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Exploring the glycan repertoire of genetically modified mice by isolation and profiling of the major glycan classes and nano-NMR analysis of glycan mixtures

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The production of mice with genetic alterations in glycosyltransferases has highlighted the need to isolate and study complex mixtures of the major classes of oligosaccharides (glycans) from intact tissues. We have found that nano-NMR spectroscopy of whole mixtures of N- and O-glycans can complement HPLC profiling methods for elucidating structural details. Working toward obtaining such glycan mixtures from mouse tissues, we decided to develop an approach to isolate not only N- and O-glycans, but also to separate out glycosphingolipids, glycosaminoglycans and glycosylphosphatidylinositol anchors. We describe here a comprehensive Glycan Isolation Protocol that is based primarily upon the physicochemical characteristics of the molecules, and requires only commonly available reagents and equipment. Using radiolabeled internal tracers, we show that recovery of each major class of glycans is as good or better than with conventional approaches for isolating individual classes, and that cross-contamination is minimal. The recovered glycans are of sufficient purity to provide a "glycoprofile" of a cell type or tissue. We applied this approach to compare the N- and O-glycans from wild type mouse tissues with those from mice genetically deficient in glycosyltransferases. N- and O-glycan mixtures from organs of mice deficient in ST6Gal-I (CMP-Sia:Galβ1–4GlcNAc α2–6 sialyltransferase) were studied by the nano-NMR spectroscopy approach, showing no detectable α2–6-linked sialic acids. Thus, ST6Gal-I is likely responsible for generating most or all of these residues in normal mice. Similar studies indicate that this linkage is very rare in ganglioside glycans, even in wild-type tissues. In mice deficient in GalNAcT-8 (UDP-GalNAc:polypeptide O-Ser/Thr GalNAc transferase 8), HPLC profiling indicates that O-glycans persist in the thymus in large amounts, without a major change in overall profile, suggesting that other enzymes can synthesize the GalNAc-O-Ser/Thr linkage in this tissue. These results demonstrate the applicability of nano-NMR spectroscopy to complex glycan mixtures, as well as the versatility of the Glycan Isolation Protocol, which makes possible the concurrent examination of multiple glycan classes from intact vertebrate tissues.

Key words: glycan/mice/glycosyltransferase/NMR

Introduction

Eukaryotic cells express several classes of oligosaccharides attached to proteins or lipids (Stults *et al.*, 1989; Ferguson, 1992; Furukawa and Kobata, 1992; Hart, 1992; Lis and Sharon, 1993; Margolis and Margolis, 1993; Hascall *et al.*, 1994; Schnaar, 1994; Varki and Freeze, 1994). Animal glycans can be N-linked via β-GlcNAc to Asn (N-glycans), O-linked via α-GalNAc to Ser/Thr (O-glycans), or can connect the carboxyl end of a protein to a phosphatidylinositol unit (GPI-anchors) via a common core glycan structure (Ferguson, 1992; Furukawa and Kobata, 1992; Hart, 1992; Lis and Sharon, 1993; Varki and Freeze, 1994; Burda and Aebi, 1999). In heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS) proteoglycans, the linkage region of the glycosaminoglycan (GAG) chain has a β-Xyl residue O-linked to Ser (Hascall *et al.*, 1994; Esko and Zhang, 1996). In contrast, keratan sulfate (KS) is a GAG chain extended from N-glycan or O-glycan cores (Margolis and Margolis, 1993; Hascall *et al.*, 1994; Iozzo, 1998). Nuclear and cytoplasmic glycosylation also exists, with the most common being β-GlcNAc-O-Ser/Thr (O-GlcNAc) (Holt *et al.*, 1987; Hart *et al.*, 1989). Glycosphingolipids (GSLs, often called glycolipids) have a glycan linked to a ceramide moiety via β-Glc (Glc-Cer) or β-Gal (Gal-Cer) (Hakomori, 1990; Zeller and Marchase, 1992; Sandhoff and Klein, 1994; Karlsson, 1995). Free glycan chains also occur, including sialyloligosaccharides (Kunz and Rudloff, 1993), hyaluronan (HA) (Laurent and Fraser, 1992), and degradation fragments of GAG chains (Bai *et al.*, 1997).

N-Glycans are grouped into high mannose, complex, and hybrid types (Furukawa and Kobata, 1992; Varki and Freeze, 1994; Stanley and Ioffe, 1995), with β-GlcNAc branches in complex N-glycans giving di- or multi-antennary units (Schachter, 1991). Vertebrate O-glycans include seven or more different core structures (Schachter and Brockhausen, 1992), and are often found in clusters on polypeptides called mucins (Verma and Davidson, 1994; Devine and McKenzie, 1992; Rose, 1992; Strous and Dekker, 1992). Cells may also contain lipid-linked oligosaccharide precursors from the N-glycan and GPI-anchor biosynthesis pathways (Ferguson, 1992; Furu-

