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Essential Roles of Carbohydrate Signals in Development, Immune Response and Tissue Functions, as Revealed by Gene Targeting

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Knockout mice lacking glycosyltransferases or sulfotransferases provide unequivocal evidence that the carbohydrate moieties of glycoproteins, glycolipids, and proteoglycans play essential roles in various biological phenomena such as development, the immune response, and tissue functions. Examples of abnormalities of null mutants include arrest of embryogenesis due to deletion of *N*-acetylglucosaminyltransferase I or glucosylceramide synthase, failure of kidney formation in heparan sulfate 2-*O*-sulfotransferase deficiency, suppressed antibody production in α-2,6-sialyltransferase deficiency, male sterility in GM2/GD2 synthase deficiency, and abnormalities in the function and stability of myelin in galactosylceramide deficiency.

Key words: glycolipids, glycoproteins, glycosyltransferases, proteoglycans, sulfotransferases.

The carbohydrate moieties of glycoproteins, glycolipids, and proteoglycans have been believed to play important roles in intercellular recognition, which regulates differentiation, development, leukocyte trafficking, the immune response, and other tissue functions. To precisely evaluate the physiological roles of the sugar moieties, it is necessary to genetically alter the sugar structure and to analyze the consequences of the alterations. The deletion of glycosyltransferases and sulfotransferases from the mouse genome by gene targeting is ideally suited for this purpose. Recently, the number of these genes deleted in this way has been increasing rapidly (Table I). This review will briefly summarize the phenotypes of the null mutant mice and their implications.

Development and other aspects of reproduction

Asparagine-linked oligosaccharides in glycoproteins are classified into the high-mannose type, which is composed of mannose and *N*-acetylglucosamine, the complex type, which in addition has galactose and usually sialic acid and fucose, and the hybrid type. The core structure of complex type and hybrid type glycans is formed through the processing of high-mannose type glycans. The first evidence that glycoprotein-bound glycans are required for development was obtained through knockout of the *N*-acetylglucosaminyltransferase I (GnTI) gene, the enzyme catalyzing the key reaction in the formation of complex type and hybrid type asparagine-linked glycans from a high-mannose type glycan, $(Man)_5(GlcNAc)_2$ (Fig. 1). All the null embryos die before embryonic day (E) 10.5 (*1*, *2*) (Fig. 2). The reason for the embryonic lethality appears to be an abnormality of vascularization. Determination of the left-right body plan is also impaired in the mutant embryos.

The fucosylated large poly-*N*-acetyllactosamine structure called embryoglycan, which belongs to the complex type, is abundant in early embryonic cells and is expected to play important roles in early embryogenesis (*3*). Poly-*N*-acetyllactoamines are glycans containing the repeated and often branched structure of Galβ1–4GlcNAc. The results of the knockout experiment mentioned above at first appeared to indicate that complex type glycans including embryoglycan are not required for preimplantation development or early postimplantation development. However, staining with lectins such as E-PHA has shown that complex type glycans are present in the preimplantation period even in GnTI-deficient embryos. On E 6.5, complex type glycans revealed by E-PHA staining are moderately less in the null embryos, and on E 7.5, the staining is markedly decreased. Therefore, whether or not a specific structure of the complex type glycan is required in early stages of mouse embryogenesis cannot be determined. The persisting complex type glycans in early embryos appear to be derived from eggs and are also formed by the maternally derived enzyme (*4*, *5*).

Subsequently, two enzymes in the pathway for the synthesis of the core portion of asparagine-linked oligosaccharides have been knocked out (*6*, *7*). Mannosidase-II catalyzes the processing reaction following *N*-acetylglucosamine transfer (Fig. 1). However, mannosidase-II deficient mice are born (*6*). In splenocytes and fibroblasts of adult mutant mice, about 50% complex type glycans are present compared to in wild-type mice. Complex type glycans are lost in erythrocytes of null mutants, and these mice show anemia with elevated level of reticulocytes. The phenotype of the mice is similar to that of human congenital dyserythopoietic anemia type II. This result indicates that the alternative processing pathway without mannosidase-II plays significant roles in both embryonic and adult cells except for

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Abbreviations: C2GnT, Core 2β-1,6-*N*-acetylglucosaminyltransferase; GnTI, *N*-acetylglucosaminyltransferase I; Dol, dolichol; Dol-P, dolichol phosphate; GPT, GlcNAc-1-phosphotransferase; GST, glucosylceramide synthase; E, embryonic day; FuTVII, fucosyltransferase VII; HAS, hyaluronic acid synthase; ST3GalI, α-2,3-sialyltransferase I; ST6Gal, α-2,6-sialyltransferase.

those of the erythrocyte lineage.

