Enzymes involved in mammalian oligosaccharide biosynthesis

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Several new sialyltransferases, *N*-acetylgalactosaminyltransferase and fucosyltransferase genes have been reported in this past year. These sequences have advanced our understanding of the structural, functional and evolutionary relationships amongst the glycosyltransferases, including their roles in selectin ligand biosynthesis. Ablation of the murine *N*-acetylgalactosaminyltransferase I gene through gene 'knock out' technology has yielded insight into the role of this gene in the developing mouse. Novel 'O-linked' protein glycosylation events described in the past year have added to the substantial known diversity in the oligosaccharide structure and glycosyltransferase repertoire of mammalian organisms.

Current Opinion in Structural Biology 1994, 4:683–691

Introduction

Eukaryotic organisms synthesize oligosaccharide chains through the actions of glycosyltransferases, coupled with oligosaccharide-chain remodeling events catalyzed by glycohydrolases. These latter enzymes, including glucosidases and mannosidases, for example (reviewed in [1]), typically operate during the early stages of protein *N*-glycoside maturation (Fig. 1). By contrast, both early and late stages of oligosaccharide chain synthesis require glycosyltransferases [2–4]. With few exceptions, each glycosyltransferase can catalyze the synthesis of a single glycosidic linkage. Because recent work has indicated that multiple distinct enzymes can synthesize identical sugar linkages, there must be roughly a few hundred distinct glycosyltransferases, and corresponding genes, to synthesize the multitude of distinct oligosaccharides identified in mammalian species.

Molecular cloning efforts initiated in 1986 [5–7] have to date yielded the discovery and characterization of more than 30 different mammalian glycosyltransferase genes [5–20,21•–23•,24–32,33••,34••,35–48,49••,50,51,52••,53,54••,55••,56•]. Each of the glycosyltransferase genes or cDNAs cloned to date predict enzymes with a common topology, consisting of a short (less than 25 residues) amino-terminal cytoplasmic domain, a single transmembrane segment, and a larger carboxyl-terminal catalytic domain (generally more than 325 residues) in the lumen of the Golgi apparatus. Several Golgi oligosaccharide-processing enzymes also share this type II transmembrane topology, suggesting that this topology has an important function in these Golgi-resident proteins. Some, though not all, mammalian glycosyltransferases are themselves subject to post-translational processing by glycosylation; many are also processed into soluble forms through proteolytic events.

Despite the topological similarity of these glycosyltransferases, sequence analysis of cloned enzymes suggests that there is generally little, if any, detectable primary sequence similarity between members of enzymatically distinct glycosyltransferase families, although glycosyltransferases within rather small catalytically related families may share primary sequence similarity. There are, for example, no discernible significant primary sequence similarities between the human H blood group α(1,2)fucosyltransferase [25] (GDP-fucose: β-D-galactoside 2-α-L-fucosyltransferase) and any of the α(1,3)fucosyltransferases [33••,34••] (GDP-fucose:β-D-N-acetylglucosaminide 3-α-L-fucosyltransferases), despite the fact that these two classes use the nucleotide sugar substrate GDP-fucose, and can operate on identical oligosaccharide precursor substrates. By contrast, the α(1,3)fucosyltransferases share substantial amounts of primary sequence similarity. This review summarizes recent progress in the structural and functional definition of mammalian glycosyltransferases through molecular cloning approaches. We also discuss recent results obtained through genetic 'knock out' of the murine *N*-acetylglucosaminyltransferase-I gene, which encodes a key enzyme in the synthesis of complex type asparagine-linked glycosides. Current understanding of the relationship between selectin ligand synthesis and

Abbreviations

EGF—epidermal growth factor; **ELFT**—ELAM-1 ligand fucosyltransferase; **Fuc-T**—fucosyltransferase; **GlcNAc-T-I**—*N*-acetylglucosaminyltransferase-I; **PCR**—polymerase chain reaction.