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kawa and Kobata, 1992; Burda and Aebi, 1999). Neutral and acidic GSLs can have a variety of extensions arising from the Glc-Cer or Gal-Cer core regions (Hakomori, 1990; Zeller and Marchase, 1992; Sandhoff and Klein, 1994; Karlsson, 1995). While core regions are unique for each class, elongation can involve similar outer chains. For example, N- and O-glycans and GSLs often carry subterminal Galβ1–4GlcNAcβ1- (so-called LacNAc) units, which can sometimes be repeated (polylactosamines), or less commonly, Galβ1–3GlcNAcβ1- or GalNAcβ1–4GlcNAcβ1- (so-called LacdiNAc) units (Smith *et al.*, 1992; Baenziger, 1996). Some of these units are capped by sialic acid (Sia), Fuc, α-Gal, or β-GlcA (Chan *et al.*, 1991; Jungalwala, 1994; Varki and Freeze, 1994). Further diversity arises from modifications like sulfation (Smith *et al.*, 1992; Hooper *et al.*, 1996) and variations of Sia structure (Troy, 1992; Varki, 1992; Rutishauser, 1996; Kelm and Schauer, 1997). GPI-anchors can also carry various side chain substituents, including phosphoethanolamine units, Man, GalNAc, or Gal residues, or even sialylated LacNAc units (Ferguson, 1992). The outer regions of proteoglycans (Margolis and Margolis, 1993; Hascall *et al.*, 1994; Salmivirta *et al.*, 1996) are unbranched GAG chains, which are polymers of a disaccharide with a hexosamine and a uronic acid (GlcA or IdoA), or in the case of KS, Gal. With the exception of HA, such GAG disaccharides can be O-sulfated at various positions. In HS, some GlcNAc residues are N-de-acetylated or N-sulfated (Salmivirta *et al.*, 1996).

Since glycans are involved in interactions with the environment, other cells or bioeffectors (Rademacher *et al.*, 1988; Ferguson, 1992; Varki, 1993; Hakomori, 1990; Schnaar, 1994; Stanley and Ioffe, 1995; Baenziger, 1996; Crocker and Feizi, 1996; Gahmberg and Tolvanen, 1996; Rutishauser, 1996; Dennis *et al.*, 1999; Reuter and Gabius, 1999), their isolation and structural characterization are of great interest. However, there is limited information about the glycan repertoire of whole cells, tissues, and physiological fluids. Indeed, most reported structures of vertebrate N- and O-linked glycans have been from readily accessible sources such as mucins, plasma glycoproteins or erythrocytes. Recently, intact mammalian tissues such as brain and lung have been submitted to total N-glycan release, aiming to generate "tissue oligosaccharide libraries" (Wing *et al.*, 1992; Norgard-Sumnicht *et al.*, 1995; Fujimoto *et al.*, 1999). In two instances (Wing *et al.*, 1992; Norgard-Sumnicht *et al.*, 1995), such libraries included many unexplained anionic molecules. Thus, the oligosaccharide repertoire of mammalian organs may be even more extensive than summarized above. Investigators who study intact tissues typically focus on a specific class of glycans, and methods for their isolation and purification usually discard the other classes (Kobata and Furukawa, 1992; Hascall *et al.*, 1994; Rudd and Dwek, 1997; Geyer and Geyer, 1998). In some situations, it would be desirable to isolate all glycan chains of all major groups of glycoconjugates in parallel. Examples are mice (Marth, 1994) or cell lines (Esko, 1991; Stanley, 1992) with genetically engineered defects in glycosylation. To compare such mutants to the wild-type situation we have developed a "Glycan Isolation Protocol" for the isolation and fractionation of all major classes of glycans from vertebrate cells or tissues, and a novel nano-NMR approach to analyzing complex glycan mixtures. These approaches have been applied here to analyze tissues from two types of mice with genetically induced changes in glycosylation.

Results and discussion

Section 1. Developing a nano-NMR approach to analyzing glycan mixtures

NMR analysis of whole glycan mixtures from fetuin by ^{1}H-NMR spectroscopy. ^{1}H-NMR spectroscopy is regarded as a powerful technique for the structural characterization of glycopeptides or free glycans obtained from glycoconjugates (Vliegenthart *et al.*, 1983; Van Halbeek, 1993, 1994). However, complete structure determination by ^{1}H-NMR requires a glycan's availability in virtually pure state. Thus, the traditional approach has been to separate out each component for individual study by NMR spectroscopy. Despite its successes, this approach is limited in several ways: (1) separating closely related carbohydrate structures from each other, to the level of >95% purity, is a laborious effort at best, and simply impossible in many cases; (2) minor components in the mixture may be lost during the separation process, or cannot be recovered in amounts sufficient for NMR analysis; and (3) the quantitative relationship between various components of a mixture may get compromised during separation, making it difficult to obtain a global overview of the structural data, including the ratios of components in the starting mixture. Thus, we felt that there is enough to be gained from analyzing mixtures of glycans by ^{1}H-NMR to offset the decrease in detailed structural information that can be obtained from such complex spectra.

