression was restored by treatment with the DNA methylation inhibitor 5'-aza 2'-deoxycytidine [74]. However, a previously reported semiquantitative RT-PCR analysis with a more conventional number of amplification cycles (30–35) in a panel of colon cancer cell lines and a normal mucosa specimen revealed a clearly detectable transcript level only in differentiated Caco-2 and normal mucosa [51]. This finding is in agreement with enzyme activity data showing that *B4GALNT2* activity is practically undetectable in colon cancer cell lines, with the exception of Caco-2 after differentiation [65]. However, even in differentiated Caco-2, the level of enzyme activity is approximately 10% that of normal colonic mucosa. The methylation level of several glyco-genes, including *B4GALNT2*, was investigated in human gastrointestinal cancers and cell lines [75]. The *B4GALNT2* gene was found to be methylated in about half of the gastric cancer cases examined and in the majority of gastric and colon cancer cell lines. Treatment of cell lines with anti DNA-methylation agents induced a weak expression of the *B4GALNT2* transcript and Sd^a antigen [75]. Altogether, these results are consistent with the view that methylation of the *B4GALNT2* genomic regulatory region plays a role in switching off gene expression during carcinogenesis of gastrointestinal tissues. This is reflected by the very low level of mRNA expression and enzyme activity in cell lines (including Caco-2), even lower than that expressed by many colon cancer surgical specimens, suggesting that *in vitro* culture further inhibits *B4GALNT2* gene expression. Removal of epigenetic marks leads to a level of enzyme activation which remains far-removed from that of the normal colonic mucosa. This suggests that other major mechanisms are involved in regulation of this gene in colorectal tissues. In particular, the set of transcription factors necessary to ensure the high levels of expression measured in normal human colon may not be properly expressed in cancer cell lines and tumor tissues. Thus, removal of the epigenetic blocks would have the effect of releasing the brakes of a vehicle, rather than igniting the engine.

5. Multiple roles of the Sd^a antigen

Studies in animal model systems have revealed a role for the Sd^a antigen in the regulation of physiological mechanisms, such as hemostasis or reproduction, as well as unexpected effects on neuromuscular diseases.

5.1. Hemostasis

Von Willebrand factor (vWf) is a plasma glycoprotein that plays an essential role in hemostasis by stabilizing coagulation factor VIII and mediating the adhesion and aggregation of platelets to sites of vascular injury. vWf is produced exclusively by megakaryocytes and endothelial cells. Von Willebrand disease comprises a heterogeneous group of bleeding disorders caused by qualitative or quantitative abnormalities of vWf. While a percentage of the cases with a quantitative reduction of vWf is due to mutation of the vWf gene, other cases are likely due to mutations of modifier genes. The plasma level of vWf in RIIIS/J mice is about twentyfold lower than that of normal C57BL6/J mice. *MvWf1* is a major modifier locus determining vWF levels in RIIIS/J mice. This locus encodes a gain of function regulatory mutation of the *B4galnt2* gene which determines a switch in the tissue expression pattern from gastrointestinal/epithelial to vascular endothelium [76,77]. The genomic region responsible for the switch lies approximately 30 kb upstream of the *B4galnt2* gene [78]. In comparison with C57BL6/J mice, in RIIIS/J mice this sequence shows 2–3% single nucleotide (SNP) divergence as well as large insertion and deletions, resulting in

an approximate size decrease of 10 kb. The endothelial expression of *B4galnt2* results in expression of the Sd^a antigen on the sugar chains of vWF and its consequent rapid clearance from the blood, accounting for the reduced vWF levels. The fact that several inbred mouse strains, including five strains derived from the wild, share this phenotype suggests that the endothelial switch is due to positive selection through a still unknown benefit [79]. However, a recent study suggested that the putative benefit could result not from the gain of *B4galnt2* in endothelia, but rather from its loss in intestine [80]. It remains to be established whether polymorphisms of the *B4GALNT2* gene are responsible for reduced vWF levels in humans.