 ISSN 0959-440X

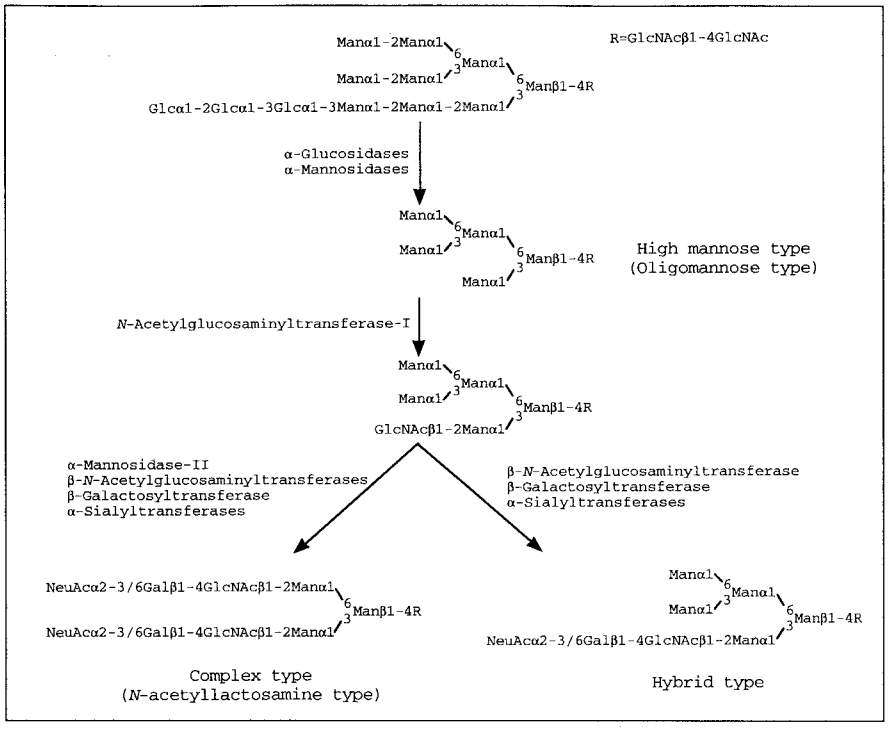

Fig. 1. The biosynthesis of *N*-linked oligosaccharides. Representative examples of each type are shown; microheterogeneity within each structural example yields many more structures than are shown here.

α(1,3)fucosyltransferases is outlined, with a discussion of recently described novel protein glycosylation modifications.

Recently isolated glycosyltransferase genes

Since the glycosyltransferases are generally non-abundant proteins, it has been difficult to purify these enzymes for molecular cloning purposes. Cloning methods have therefore been developed to allow the isolation of glycosyltransferase genes without the need for protein purification (reviewed in [3–5]). These include expression-cloning approaches, low-stringency hybridization methods, and polymerase chain reaction (PCR) cloning with primers derived from sequences conserved in catalytically similar glycosyltransferases. An expression cloning method originally developed by Seed *et al.* [57,58] (see also [59]) has been applied, with some modification, to glycosyltransferase cloning [15,24,26,29,34••,39,42,43]. This approach generally involves the screening of mammalian cDNA expression libraries transfected into a mammalian host cell, using a genetic selection or screen for a novel glycosylation phenotype. Selections or screens rely on antibodies or lectins capable of detecting a novel surface-localized glycosidic modification corresponding to the glycosyltransferase of interest. Although this method can select directly for a functional cDNA, its successful use requires host cells with the proper glycosylation phenotype (i.e. competent in the synthesis of the desired enzyme's precursor substrates, but deficient in the desired enzyme itself), and reagents that can detect, or select for, the oligosaccharide product of the desired enzyme.

Rapid and technically straightforward low-stringency hybridization methods have also been used to isolate new members of gene families encoding catalytically similar glycosyltransferases [28,30,32,33••]. This approach cannot be expected to yield genes corresponding to glycosyltransferases with catalytic properties distinct from the probe enzyme, however, because interfamily sequence comparisons suggest that these enzymes will maintain primary sequences virtually entirely distinct from the probe sequence. PCR cloning approaches have been used to circumvent this difficulty in some instances, where previous sequence comparisons have identified short conserved sequence motifs amongst a group of enzymes with shared, and distinct, catalytic properties.