Mixtures of 2 to 3 glycans have been previously examined by NMR for full structural characterization (see, for example, Spellman *et al.*, 1989, 1991; Watson *et al.*, 1994), but only when complete separation had not been possible. The larger the number of component glycans in a mixture, the less information can be gained about each individual component. On the other hand, global structural information about the presence of certain epitopes and their relative ratios in a mixture of glycans should be attainable from the spectra of mixtures. For example, we reasoned that it should be possible to obtain partial structural information from ^{1}H-NMR spectra of whole complex mixtures of N- and O-glycans, particularly in cases where the purpose is to compare two closely related glycoprotein/glycopeptide samples. To deal with the issue of limited amounts of glycan samples, we used nano-^{1}H-NMR spectroscopy (at 500 MHz) which we have shown previously (Manzi *et al.*, 1995) to be capable of improving sensitivity up to 10-fold over conventional, 5 mm probe NMR at the same magnetic field strength. To test the concept, we initially studied bovine fetuin, a well-known glycoprotein with at least 30 structurally unique N- and O-glycan chains that have been extensively studied by many laboratories using diverse analytical methods (Baenziger and Fiete, 1979; Nilsson *et al.*, 1979; Takasaki and Kobata, 1986; Green *et al.*, 1988; Hardy and Townsend, 1988; Cumming *et al.*, 1989; Hermentin *et al.*, 1994; Ishii-Karakasa *et al.*, 1997; Kotani and Takasaki, 1997; Chen *et al.*, 1998; Mechref and Novotny, 1998). We obtained whole mixtures of N- and O-glycans from fetuin (Sigma) via automated hydrazinolysis in the N- and O-mode, followed by re-N-acetylation and clean-up. Starting from 2 mg of fetuin, the typical yield was ~100 μg

of mixed N- and O-glycans. Figure 1 shows the spectrum obtained when this glycan mixture was analyzed by nanoprobe ^{1}H-NMR spectroscopy at 500 MHz. The presence and relative abundance of certain structural elements could be assigned from the structural reporter group signals that remained individually observable, despite the complexity of the mixture. The following structural features could be confidently assigned: (1) the overall ratio of Siaα2–3Gal versus Siaα2–6Gal structural elements was determined to be 2:1, by integration of the triplets corresponding to H3ax of α2–3 and α2–6 Sia, at δ 1.80 and 1.72, respectively, and independently of the H3eq signals at δ 2.76 and 2.67, respectively; (2) the ratio of di-antennary versus tri-antennary N-glycans was estimated to be 1:3, based on the relative intensities of the Man H2 signals at δ 4.25 and 4.21, respectively; (3) Fuc, in either α1–6 or α1–3 linkage to GlcNAc residues or α1–2 linkage to Gal residues was undetectable, by virtue of the absence of Fuc CH_3 signals in the $1.17<\delta<1.28$ region; (4) Neu5Gc was not present, as judged from the absence of the typical, sharp CH_2 singlet at δ 4.11–4.12 (see below); and finally, (5) the apparent ratio of N- to O-glycans was found to be 3:1, based on integration of the H1 doublets of the α-anomers of the reducing-end GlcNAc and GalNAc residues at δ 5.18 and 5.21, respectively. (To reconcile this with the theoretical ratio of 1:1, it is not sufficient to just take into account that the ratios of α:β anomers for N- and O-glycans are 67:33 and 55:45, respectively (Herlant-Peers *et al.*, 1981; Spellman *et al.*, 1991); it is likely that the relative yield of intact O-glycans in the automated hydrazinolysis procedure was lower than that of the N-glycans). On the other hand, the spectrum did not give unambiguous evidence regarding the occurrence of the Neu5Acα2–6(Galβ1–3)GlcNAc structural element, which is known to be present in a small proportion of the triantennary N-glycans (Green *et al.*, 1988; Cumming *et al.*, 1989); nor was it possible to distinguish between Siaα2–3Galβ1–3 and Siaα2–3Galβ1–4 elements, or between Siaα2–6Gal and Siaα2–6GalNAc units. The NMR parameters for each of the aforementioned elements are known to be unique (Vliegenthart *et al.*, 1982; Bernard *et al.*, 1983; Lecat *et al.*, 1984; Green *et al.*, 1988), yet their differences are apparently too subtle to be observable in the spectrum of this mixture. Nonetheless, the data indicate that, at the level of glycan complexity present in a glycoprotein like fetuin, it is possible to study a whole mixture of N- and O-glycans by ^{1}H-NMR spectroscopy and obtain substantial structural information.