The initial reactions in the synthesis of asparagine-linked glycans proceed with the dolichol (Dol)-bound form. GlcNAc-1-phosphotransferase (GPT) catalyzes the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichol phosphate (Dol-P) to form GlcNAc-P-P-Dol, which is the first lipid bound sugar in the pathway (Fig. 1). The embryos deficient in this enzyme complete preimplantation development and implantation, but shortly thereafter cell degeneration becomes apparent among both embryonic and extraembryonic cell types (*7*) (Fig. 2). This demonstrates the necessity of asparagine-linked oligosaccharides, either the complex type, hybrid type or high-mannose type, in early post-implantation development. Again the results of this experiment do not mean that asparagine-linked glycans are not required for preimplantation embryogenesis. Staining with E-PHA and also with concanavalin A reveals that asparagine-linked oligosaccharides reacting with these lectins persist in the mutant preimplantation embryos. Therefore, the second GPT gene may be present and/or a parental GPT source is available to the embryos.

Mice deficient in β-1,4-galactosyltransferase are born normally and are fertile (*8*, *9*). This result was surprising since the Galβ1-4GlcNAc structure is common in complex type asparagine-linked oligosaccharides, mucin oligosaccharides and glycolipids. However, residual β-1,4-galactosyltransferase and its products were found in the null mutant mice (*9*, *10*), and this finding promoted the discovery of a series of additional β-1,4-galactosyltransferases (*11*). The β-1,4-galactosyltransferase deficient mice show retarded growth and frequently die during the neonatal period (*8*, *9*). The neonatal lethality is ascribed to pituitary insufficiency due to the non-galactosylation of pituitary hormones (*8*) or to augmented proliferation and abnormal differentiation of epithelial cells (*9*).

A zona pellucida glycoprotein, ZP3, reacts with sperm and induces the acrosome reaction. The cell-surface localized long isoform of β-1,4-galactosyltransferase produced through alternative splicing has been considered to be a sperm receptor of ZP3. That β-1,4-galatosyltransferase deficient mice are fertile prompted reexamination of the role of cell-surface β-1,4-galactosyltransferase in fertilizaion (*12*). Sperm from the null mutants binds less ZP3, is unable to undergo the acrosome reaction in response to ZP3, and is less efficient in penetrating the egg coat (*12*). Therefore, the acrosome reaction involving β-1,4-galactosyltransferase is not essential for fertilization, but appears to facilitate it.

In the egg-sperm interaction, the α-galactosyl residue in

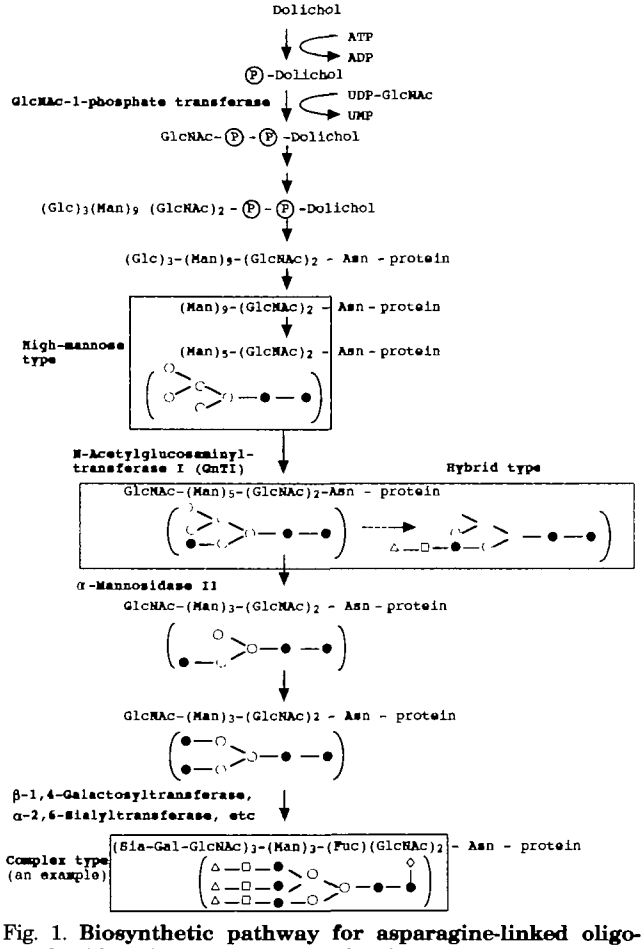

Fig. 1. **Biosynthetic pathway for asparagine-linked oligosaccharides.** The enzymes indicated to the left of arrows have been knocked out, Ⓟ, phosphate; ○, Man; ●, GlcNAc; □, Gal; △, Sia; ◇, Fuc.