Sialyltransferases

Biochemical experiments have indicated that mammalian organisms may encoded numerous sialyltransferases. Sequence analysis of the first several cloned sialyltransferases identified a conserved peptide motif in these enzymes (Fig. 2), embedded within otherwise dissimilar protein sequences. Paulson and co-workers [48] used this observation to design oligodeoxynucleotide PCR primers with degenerate sequences corresponding to the ends of the 'sialyl' motif [45]. These primers were then used with the PCR to amplify novel sequences from cDNA libraries. In one instance, this approach yielded a PCR product with a novel sequence, from a human placenta cDNA library. A cDNA subsequently isolated from the library with the PCR product was shown by sequence analysis and expresson studies to encode a novel α(2,3)sialyltransferase, termed STZ [54••].

The enzyme STZ can efficiently sialylate both glycoprotein and glycolipid substrates, to form the terminal sequences NeuAcα2-3Galβ1-3Gal*N*Ac and NeuAcα2-3Galβ1-4Glc*N*Ac. This enzyme cannot form the product NeuAcα2-3Galβ1-3Glc*N*Ac, nor can it operate on terminal Lewis x or Lewis a trisaccharide determinants to form sialyl Lewis x or sialyl Lewis a moieties. These results lend additional support to previous observations (cited in [28]) indicating that α2-3sialylation precedes a regulated α1-3fucosylation event in the ordered synthetic process of these two selectin ligands.

The Paulson group have also used this PCR approach to isolate another novel cDNA, termed STX, from a newborn rat brain cDNA library [49••]. The cDNA sequence predicts a protein with a type II topology and primary structure consistent with those of other sialyltransferases. However, attempts to demonstrate that the recombinant protein manifests sialyltransferase activity have failed so far, even though a relatively wide variety of acceptor substrates have been tested. Nonetheless, it is very interesting that the STX gene is transcribed in newborn rat brain, but not in the adult brain, nor in other organs, including the kidney, liver, spleen, intestine, submaxillary gland and lung. These observations suggest that this putative sialyltransferase may play a critical role in the developing central nervous system, an organ where substantial metabolic activity is devoted to ganglioside biosynthesis.

Tsuji and collaborators [55••] also used the PCR method to isolate novel sialyltransferase genes, including a chicken Gal*N*Ac α2,6-sialyltransferase sequence and two distinct murine and rat α(2,3)sialyltransferase genes [51,56•]. One of the products of the chicken sialyltransferase is the sialyl Tn determinant (NeuAcα2-6Gal*N*Acα1-Ser/Thr). This antigen has been identified in mammalian species as a 'cancer-related' epithelial cell antigen, and should be interesting to explore the expression, regulation, and function of the human counterpart(s) of this gene in human carcinomas.

One of the murine α(2,3)sialyltransferases cloned by Tsuji *et al.* (ST3GalA.1) [55••] is apparently the homologue of a porcine submaxillary gland Galβ1,3Gal*N*Acα-2,3sialyltransferase reported previously [47]. The other α(2,3)sialyltransferases, isolated from both mouse and rat, are novel Galβ1,3Gal*N*Ac α(2,3)sialyltransferases (ST3GalA.2). Although ST3GalA.1 and ST3GalA.2 exhibit roughly similar acceptor substrate specificities, they maintain distinct tissue-specific expression patterns. The *ST3GalA.1* gene is abundantly expressed in submaxillary glands, for example, whereas the *ST3GalA.2* transcripts are most prominent in the mouse brain and liver.

Sasaki *et al.* [52••] have recently reported the use of a modified expression-cloning method in the isolation of a human Galβ1,3/1,4Glc*N*Acα2,3-sialyltransferase gene. These investigators used a cytotoxic lectin [*Ricinus communis* agglutinin (RCA_{120}), which binds to β-galactosides], to select for a cDNA encoding a sialyltransferase that extensively masks the surface-localized β-galactoside toxin receptors on the transfected, lectin-susceptible mammalian host. The resulting cloned cDNA encodes an α(2,3)sialyltransferase capable of using both Galβ1-3Glc*N*Ac and Galβ1-4Glc*N*Ac. The sequence of this enzyme is essentially identical to the α(2,3)sialyltransferase cloned by Kitagawa and Paulson [54••], who reported that this enzyme is unable to use Galβ1-3Glc*N*Ac. This discrepancy has not yet been resolved.