Section 2. Development of a glycan isolation protocol for tissue and cells

To apply this novel NMR approach towards comparing glycosylation in normal and mutant mouse tissues, it is necessary to obtain mixtures of N- and O-glycans in a relatively pure state (i.e., free of other glycan types and of other ^{1}H-containing cellular molecules). Also, mutations in glycosyltransferases could cause expected or unexpected changes in other classes of glycans, e.g., in GSLs. Thus, in developing a generally applicable approach, we decided to attempt the concurrent extraction, fractionation and isolation of all of the major classes of glycans from cells and tissues. The envisaged approach would allow the characterization of any cell type or tissue by the relative proportion of structural species within each of the major classes of glycans that it harbors, i.e., it would provide a

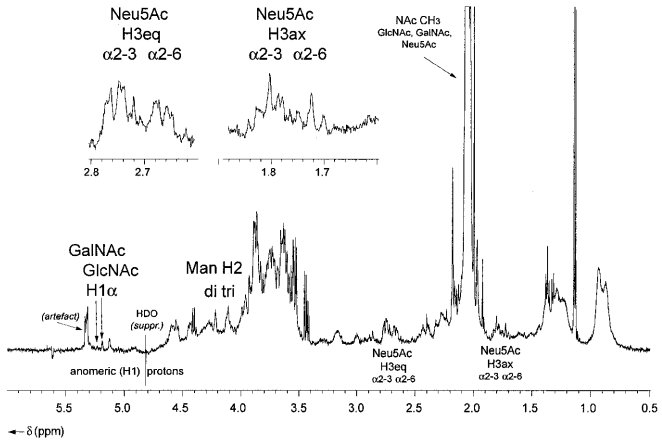

Fig. 1. ^{1}H-NMR spectrum of the "whole mixture" of N- and O-glycans released from bovine fetuin (2 mg) by hydrazinolysis. The spectrum was recorded at 500 MHz and 27°C, in 1000 transients, using a ^{1}H-nanoprobe holding the sample in 40 µl D_2O (pD 6.5). The HDO signal (at δ 4.76) was selectively suppressed by presaturation. Monosaccharide and Sia composition analyses indicated that the sample contained ~100 µg of glycan material. Assignments are given for selected structural reporter group signals, including those of some H1and the Man H2, and in the inserts for Neu5Ac H3eq and H3ax atoms.

"glycoprofile" of the cell or tissue. Alternatively, if the immediate aim is to isolate a particular class of glycans, the approach should leave the other classes in a form that could be stored and examined at a later time, as needed. Another goal was to design an approach that, with minimal modification, would be generally applicable to a wide range of biological samples (i.e., cells, organs, body fluids) where combinations of different glycoconjugates are present. The ideal protocol should satisfy the following criteria: maximum possible recovery of each group of glycans, without any selective losses of particular subsets present in the starting material; minimal contamination of one group of glycoconjugates with another; assure stability of the most labile sugar residues (e.g., Sias), and the most labile modifications (i.e. sulfate esters, phosphate esters, O-acetyl esters); and individual fractions should be obtained in a form that does not impair subsequent glycan release and/or profiling.

The Glycan Isolation Protocol described here comes close to satisfying these criteria. The overall scheme is outlined in Figure 2, and described in detail under *Materials and methods*. It involves the initial extraction of GSLs and other glycolipids (along with phospholipids and sterols), SDS-mediated solubilization and subsequent proteolysis of denatured glycoproteins and proteoglycans, recovery of GPI-anchors as lipid-linked glycopeptides, and finally, the separation of N- and O-glycan containing glycopeptides from high-charge GAG-containing glycopeptides. Glycans from each fraction can subsequently be released and profiled, and selected components of each can be subjected to detailed analysis. Along the way, fractions containing low-molecular-weight molecules like monosaccharides, sugar nucleotides, free oligosaccharides, and small glycopeptides can be saved separately. Certain variations and optional extensions of the protocol are feasible (see Figure 3). As described below, the approach was validated using a set of radiolabeled standards representing each of the major classes