TABLE I. **Gene targeting of glycosyltransferases and related enzymes.**

Enzyme	Deleted structure	Reference
β-1,4-*N*-Acetylgalactosaminyltransferase (GM2/GD2 synthase)	GalNAcα1-4Galβ1-4Glc-Cer 3 b NeuAcα2	*17, 18, 35*
GlcNAc-1-Phosphotransferase (GPT)	*cf.* Fig. 2	*4, 7*
N-Acetylglucosaminyltransferase I (GnT I)	*cf.* Fig. 2	*1, 2*
Core 2 β-1,6-*N*-acetylglucosaminyltransferase (C_2GnT)	Galβ1-3 (GlcNAcβ1-6)GalNAc	*30*
Fucosyltransferase VII (FuTVII)	Galβ1-4 (Fucα1-3) GlcNAc	*29*
β-1,4-Galactosyltransferase	Galβ1-4GlcNAc	*8, 9*
α-1,3-Galactosyltransferase	Galα1-3Galβ1-4GlcNAc	*13, 14*
Galactosylceramide synthase	Gal-Cer	*37*
Glucosylceramide synthase	Glc-Cer	*16*
α-Mannosidase II	*cf.* Fig. 2	*3*
α-2,3-Sialyltransferase I (ST3Gal I)	NeuAcα2-3Galβ1-3GalNAc	*31*
α-2,6-Sialyltransferase (ST6Gal)	NeuAcα2-6Galβ1-4GlcNAc	*36*
N-Deacetylase/*N*-sulfotransferase	[(2-SO_3^-)IdoAα1-4(6-SO_3^-) GlcNSO_3-α1-4]$_n$	*39, 40*
Heparan sulfate 2-*O*-sulfotransferase	[(2-SO_3^-)IdoAα1-4(6-SO_3^-) GlcNSO_3-α1-4]$_n$	*20*
Hyaluronic acid synthase 2 (HAS2)	(GlcAβ1-3GlcNAcβ1-4)$_n$	*24*

The deleted sugar or sulfate residues are underlined.

ZP3 was also considered to be important. The null mutant mice lacking α-1,3-galactosyltransferase cannot produce α-galactosyl residues in the Zona pellucida, as judged from the reactivity to *B. simplicifolia* isolectin B_4, but are still capable of reproduction (*13*). This finding is not consistent with the proposed role of the α-galactosyl residue in fertilization. Interestingly, the knockout mice develop cataracts, although they have normal organs (*14*).

The null mutant mice deficient in α-1,3-galactosyltransferase are good model animals, since the Galα1-3Gal antigen is the major xenoantigen in the case of pig-to-man organ transplantation. The hearts from genetically manipulated mice, which are devoid of the α-galactosyltransferase and overexpress complement regulatory factors, are less damaged by human serum as compared to those from control mice (*15*), implying that similarly manipulated pigs, if they become available, might be a source of organs for xenotransplantation.

Glucosylceramide synthase (GST) forms glucoylceramide, which serves as the precursor of both gangliosides and globo-series glycolipids (Fig. 3). Until E 6.5, null mutant embryos as to glucosylceramide synthase are indistinguishable from wild-type embryos, while before E 9.5 all null embryos die (*16*) (Fig. 2). Greatly enhanced apoptosis centered in the ectoderm at the gastrulation stage (E 7.5) appears to be the primary cause of the embryonic death. Embryonic stem cells homozygous for the deletion can differentiate into neuronal cells and erythrocytes *in vitro*. Upon subcutaneous inoculation, the mutant cells differentiate, but the components of well differentiated tissues such as smooth muscle and cartilage are less than in tumors derived from wild-type embryonic stem cells. These results verify that glycosphingolipids are indispensable for embryogenesis and cell differentiation.