N-acetylgalactosaminyltransferases

Synthesis of O-linked oligosaccharides on cell surface and secreted proteins is initiated by the covalent modification of some serines or threonines with *N*-acetylgalactosamine. This reaction is catalyzed by one (or possibly more) UDP-Gal*N*Ac:polypeptide *N*-acetylgalactosaminyltransferases. Two groups have independently reported the isolation of cDNA clones encoding the bovine form of a UDP-Gal*N*Ac:polypeptide *N*-acetylgalactosaminyltransferase purified from bovine colostrum, and expressed in the small intestine [22•] or placenta [23•]. This enzyme is predicted to maintain the type II transmembrane topology typical of other glycosyltransferases. Initial characterization of the acceptor substrate specificy of the recombinant form of the enzyme indicates that it transfers Gal*N*Ac to threonine residues at a rate more than 50-fold greater than transfer to serine residues in similar peptide sequence con-

hST3(G3/4GN)	157	GRRGIIVGNGGVLANKSKGSRIDDYDIVVRLNSAPVKGFEKDVGSKTT	204
mST3(G3GalNAc)-I	136	GRRGAVVGNSGNLKDSSYGPEIDSHDFVLRMNKAPTVGFEADVGSRTT	183
mST3(G3GalNAc)-II	149	GRRGAVVGNSGNLRGSGYGQEVDSHNFIMRMNQAPTVGFEKDVGSRTT	196
hST3(G4GN/3GalNAc)	116	GRRGVVVGNGHRLRNSSLGDAINKYDVVIRLNNAPVAGYEGDVGSKTT	163
cST6(GalNAc)	337	GISGAVVGNGGILNNSGMGQEIDSEDYVFRVSGAVIKGYEKDVGTKTS	384
hST6(G4GN)	181	WGRGAVVSSAGSLKSSQLGREIDDHDAVLRFNGAPTANFQQDVGTKTT	228
rSTX(?)	154	FQTGAIVGNSGVLLNSGCGQEIDTHSFVIRCNLAPVQEAYRDVGLKTD	201

Fig. 2. Comparison of amino acid sequences of sialyltransferases in the 'sialylmotif'. Their substrate specificities are shown in parenthesis: G, galactose; GN, *N*-acetylglucosamine. Numbers on their names indicate linkage positions of glycosidic bonds. References are (from top): hST3(G3/4GN), [45]; mST3(G3GalNAc)-I, [46]; mST3(G3GalNAc)-II, [51]; hST3(G4GN/3Gal*N*Ac), [49••]; cST6(Gal*N*Ac), [50]; hST6(G4GN), [40]; rSTX, [44]. Amino acids conserved in any three sequences are emphasized by outlined letters.

texts [22•,23•]. These observations suggest the existence of additional UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases.

An expression-cloning approach has been used recently to isolate a murine cDNA encoding a β(1,4)*N*-acetylgalactosaminyltransferase that operates on glycoproteins with the terminal oligosaccharide NeuAcα2,3Galβ1,4-GlcNAcβ1,3Gal [21•]. This enzyme forms an oligosaccharide epitope defined previously as the human Sd^a blood group antigen [NeuAcα2,3(GalNAcβ1,4)Galβ1,4-GlcNAcβ1,3Gal]. This latter antigen has also been defined independently as a functionally significant epitope (known as CT1 or CT2) on murine cytotoxic T cells. This murine CT β(1,4)*N*-acetylgalactosaminyltransferase shares a substantial degree of primary sequence similarity with a previously cloned human β(1,4)*N*-acetylgalactosaminyltransferase that creates a glycolipid antigen known as GM2 [20]. GM2 and the Sd^a antigen share the identical terminal tetrasaccharide moiety, but differ in their underlying substructure (glycolipid versus glycoprotein, respectively). An analysis of these two enzymes should provide information on their peptide sequences that allow discrimination between substrates with identical trisaccharide termini presented on otherwise dissimilar molecules. It will also be interesting to see whether the human Sd^a blood group locus corresponds to the mouse sequence, and to explore the functional role of the CT1/CT2 epitope in immune cells.