5.2. Reproduction

The Sd^a antigen is expressed by porcine primordial germ cells and by a subpopulation of spermatogonia in cattle, pigs, horses and lama [81], and also by the N- and O-glycans of murine zona pellucida glycoproteins [82], indicating that both male and female gametes can express this antigen whose biological significance remains to be elucidated. Moreover, the major N-glycan of bovine pregnancy-associated glycoproteins, which are secretory proteins of trophoblast cells in ruminants, is a large structure whose four antennae are terminated by the Sd^a antigen [83].

In primary cultures of mouse endometrial cells, the *B4galnt2* mRNA is up-regulated by progesterone and down-regulated by estrogens [84], while in mouse uterine tissues the expression of *B4galnt2* mRNA starts at the peri-implantation stage (embryonic day 3.5) and peaks at embryonic day 10.5, in parallel with the level of placental progesterone production. *B4galnt2* expression decreases as estrogen increases during pregnancy. *In vivo* down-regulation of *B4galnt2* by siRNA treatment effectively reduced the number of implanted embryos [84]. Transfection of a mouse full-length *B4galnt2*-GFP chimera in the blastocyst trophectoderm cells in blastocyst revealed a plasma membrane localization in both cases [85]. By contrast, in uterine tissues of pregnant mice, the *B4galnt2* protein appeared to be localized in cytoplasm (including organelles) and not on the plasma membrane. Attachment of the blastocyst to endometrial cells *in vitro* can be inhibited by either antibodies against *B4galnt2* or lectins recognizing the Sd^a epitope [85]. These data indicate that the Sd^a antigen is likely involved in embryo attachment.

Glycodelin is a human glycoprotein existing in four isoforms: glycodelin-A (GdA) from the amniotic fluid; glycodelin-F (GdF) from follicular fluid; glycodelin-C (GdC) from cumulus matrix, and glycodelin-S (GdS) from seminal plasma. The four molecules share the same peptide and differ only for the N-linked chains. In particular, the major complex type GdS glycans are bi-antennary structures very rich in fucosylated structures, such as Lewis x and Lewis y antigens. A portion of the N-linked chains of all three female glycodelins are decorated by the Sd^a antigen [86]. However, while this is the only sialylated structure in GdC, both GdF and GdA contain α 2,6-sialylated glycans. Both GdF and GdA display immunosuppressive activity which is lacking in GdS and GdC and is likely due to α 2,6-sialylated structures. The immunomodulatory activity of GdA protects the fetus from the maternal immune response through mechanisms including the induction of T-cell apoptosis, skewing of T-cell differentiation toward the Th-2 phenotype and modulation of the activities of other immune cells such as natural killer, B-, and dendritic cells. In gestational diabetes mellitus, a decrease of the expression of high mannose chains and of the α 2,6-sialylation of GdA N-linked chains and an increase in the structures carrying the Sd^a antigen were observed. These changes in GdA glycosylation were accompanied by a reduction of GdA immunosuppressive activities [87].

5.3. Neuromuscular tissues

The presence of GalNAc-containing carbohydrates at the neuromuscular junction was formerly detected with GalNAc-specific lectins from *D. biflorus* and *V. villosa* in a variety of vertebrates (human, mouse,

rabbit, guinea pig, chick, frog, axolotl, snake, fish, lamprey), and found to be associated with both the enzyme acetylcholine esterase and a glycolipid [88,89]. It was subsequently reported that these GalNAc residues play a role in the clustering of acetylcholine receptors (AChR) induced by a large proteoglycan, called agrin, during organization of the neuromuscular synapse [90]. In addition, GalNAc was found in both the presynaptic motor neuron [91,92], where it is carried by the G_{M2} ganglioside and recognized by the CT1 antibody, and in the postsynaptic structures, where it is carried by glycoproteins and long sugar chain gangliosides and is recognized by the CT2 antibody [93]. The expression of CT1 and CT2 antigens in the pre- and postsynaptic termini is dependent on the expression of *B4galnt1* and *B4galnt2* respectively [93]. Moreover, the different reactivity of CT1 and CT2 antibodies is at least in part explained by the preference of CT1 for Neu5Ac and of CT2 for Neu5Gc [94]. In *B4galnt2* KO mice, an intracellular accumulation of AChR within endosomes of skeletal myofibers was observed, suggesting that the Sd^a antigen is necessary for proper AChR routing. Instead, intracellular transport of AChR was normal in *B4galnt1*-KO mice [93].