β-1,4-*N*-Acetylgalactosaminyltransferase (GM2/GD2 synthase) is the key enzyme in the formation of complex gangliosides (Fig. 3). Null mutant mice deficient in this enzyme lack all complex gangliosides, but apparently develop normally (*17*). Therefore, some glycolipids among globo-series glycolipids, simple gangliosides, lactosylceramide, and glucosylceramide are essential for embryogenesis. The null mutant male mice as to GM2/GD2 synthase are, however, sterile and do not produce sperm (*18*). The levels of testosterone in the serum of these mice is very low, although testosterone production is unaffected. Testosterone accumulates in interstitial Leydig cells, indicating that complex gangliosides are essential for the transport of testosterone to the seminiferous tubules and bloodstream from Leydig cells.

Glycosaminoglycan chains are also required for embryogenesis. Heparan sulfate 2-*O*-sulfotransferase transfers sulfate to the 2 position of the iduronic acid residue of heparan sulfate (*19*). When the 2-sulfotransferase gene is disrupted by insertion mutagenesis, the homozygous mutant mice die in the neonatal period (*20*). Surprisingly, kidneys are absent in the mutant mice. Closer analysis has revealed that mesenchymal condensation around the ureteric bud and initiation of branching morphogenesis are

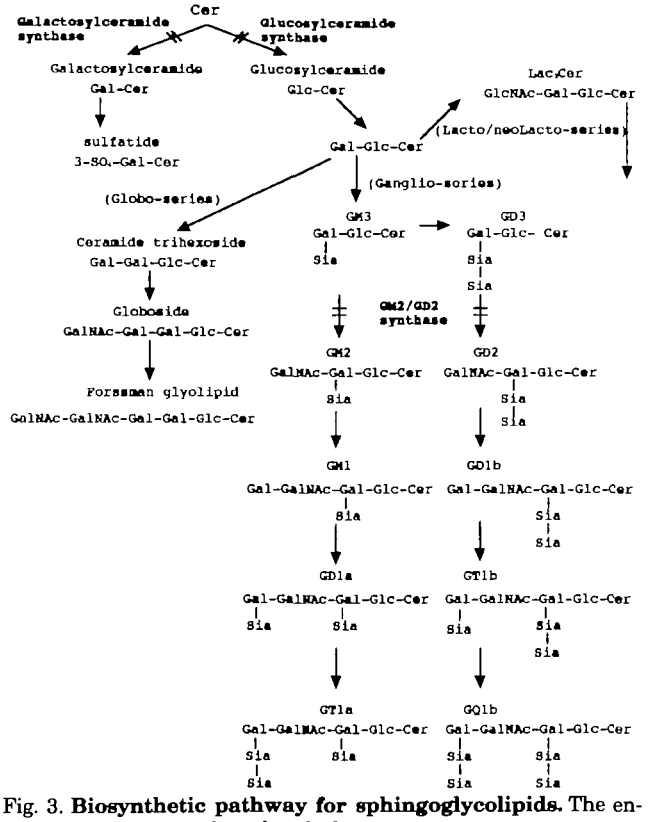

Fig. 3. **Biosynthetic pathway for sphingoglycolipids.** The enzymes indicated have been knocked out.

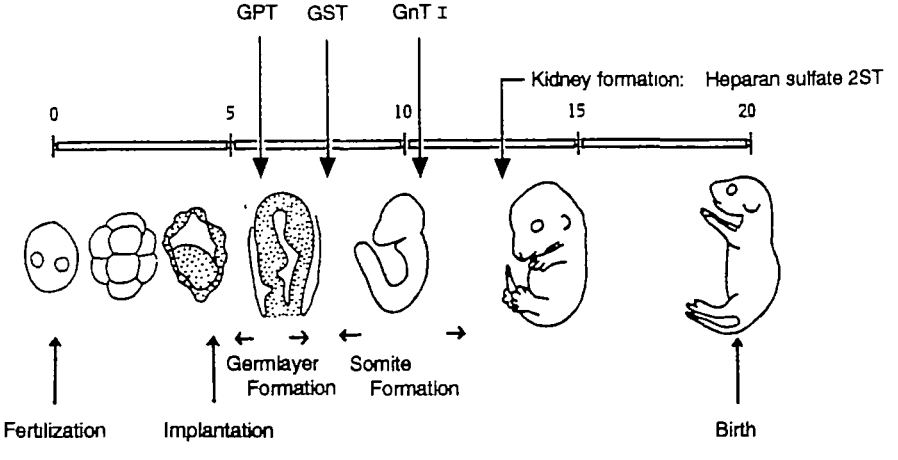

Fig. 2. **Blockage of mouse embryogenesis by deletion of a specific enzyme for glycan formation.** GPT, GlcNAc-1-phosphotransferase; GST, glucosylceramide synthase; GnTI, *N*-acetylglucosaminyltransferase I; ST, sulfotrans-ferase.