Ablation of the GlcNAc transferase-I gene in mice

The gene targeting method developed by Capecchi *et al.* [60,61] is a powerful technique that is widely used to study biological function(s) of molecules whose expression is regulated by a cloned gene. Two groups have independently used this approach to explore complex asparagine-linked (*N*-linked) oligosaccharides. A key regulatory step in the biosynthesis of these molecules is catalyzed by the glycosyltransferase termed *N*-acetylglucosaminyltransferase-I (GlcNAc-T-I; the product of the mgat-I locus; Fig. 1) [35,36,38]. These investigators created mice deficient in the *GlcNAc-T-I* gene through standard gene targeting procedures [62••,63••]. Mice heterozygous for one null mgat-I allele develop normally, and are without any obvious abnormal phenotype. By contrast, homozygosity for the null *mgat-1* allele yields a lethal embryonic phenotype. These mice die at approximately 11.5 days of gestation. Homozygous null embryos have no detectable GlcNAcT-I activity, and are also (as expected) completely deficient in hybrid and complex type *N*-linked oligosaccharides. Blastocyst and morula formation, compaction and implantation proceed normally without GlcNAc-T-I expression, suggesting that complex and hybrid type *N*-linked oligosaccharides are not essential for early mouse development. Null embryos are growth-retarded, however, have less than the normal number of somites and exhibit a defect in neuropore closure. Approximately half of the homozygous null embryos also exhibit a phenotype known as situs inversus, which apparently results from an inversion of the normal developmental rotational process that yields organ asymmetry. The mechanisms responsible for each component of this pleiotropic phenotype are not yet understood. These may eventually be shown to include disruption of essential *N*-linked oligosaccharide-dependent cell–cell recognition events during mid-embryogenesis, or a requirement for *N*-linked oligosaccharides in the proper folding, intracellular trafficking or turnover of developmentally important glycoproteins. In any event, most glycobiologists will be pleased that mature *N*-oligosaccharides (complex and hybrid types, at least) are necessary for proper mammalian development.

Fucosyltransferases and selectin ligand biosynthesis

Leukocyte adhesion to E- and P-selectins is an early and important step in the process of leukocyte extravasation. The sialyl Lewis x tetrasaccharide is an essential component of the counterreceptors for these two selectins (reviewed in [64,65]). The final step in the biosynthesis of the sialyl Lewis x tetrasaccharide is catalyzed by α(1,3)fucosyltransferases (Fuc-T) operating on α(2,3)sialylated *N*-acetyllactosamine type oligosaccharides [66] (Fig. 3). As noted above, there is as yet no precedent for an α(2,3)sialyltransferase activity capable of sialylating the Lewis x tetrasaccharide to form the sialyl Lewis x antigen (Fig. 3).

Five distinct human α(1,3)fucosyltransferase genes have been cloned and characterized [26–32, 33••,34••] (Table 1). Two of these, termed Fuc-TIV and Fuc-TVII, are transcribed in HL-60 cells [67•] and other leukocyte cell lines that express selectin ligands [27,28,33••,34••]. They are thus candidates for fucosyltransferases that control selectin-ligand biosynthesis in leukocytes. By contrast, transcripts corresponding to the Fuc-TIII, Fuc-TV and Fuc-TVI genes are not generally expressed to a significant degree in these cells. These observations, and genetic analysis of the Fuc-TIII and Fuc-TVI loci , exclude these latter three genes from such consideration.