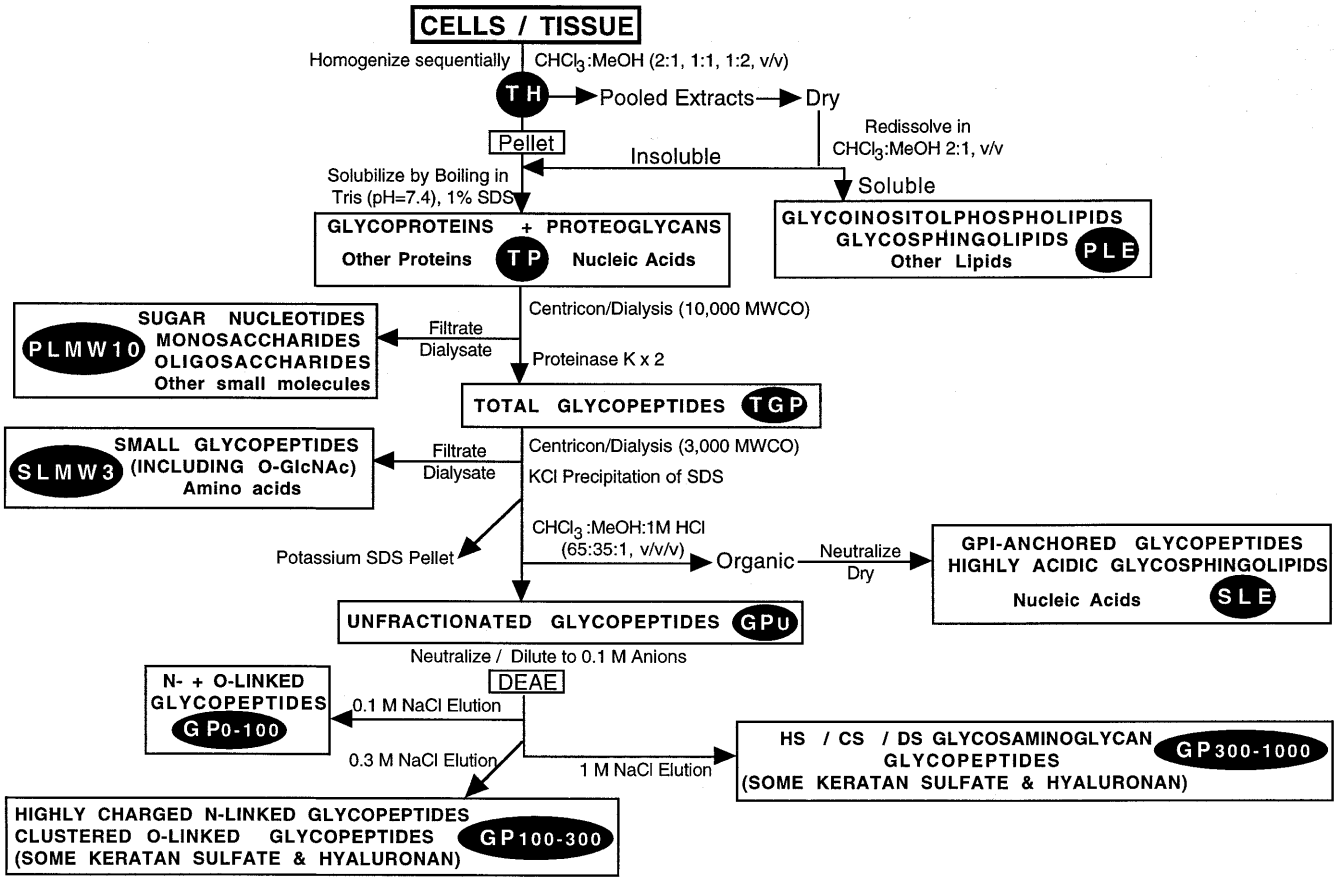

Fig. 2. Schematic outline of the Glycan Isolation Protocol for preparation of the major classes of glycans. The steps involved in the optimized protocol are shown. For details of each step see *Materials and methods* and *Results and discussion*. The nomenclature of the individual fractions is as follows: TH, Total homogenate obtained after the initial homogenization of the sample in organic solvents; PLE, primary lipid extract, obtained after removing all material insoluble in organic solvents; TP, total glycoproteins and proteoglycans, after solubilization of the delipidated pellet in SDS; PLMW10, primary low-molecular-weight fraction obtained by ultrafiltration of the SDS extract against a 10,000 MW cutoff membrane; TGP, total glycopeptides, after Proteinase K digestion, before ultrafiltration; SLMW3, secondary low-molecular-weight fraction obtained by ultrafiltration of the Proteinase K digest against a 3,000 MWCO membrane; SLE, secondary lipid extract, obtained by organic extraction after SDS precipitation; GP_U, total unfractionated glycopeptides, after secondary organic extraction, before DEAE; GP_{0-100}, glycopeptides running through/eluting from DEAE in 100 mM NaCl; $GP_{100-1000}$, glycopeptides eluting from DEAE in 1000 mM NaCl after 100 mM NaCl elution; $GP_{100-300}$, glycopeptides eluting from DEAE in 300 mM NaCl after 100 mM NaCl elution; $GP_{300-1000}$, glycopeptides eluting from DEAE in 1000 mM NaCl after 300 mM NaCl elution.

of glycans to be separated from each other. First, we will briefly outline the rationale for the major decisions taken in setting up the approach.

Determining the extraction sequence and other conditions. Using normal mouse thymus, we initially tested two alternatives: extracting lipids first and then solubilizing the proteins for glycopeptide preparation; or preparing glycopeptides first and later partitioning the lipids into an organic solvent. With the second approach, ~40% of glycopeptides were extracted with the lipids. Lipid extraction was therefore established as the first step. An added advantage is that all biologically active proteins (e.g., glycosidases) from a tissue would be inactivated by organic solvent denaturation at the outset. Also, GPI-anchors attached to proteins would not be extracted, allowing separation at a later stage, after proteolysis. A minor disadvantage is the possibility that some highly charged GSLs might not be extracted in the initial organic phase. However, these would be recovered after proteolysis in a second acidified organic extract, along with GPI anchors. During the initial phases of developing the approach, the recovery of radiolabeled standards representing each of the major classes of glycans was studied at various steps, either when used as pure populations or when spiked into a typical tissue (kidney or brain) prior to extraction. This allowed rational decisions to be made regarding the modification of various steps, to maximize recovery and separation. By maintaining pH 7.4 and room temperature (RT) throughout the protocol we also prevented losses of labile groups like O-acetyl esters (Varki and Diaz, 1984).