The dystrophin-associated protein complex (DAPC) comprises several proteins whose ultimate role is to connect the F-actin cytoskeleton of the muscular fibers with the laminin of the extracellular matrix. The DAPC is formed by intracellular (dystrophin, dystrobrevins), transmembrane (β -dystroglycan) and extracellular (α -dystroglycan) proteins [95]. Mutations of the genes encoding DAPC proteins destabilize this complex, leading to loss of membrane integrity and ultimately to muscular dystrophy. The best known of these conditions is Duchenne muscular dystrophy (DMD), a devastating human X-linked disease caused by the lack of dystrophin. A good murine model of DMD, not expressing dystrophin, is the *mdx* mouse.

α -Dystroglycan carries a very specific type of O-glycosylation in which a mannose residue is linked to serine or threonine and is further elongated with GlcNAc, Gal and sialic acid monosaccharides, giving rise to a structure analogous to the antennae of N-linked chains [96]. The importance of this structure is demonstrated by the extremely severe phenotypes of dystrophic syndromes, such as Walker–Warburg disease and muscle–eye–brain disease, in which the addition of O-linked mannose or N-acetylglucosamine, respectively, is impaired [97,98] because of the deficiency of the cognate glycosyltransferases. Owing to the very restricted expression of *B4galnt2* inside the neuromuscular junction, these α 2,3-sialylated lactosaminic type O-mannose linked chains of α -dystroglycan are decorated by β 1,4-linked GalNAc only at the neuromuscular junctions [88,91,99] in adult mice. Another molecule of the neuromuscular junction which can be decorated by the Sd^a antigen is agrin. This modification increases agrin binding to myotubes and enhances its AChR clustering activity [100].

When transgenic mice ectopically overexpressing *B4galnt2* in skeletal muscle fibers (outside the neuromuscular junction) were crossed with *mdx* mice, the resulting phenotype displayed a near complete reversion of dystrophy [101]. In addition, *B4galnt2* expression in the skeletal muscle induced reversion of the dystrophic phenotype in a variety of other murine models of dystrophy, including DGS654A, in which processing of the dystroglycan molecule is inhibited because of a point mutation [102], MDC1A, in which α 2-laminin is deficient [103] and mice lack α -sarcoglycan [104]. Ectopic expression of *B4galnt2* also showed protection in a model of muscle damage induced by response to eccentric contraction [105].

To understand the mechanisms linking *B4galnt2* expression and reversion of the dystrophic phenotype, it should be remembered that while several proteins of DAPC are localized both on the muscular fibers and at the neuromuscular junctions, others are present in either the fibers or the junctions. At least two differences between muscular fibers and neuromuscular junctions, illustrated in Fig. 4, are of particular relevance. First, in the neuromuscular junctions the role played by dystrophin in connecting F-actin filaments with cell membranes is also played by utrophin, a dystrophin paralogue. Second, in the neuromuscular junctions (not in muscular fibers) the binding of α -dystroglycan

occurs for both laminins and agrin. Thus, an alternative protein scaffold exists at the neuromuscular junction with structures and functions similar and distinct from those present on muscle fibers. When *B4galnt2* is extrasynaptically expressed in muscle fibers, the α 2,3-sialylated lactosaminic *O*-mannose-linked glycans of α -dystroglycan become modified by β 1,4-linked GalNAc, giving rise to a structure which is a better ligand for laminins and agrin [106]. The forced expression of *B4galnt2* results in two main effects: i) inhibition of muscle growth [107] and ii) increased expression of the extracellular matrix proteins normally confined to the synapse (laminins α 4 and α 5, NCAM and utrophin) [99,101]. The first effect is due to activation of myostatin signaling [107], while the second is due to increased expression of the mRNA of genes encoding DAPC proteins, including agrin, laminin α 5, utrophin and integrin α 7 [106].