Fuc-TIV [28,29] is also known as ELFT (ELAM-1 ligand fucosyl transferase) [27]. Characterizations of the *in vitro* catalytic properties of Fuc-TIV/ELFT indicate that it does not efficiently form the sialyl Lewis x tetrasaccharide from 3′-sialyl *N*-acetyllactosamine. Likewise, Fuc-TIV does not yield cell surface sialyl Lewis x expression when expressed in some cultured cell lines that can be converted to sialyl Lewis x positivity by transfection with other α(1,3)fucosyltransferase genes [28,29]. By contrast Goelz *et al.* [27] reported that Fuc-TIV/ELFT determines expression of E-selectin ligands and the sialyl Lewis x antigen when expressed in a dihydrofo-

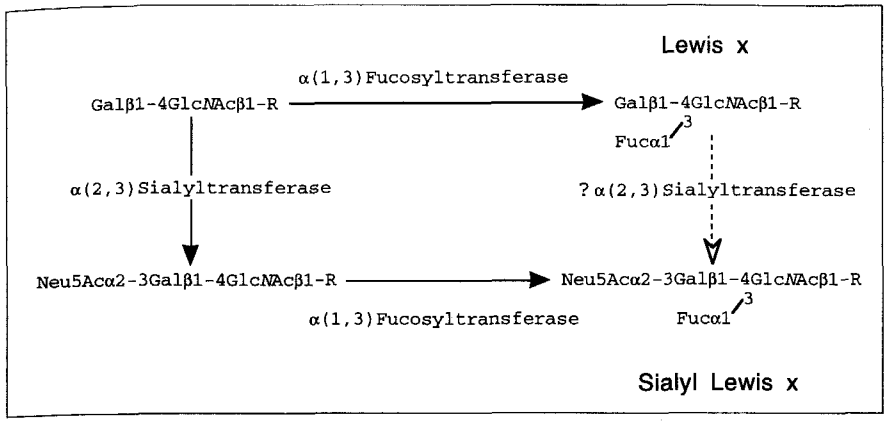

Fig. 3. The biosynthetic routes of the sialyl Lewis x determinant. The broken line indicates a hypothetical route that would require a sialyltranferase with activities not found in any known sialyltransferase.

late reductase-resistant Chinese hamster ovary cell line. Goelz *et al.* [68••] hypothesize that these discrepancies can be accounted for by differences in the glycosylation phenotype of host cells used in these types of transfection experiments. The biochemical basis of these differences is not yet known, nor is it known whether human leukocytes maintain a glycosylation phenotype capable of supporting Fuc-TIV-determined sialyl Lewis x expression.

The recent molecular cloning of another human α(1,3)fucosyltransferase gene, termed Fuc-TVII, suggests another, Fuc-TIV-independent, route for sialyl Lewis x expression in leukocytes [33••,34••]. The Fuc-TVII gene is expressed in several types of cultured human leukocytic cell lines. *In vitro*, the corresponding enzyme can efficiently use sialyl N-acetyllactosamine as an acceptor substrate to form the sialyl Lewis x tetrasaccharide. Furthermore, expression of this enzyme in COS-7 and Chinese hamster ovary cells leads to surface expression of the sialyl Lewis x antigen. Although these observations suggest two possible routes for the biosynthesis of E- and P-selectin ligands, the relative contributions of these routes to this process remains to be explored, as does the possibility that there are other paths also, using other unknown enzymes.

Novel *O*-linked oligosaccharides

Many mammalian glycoproteins contain complex carbohydrates linked through *O*-glycosidic linkages to some serine or threonine residues (Fig. 4). Although the understanding of the *O*-glycoside biosynthesis is less advanced than that of *N*-linked oligosaccharides, *O*-glycosides are believed to be as biologically important as the *N*-glycosides. L- and P-selectin oligosaccharide ligands are born on *O*-glycosides of mucin-like glycoproteins [69–75], for example, and Glc*N*Ac moieties linked directly to serine or threonine residues on many nuclear and cytoplasmic proteins are thought to be important regulators of protein–protein interactions (reviewed in [76]; see also Hayes and Hart, pp 692–696).

Recent advances in analytical methods for oligosaccharide structures have led to the discovery of several novel types of *O*-glycosides. Hase *et al.* [77–80] (see also [81]) have recently reported a xylosylglucose type sugar chain found on several blood clotting glycoproteins (Fig. 4). Although β-linked xylose residues are widely distributed as a core residue on proteoglycans, the xylosylglycans reported by Hase *et al.* are the first examples of α-linked xylose residues in mammalian glycoproteins. Each of

Table 1. Human α(1,3)fucosyltransferases.