Separating protein- or lipid-bound glycans from soluble glycans. Cells and body fluids contain pools of monosaccharides, sugar phosphates and sugar nucleotides and occasionally, free glycans. Such small molecules are best removed or separated from protein-bound glycans prior to protease treatment, either by dialysis using a large molecular weight cut-off (MWCO) membrane, or by membrane ultrafiltration. We found no

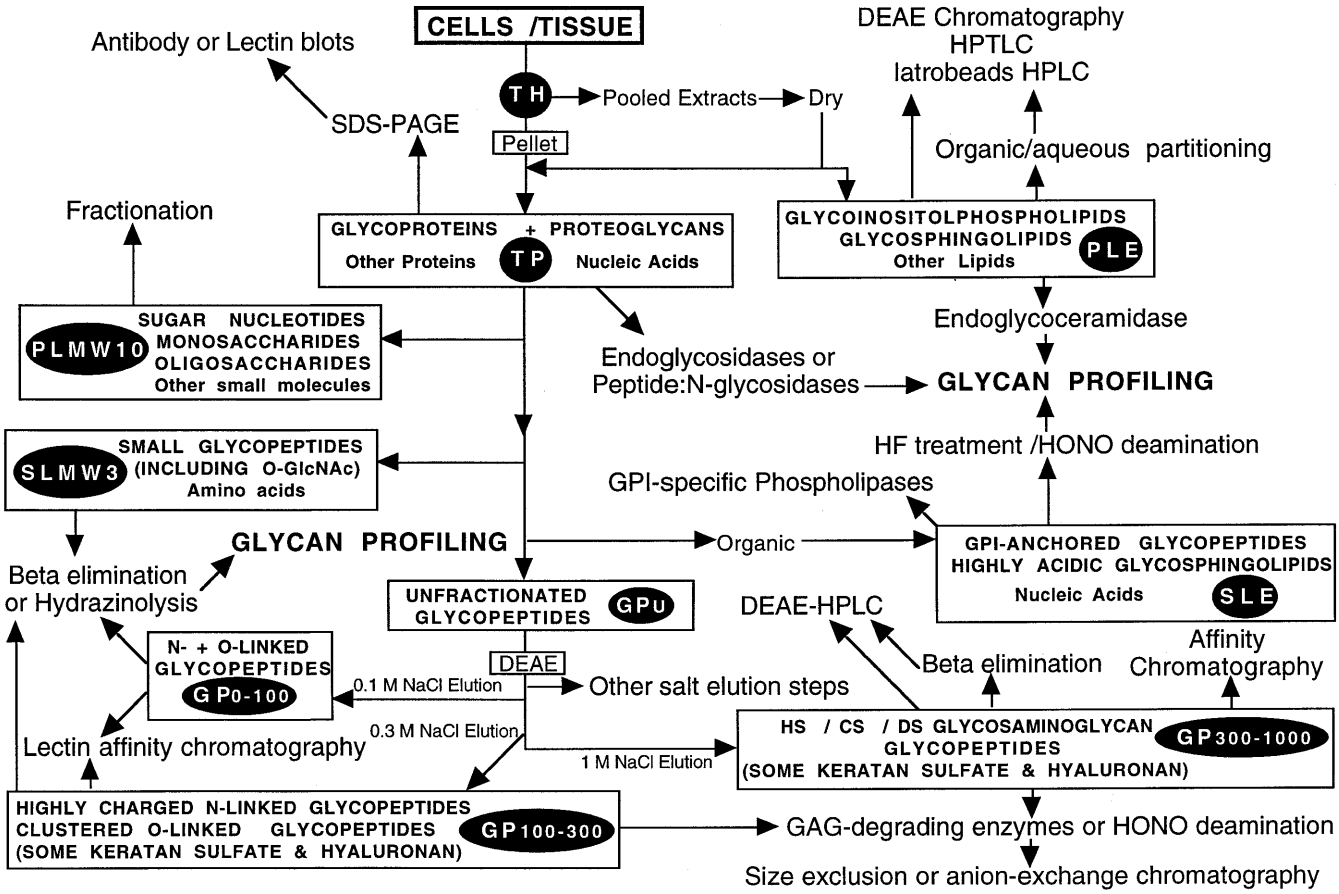

Fig. 3. Options for extending or modifying the Glycan Isolation Protocol. The major steps involved in the optimized protocol are shown (as in Figure 2), along with some of the possible options for further purification and/or profiling of each fraction. For further details, see *Materials and methods*.

difference between conventional dialysis and ultrafiltration on membrane cartridges for elimination of small molecules, or for the subsequent separation of small glycopeptides from the larger species (see below). Ultrafiltration is advantageous as the initial step when analyzing biological fluids that contain free oligosaccharides (e.g., milk) allowing them to be recovered in a reasonable volume for further fractionation and analysis. Likewise, during isolation of small O-type glycopeptides after the proteolysis step, it is convenient to have them recovered in the small volume obtained by ultrafiltration. If recovery of either of these low-molecular-weight fractions is not of interest, conventional dialysis is simpler, albeit with greater risk of accidental sample loss.