Although the mechanism(s) linking *B4galnt2* expression in skeletal muscles and resistance to dystrophy are not completely elucidated, at least part of this resistance is due to the Sd^a antigen on several DAPC proteins, the most important of which is probably α -dystroglycan, allowing their localization along the muscular fibers, not only at the neuromuscular junction. The presence of utrophin at sites in which dystrophin is usually present allows utrophin to act as a dystrophin “surrogate”. The ectopic extrasynaptic localization of utrophin in muscular fibers has also been observed in adult mice upon electrotransfer of plasmid DNA carrying an expression cassette of *B4galnt2* [108]. However, when *B4galnt2* expression in muscular fibers was mediated after birth by transfer with an adeno-associated virus vector, muscles develop normally, while the dystrophy is still prevented through an utrophin-independent [109] mechanism. This finding suggests that mechanisms other than that described above contribute to *B4galnt2*-induced dystrophy prevention.

5.4. Phenotype of *B4galnt2* knockout mice

The *B4galnt2* KO mouse was generated by Core G of the Consortium for Functional Glycomics (<http://www.functionalglycomics.org>). The preliminary survey of its phenotype can be summarized as follows: there are no obvious histological changes found in major organs and tissues. In the blood, increased frequencies of Gr-1 + neutrophils and CD138 + (syndecan-1) plasma cells and a decrease of CD3 +, CD62L + T-cells were observed. In the bone marrow, there was a decrease of CD22 +, CD19 + B-cells. A double level of serum alkaline phosphatase was the major serological alteration of KO mice. KO mice exhibited an increased startled response (response to a sudden stimulus).

6. Concluding remarks

Some crucial questions remain open despite long and intense investigation in the field.

First, through which mechanisms are the tissue- and stage-specific expression of B4GALNT2 and the Sd^a antigen obtained? It is widely accepted that B4GALNT2 is the only enzyme able to synthesize the Sd^a antigen and that this enzyme is the product of a single genetic locus. Thus, the most likely mechanism to achieve tissue-specific enzyme expression is through the alternative use of different *B4GALNT2* gene promoters regulated by different sets of transcription factors. This point is of special relevance to explain the fact that about half the people with Sd^a erythrocytes secrete Sd^a antigen in the urine [8,9]. This point is also related to the very high level of enzyme expression achieved by normal colonic mucosa and its dramatic down-regulation in cancer. The removal of epigenetic marks, such as methylation of the *B4GALNT2* promoter, in colon cancer cell lines does not appear to be sufficient to restore the high level of expression observed in normal colon, suggesting a crucial role for transcription factors.

Second, what is the role of the enzyme's long isoform? Such a long cytoplasmic tail is likely to be involved in very specific phenomena, including a peculiar intracellular localization. Both the long and the short protein isoforms are enzymatically active [53] and are able to synthesize the Sd^a antigen in transfected cells. Owing to the fact that the addition of β 1,4-linked GalNAc must take place after the addition of α 2,3-linked sialic acid, the B4GALNT2 must be localized, at least in part, in a Golgi compartment not preceding those containing α 2,3-sialyltransferases. However, other less conventional intracellular localizations should be carefully considered.

Third, what is the role of the Sd^a antigen in pathogen resistance? Bacterial adhesins may be specific for α 2,3-sialylated glycoconjugates, and a β 1,4-linked GalNAc residue may hinder the attachment of pathogenic bacteria expressing these adhesins. Consistent with this hypothesis is the localization of the Sd^a antigen in tissues and organs, such as colon and kidney, in close contact with the external environment.