	Fuc-TIII	Fuc-TIV (ELFT)	Fuc-TV	Fuc-TVI	Fuc-TVII
Classification	Lewis blood group type	Myeloid type	–	'Plasma type'	Leukocyte type
Potential *N*-glycosylation sites	2	2	4	4	2
Human chromosome	19	11q21	19	19	9
Catalytic products	Le^x, sLe^x, Le^a, sLe^a, VIM-2	Le^x, VIM-2	Le^x, sLe^x, VIM-2	Le^X, sLe^x	sLe^x
References	[26]	[27–29,90]	[30]	[32]	[33,34]

the xylosylglucose-type oligosaccharides found to date occur on the epidermal growth factor (EGF)-like domains on clotting factors or structurally-related glycoproteins [77–80]. The α(1,3)xylosyltransferase(s) and/or peptide: α-glucosyltransferases responsible for biosynthesis of these oligosaccharides are most probably expressed in hepatocytes, because the liver is the major site of synthesis of these particular coagulation factors. *O*-linked fucose residues have also been described on the EGF-like domains of some glycoproteins [82–85,86•,87••,88••]. The positions of attachment of fucose residues differ from the xylosylglucose attachment sites, and the spectrum of glycoproteins that display *O*-linked fucose residues differ from the set of proteins modified by xylosylglucose moieties [77–81]. Some of these *O*-linked fucose moieties are substituted with an *N*-acetylglucosamine residue, which is in turn modified by galactose and then sialic acid [87••,88••] (Fig. 4). The enzymatic basis for synthesis of these structures is an exciting and unexplored area, which will certainly lead to the discovery of novel peptide: α-fucosyltransferase, and related enzymes. The function(s) of these novel glycosidic structures also remains to be explored.

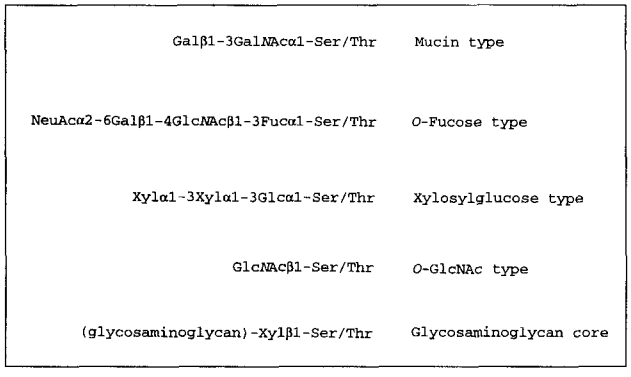

Fig. 4. Structures of *O*-glycosides linked to serine or threonine residues. Numerous other mucin-type oligosaccharide structures have also been described.

Future prospects and conclusions

Given the large number of predicted mammalian glycosyltransferases, continued growth can be expected in the number of these enzymes for which structures and biochemistry are defined through molecular cloning efforts. While the approaches discussed here will certainly continue to be useful, it may be necessary to develop novel techniques to obtain new glycosyltransferase genes that cannot be cloned with existing methods. This might include cloning methods based on the selection of, or screening for, an enzyme activity, instead of using selections or screens that are dependent upon synthesis of a new surface carbohydrate determinant recognized by antibodies or lectins.

We can also expect to see the discovery of additional novel oligosaccharide structures, analagous to the *O*-fucose and xylosylglucose type *O*-glycosides discussed above. These advances will be made possible by the use of recently developed, highly sensitive, methods for oligosaccharide structural analysis. These methods include matrix-assisted laser desorption mass spectrometry, for example, which can analyze picomole amounts of sample [89,90], and two-dimensional mapping of fluorescent-tagged sugar chains, which can detect femtomole amounts of glycoconjugate molecules [91,92].

In conclusion, the emerging array of cloned oligosaccharide-processing enzyme genes, coupled with new analytical technologies and molecular genetic approaches to the study of oligosaccharide function (e.g. transgenesis and gene targeting), open a bright and exciting future for glycobiology.

Acknowledgements

This work was supported in part by NIH grant 1R01GM47455. John Lowe is an Associate Investigator of the Howard Hughes Medical Institute.

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