Approaches for glycopeptide production. To obtain reliable separations based on intrinsic properties of the glycans, the size of attached peptide needs to be minimized. Initial experiments using mouse brain or kidney as carriers for radiolabeled standards indicated similar effectiveness of Proteinase K and Pronase, as long as SDS was used to ensure complete solubilization. Recombinant Proteinase K was adopted since, unlike Pronase, it has no contaminating enzymatic activities. In the absence of SDS, complete solubilization of the proteins denatured by organic extraction was difficult. Also, while recovery and distribution of N- and O-glycans from a standard glycoprotein ([^{3}H-sialyl]fetuin) was good even without SDS, radiolabeled GPI-anchor glycopeptides and mucin-type glycopeptides were recovered in the wrong fractions (data not shown). The poor recovery of GPI-anchor glycopeptides in the acidic organic extract is probably because attached proteins could not be completely digested without SDS denaturation.

Essentially complete solubilization of the delipidated samples was observed with 1% SDS for all cells and tissues studied. Furthermore, SDS could be effectively removed as a potassium salt precipitate after proteolysis. We did explore alternatives to SDS such as reduction-alkylation with dithiothreitol followed by iodoacetamide. While subsequent GPI extraction after proteolysis occurred at acceptable levels, the recovery of the ^{35}S-labeled GAG-type glycopeptides was surprisingly reduced, evidently due to a ~20% loss of sulfate esters. Although this was not observed with dithiothreitol alone, it was not practical to maintain a reducing agent throughout the protocol (including the DEAE column elution). Also, random exposure to an oxidizing environment could allow artifactual disulfide bond formation between free thiols. Finally, the reducing agent could not be easily eliminated from the fraction containing the small O-type glycopeptides. For all these reasons, reduction alone or reduction-alkylation were not

viable alternatives or adjuncts to denaturation of nonreduced proteins by SDS. Of course, reduction-alkylation could always be done on the isolated DEAE glycopeptide fractions of interest at the end, if this were necessary to optimize certain separations.

Recovery and distribution of sialic acids and other monosaccharides in different fractions obtained using the optimized protocol. To demonstrate the feasibility and efficacy of the approach, we studied two types of tissues from the mouse: brain (a "soft" tissue, very rich in lipids) and kidney (a "hard" tissue, enriched in epithelial cells and extracellular matrix). The optimized protocol was applied to such samples, and aliquots obtained at each step (see Figure 2) were monitored for total Sia content (by the DMB-HPLC assay after saponification of O-acetyl esters and mild acid hydrolysis). Aliquots of the final fractions were also subjected to strong acid hydrolysis and studied for monosaccharide content by HPAEC-PAD (this was not feasible with some of the less pure intermediate fractions, because of interfering impurities). Representative results of these analyses are shown in Tables I and II. The distribution of Sias, neutral monosaccharides and hexosamines was generally appropriate for the predicted composition of the individual fractions. Because of the sensitivity of the DMB-HPLC assay and the lack of interference by other molecules, routine monitoring of Sia recovery was most useful to ascertain protocol reproducibility at different steps. Overall glycan recoveries from the starting homogenate ranged from 45–85%, with improved recoveries typically seen after an operator had gained experience with the protocol.

Recovery and distribution of standard glycoconjugates submitted to the optimized protocol. Bovine fetuin (which contains various kinds of sialylated O-glycans and N-glycans) was radiolabeled in the Sia side chains via mild periodate/$NaB[^3H]_4$ reduction, and then either subjected directly to automated hydrazinolysis for release of glycans, or mixed with unlabeled brain tissue homogenate and put through the optimized protocol, followed by automated hydrazinolysis of the glycopeptide fractions. As expected, essentially all of the glycans were recovered either in the second ultrafiltration (SLMW3, the small O-glycans, containing ~1/3 of the recovered radioactivity), or in the fraction eluted with 100 mM NaCl from DEAE ($GP_{0–100}$, comprising the remainder of the O-glycans and all the N-glycans, containing the remaining ~2/3 of the recovered radioactivity). The combined HPAEC profile of recovered glycans was similar to that of the starting material, indicating no major selective loss or structural change of any of the N- or O-glycans during the process (data not shown).