Some studies advocate the translational use of B4GALNT2 in disease treatment. In particular, prevention of the metastatic phenotype of colon cancer cells by down-regulation of sLe^{x/a} antigens will require further validation in experimental systems before being considered for therapeutic human use. Gene therapy protocols based on the overexpression of B4GALNT2 to treat patients with various forms of muscular dystrophy are currently under development and hopefully their efficacy will

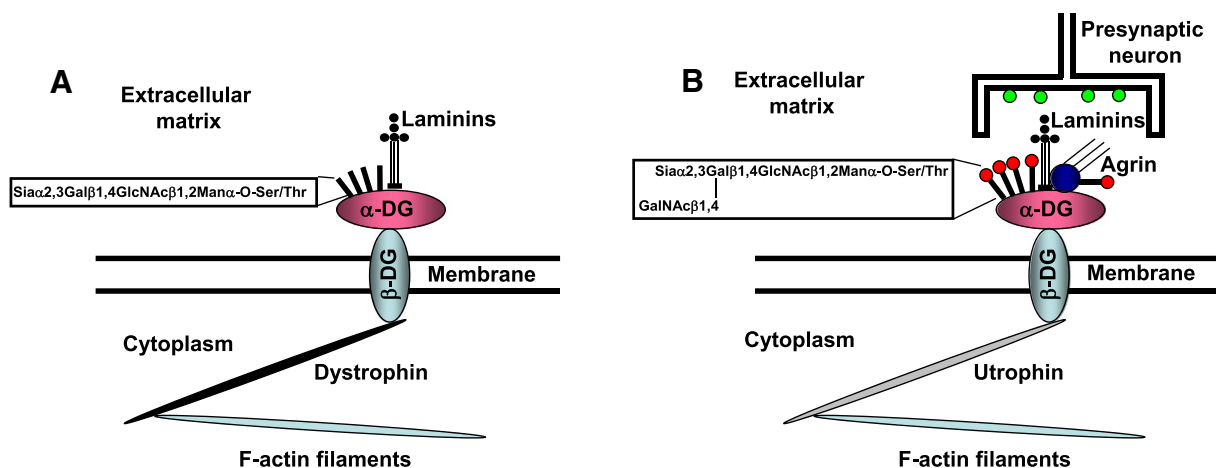


Fig. 4. Simplified representation of dystrophin-associated protein complex (DAPC). A: DAPC of muscular fibers; B: DAPC in neuromuscular junctions. Several DAPC components are omitted for simplicity. The *O*-linked chains decorating α -dystroglycan (α -DG) in muscular fibers (A) are mainly *O*-mannosyl-linked α 2,3-sialylated lactosaminic type chains. In the neuromuscular junctions (B) these chains are terminated by β 1,4-linked GalNAc (red circles), forming the Sd^a antigens, which is recognized by CT2 antibody. In the presynaptic terminus, β 1,4-linked GalNAc is associated with ganglioside G_{M2} (green circles) and is recognized by antibody CT1.

soon be evaluated in clinical trials (<http://www.nationwidechildrens.org/muscular-dystrophy-podcast>).

Sd^a antigen detection has frequently been based on the antibodies or lectins summarized in Table 2. Although these tools remain invaluable for most analyses, they do not provide information on the structures carrying the antigen, which are often complex carbohydrate chains. Furthermore, lectin-based detection of GalNAc residues is limited by cross-reactivity with other α - or β -GalNAc residues. MALDI-TOF mass spectrometry technology is the “state of the art” for complex carbohydrate analysis [110,111] and has recently been successfully used to elucidate the structure of the N-linked chains of glycodelin [86,87] and the O-linked carbohydrate chains of mucins in the adult and fetal human gastrointestinal tract [64,112]. Future use of modern structural analysis techniques will yield a more complete picture of the modulation of Sd^a antigen expression in physiological and pathological conditions.

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