arrested in the mutants. Heparan sulfate is known to be required for signaling from fibroblast growth factors. Furthermore, genetic studies on *Drosophila* have revealed that heparan sulfate proteoglycans are important in development and that Wnt signaling requires heparan sulfate (*21*, *22*). Therefore, the absence of the specific 2-*O*-sulfate group in heparan sulfate of the mutant embryos is considered to interfere with the signaling required for kidney formation.

Perlecan is a heparan sulfate proteoglycan found in the basement membrane. About 40% of embryos deficient in perlecan die around E 10.5 with defective cephalic development (*23*). The remaining perlecan-deficient mice die just after birth, with skeletal dysplasia. The entirely different phenotypes of heparan sulfate 2-sulfotransferase-deficient mice and perlecan-deficient mice are consistent with the diverse roles of individual heparan sulfate proteoglycans and with the importance of the fine structures of the glycan chains in the function.

Hyaluronic acid in vertebrates is formed by hyaluronic acid synthase (HAS). Among three HAS, HAS 2 is essential for mouse embryonic development: HAS 2 null mutant embryos are lethal (*24*). CD44 is a hyaluronic acid receptor, and is widely expressed in various adult and embryonic tissues. However, CD44-deficient mice are not embryonic lethal: the vital functions of hyaluronic acid during embryogenesis may not be mediated by CD44 (*25*). The CD44-deficient mice exhibit an altered distribution of hematopoietic progenitors and develop exaggerated granuloma responses to *Cryotosporidium pavum* infection (*25*).

Leukocyte function

Selectins control leukocyte trafficking by mediating the initial adhesion of leukocytes to endothelial cells (*26*). L-Selectin on lymphocytes recognizes its ligand on high endothelial venules and regulates lymphocyte homing. E- and P-selectins expressed on endothelial cells upon inflammation recognize their ligands on leukcocytes and are involved in leukocyte recruitment to inflammatory sites. Sialyl Le^x is important in the ligand structures for all selectins. In the case of L-selectin, the sulfated structure 6-sulfo sialyl $Lewis^x$ serves as the ligand (*27*, *28*). Gene knockout of fucosyltransferase VII (FuTVII) has demonstrated that FuTVII is essential in the synthesis of ligands for L-, E-, and P-selectins (*29*). Selectin ligand deficiency in the null mutant mice is distinguished by blood leukocytosis, impaired leukocyte extravasation in inflammation and faulty lymphocyte homing.

Core 2 β-1,6-*N*-acetylglucosaminyltransferase (C2GnT) is commonly involved in the biosynthesis of serine/threonine-linked oligosaccharides (*O*-glycans). Mice lacking C2GnT exhibit a restricted phenotype with neutrophilia (*30*). Furthermore, loss of core 2 oligosaccharides reduces the ligands for E-, L-, and P-selectins assayed *in vitro*. Reflecting this reduction, neutrophil recruitment to sites of inflammation is impaired. However, lymphocyte homing to lymph nodes is not affected in the mutant mice. This result indicates that functional ligands for E- and P-selectins are on *O*-glycans with the core 2 structure, while L-selectin ligands are also present in other structures such as complex type glycans.

α-2,6-Sialyltransferase (ST6Gal) transfers sialic acid to the 6-position of the galactose residue in the *N*-acetyllactosamine terminus. The resulting Siaα2-6Galβ1-4GlcNAc structure is commonly found in complex-type glycans. The ST6Gal-deficient mice are born at a normal frequency and are fertile. The histology of various organs is normal, and a normal hematological profile is observed. However, in the mutant mice, B cell function is severely suppressed. The null mutant mice exhibit reduced serum IgM levels, and impaired B cell proliferation on IgM and CD40 crosslinking. The production of antibodies to both a T cell-independent antigen and a T cell–dependent antigen is severely affected in the mutant mice. CD22, which is a cell-surface located lectin recognizing sialic acid, is involved in regulation of the immune response in B cells. However, the phenotype of ST6Gal-deficient mice is more severe than that of CD22-deficient mice (*32–34*). Thus, sialic acid α2-6 linked to galactose is involved in B cell activation as the result of recognition by CD22 and also by other molecule(s).

In knockout mice lacking GM2/CD2 synthase, the sizes and cell numbers of the spleen and thymus are significantly reduced. T cells from the spleens of mutant mice show reduced proliferation upon IL-2 stimulation compared to in wild-type mice (*35*). The expression levels of IL-2 receptor α, β, and γ are almost equivalent in the mutant spleen cells and wild-type cells, but the activation of JAK1, JAK3, and SAT5 after IL-2 treatment is reduced in the mutant cells. This result suggests that complex gangliosides are necessary for proper functioning of the IL-2 receptor.

ST3GalI is a sialyltransferase forming a Siaα2-3Gal linkage on the serine/threonine linked disaccharide Galβ1-3GalNAc. Null mutation of the enzyme results in a deficiency of cytotoxic $CD8^+$ T lymphocytes by apoptosis (*36*).

As above, glycans with sialic acid residues play various important roles in the physiology of lymphocytes.

Functions of other tissues

Null mutant mice deficient in GM2/GD2 synthase show an apparently normal brain morphology (*17*). However, the neuronal conduction velocity from the tibial nerve to the somatosensory cortex is slightly reduced in the mutant mice as compared to in the wild-type mice. Upon aging, the knockout mice show marked degeneration and demyelination of the peripheral nerves, and poor regeneration of resected hypoglossal nerves, verifying that gangliosides are important in the maintenance and repair of nerve tissues (*37*, *38*).

Galactosylceramide synthase (ceramide galactosyltransferase) is essential in the formation of galactosylceramide and sulfatide (Fig. 3), both of which are abundantly present in myelin. Null mutant mice lacking the galactosyltransferase can still form myelin. However, these mice exhibit severe generalized tremoring, mild ataxia and conduction deficits (*39*). Moreover, the aged deficient mice develop progressive hindlimb paralysis and extensive vacuolation of the ventral region of the spinal cord. Therefore galactosylceramide and/or sulfatide is important for the function and maintenance of myelin.

De-*N*-acetylation and *N*-sulfation are critical steps in the biosynthesis of heparin and heparan sulfate. *N*-Deacetylase/*N*-sulfotransferase (NDST) catalyzes this reaction. There are three NDSTs, namely NDST-1, NDST-2, and NDST-3. Knockout of NDST-2 results in a loss of heparin in mast cells, but the heparan sulfate level in the liver is unchanged (*40*, *41*). Therefore, NDST-2 is the enzyme involved

in heparin biosynthesis, and NDST-1 and NDST-3 might compensate for the loss of NDST-2 in the synthesis of heparan sulfate. The mast cells in skeletal muscle of the null mutant mice fail to store specific granule proteases.

Decorin and biglycan are small, leucine-rich secreted proteoglycans. Deletion of the decorin gene results in an abnormal collagen fibril morphology and skin fragility (*42*). Mice deficient in the biglycan gene look normal at birth, but their growth rate is reduced, and the bone mass is decreased (*43*). Probably, glycosaminoglycan chains in these molecules are involved in their function, but this point remains to be clarified.

Comments

Precise analysis of carbohydrates remaining in the tissues of knockout mice is critically important, since the involvement of multiple enzymes in the formation of a glycosidic linkage has been revealed to be rather common. If the material to be analyzed is too small, immunohistochemical analysis with antibodies or lectins may be able to replace chemical analysis.

The demonstration of a defect after deletion of a glycosyltransferase is usually not the end of a story but the beginning of it. Except for a few examples such as selectin-ligand interactions, the molecular consequence of the altered carbohydrate structure as to the phenotype of the mutant mice is only partly understood. Thus, studies at the cellular and biochemical levels with specimens from knockout mice will be required in subsequent stages.

Drosophila and *C. elegans* are also useful organisms for revealing the *in vivo* function of glycoconjugates, because of the advancement of gene deletion techniques for these organisms and their short lifespans. Indeed, the critical roles of heparan sulfate proteoglycans during development have also been demonstrated in the *Drosophila* system (*21*, *22*). However, it should be remembered that many glycan structures are not conserved in vertebrates and invertebrates. Gene targeting in the mouse will continue to provide exciting results concerning the functions of glycans in intercellular recognition.

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