To directly monitor the recovery of individual classes of glycans in the presence of total tissue materials, 10,000 c.p.m. of radiolabeled standards representing each major class of glycan (see *Materials and methods*) were added to separate aliquots of the minced tissues (40–70 mg wet weight brain/aliquot, 35–60 mg wet weight kidney/aliquot), and the mixtures were submitted to the optimized protocol. As shown in Table III, the distribution of radioactivity indicates a reasonable recovery of the standards in the expected fractions, with relatively little cross-contamination (some differences in relative distribution do occur, depending upon whether the "carrier" tissue is brain or kidney). An exception was that a portion of the mucins were recovered in the $GP_{100–1000}$ fraction, instead of the $GP_{0–100}$ fraction. This is presumably because the clustered O-linked glycans on the mucins have a charge density similar to that of the GAGs (this problem can be partly alleviated by adding a 300 mM NaCl elution step prior to the 1 M NaCl step, see Figure 2, and below). The lowest yield was seen for the GPI anchors, where about ~50% of the starting material was found in the SLE fraction. However, this is not much worse than the yield obtained using the conventional protocol (extraction with Triton X-114) for isolating these glycans (~70% in our hands, data not shown). We also noted that the standard ^3H-labeled GAGs derived from CHO cells (namely, CS and HS glycopeptides) underwent some degradation during the protocol. As shown in Table III, ~20% of the radioactivity was found in the PLMW10 fraction, instead of in the $GP_{100–1000}$ fraction as expected. This is not an artifact due to the quality of the standard or to fragmentation occurring during the homogenization (based on control data with pure standards, not shown). Thus, significant degradation had occurred during the short period of time after mixing the standard into the minced tissue, and prior to adding organic solvent (typically 5–10 min), presumably due to endogenous GAG-degrading enzymes. We have not pursued this matter further, but it indicates that endogenous glycan-degrading enzymes must be considered, especially at the early stages of the protocol.

Table I. Recovery of sialic acids from mouse brain and kidney tissues at each step of the optimized Glycan Isolation Protocol

Fraction	Total sialic acids in fraction (nmol/100 mg wet tissue)		
	Prep 1	Prep 2	Prep 3
Brain			
PLE	111.0	65.0	102.0
TP	80.0	66.0	84.0
PLMW10	3.0	1.2	2.6
SLMW3	0.8	0.6	5.1
SLE	0	3.3	1.1
$GP_{0–100}$	36.0	22.5	39.0
$GP_{100–1000}$	1.2	1.3	5.4
Kidney			
PLE	69.0	39.0	41.0
TP	102.0	47.0	88.0
PLMW10	0.1	0	2.6
SLMW3	7.1	2.0	2.4
SLE	0.9	0.3	0
$GP_{0–100}$	63.0	26.0	27.0
$GP_{100–1000}$	7.4	4.3	4.6

Samples of normal mouse brain (300–450 mg wet weight) and kidney (150–350 mg wet weight) were subjected to the optimized Glycan Isolation Protocol (Figure 2). Aliquots at each step were subjected to base treatment, mild acid hydrolysis and derivatization with DMB to analyze Neu5Ac plus Neu5Gc content ("total sialic acids") as described under *Materials and methods.* See Figure 2 for abbreviations.

Table II. Monosaccharide composition of final fractions obtained after subjecting mouse brain and kidney tissues to the optimized Glycan Isolation Protocol

Fraction	Monosaccharides (mole %)					
	Fuc	GalN	GlcN	Gal	Glc	Man
Brain						
PLE	1.6	8.5	0	79.9	9.9	0
SLE	0	13.0	4.3	37.0	18.6	27.2
GP_{0-100}	8.7	2.8	33.7	23.6	11.4	19.9
$GP_{100-1000}$	2.8	1.1	12.3	32.4	32.2	19.3
Kidney						
PLE	2.7	5.3	8.2	39.7	37.9	6.2
SLE	4.4	12.0	15.7	28.5	16.5	23.2
GP_{0-100}	13.4	1.8	31.6	27.1	1.8	24.3
$GP_{100-1000}$	1.5	1.4	7.0	31.5	33.4	25.2

Aliquots of final fractions obtained from mouse brain and kidney samples subjected to the optimized protocol (Figure 2) were analyzed for monosaccharide composition by HPAEC-PAD after strong acid hydrolysis, as described under *Materials and methods*. The data represent the average of three separate purifications. Xylose was also detected in small quantities, primarily in the $GP_{100-1000}$ fractions (data not shown, precise quantitation is difficult because of overlap with the larger mannose peak). Uronic acids were detected only in the $GP_{100-1000}$ fraction (not shown). Glucose is an expected environmental contaminant in these analyses, and its relative quantity is high when analyzing very small quantities of sample (e.g., $GP_{100-1000}$). However, in some fractions, glucose is expected as a normal component of the major glycoconjugate class (e.g., PLE). See Figure 2 for abbreviations.

Overall, these data indicate that with minor imperfections, the optimized Glycan Isolation Protocol yields acceptable recovery and level of purity when known standards are added to tissues prior to fractionation. It is reasonable to expect that the endogenous glycans will behave in a similar fashion to the added-back standards. Further evidence that this is indeed the case is provided in the companion paper (Norgard-Sumnicht *et al.*, 2000), where metabolically radiolabeled endogenous glycans from cultured cells are recovered using the same protocol. Studies were also performed using other glycoconjugates such as HA and KS, monitoring recoveries through the individual steps with appropriate assays (data not shown). The overall conclusion from these studies is that the Glycan Isolation Protocol as described under *Materials and methods* is capable of achieving most of the original objectives: near-complete separation of the various classes of glycans, with a recovery of each of them comparable to conventional methods optimized for the isolation of individual classes. However, some critical comments are in order. First, small free oligosaccharides and sugar nucleotides partition partially into the PLE fraction. If desired, dialysis or ultrafiltration can be used to recover these molecules from this fraction. Second, glycopeptides with clustered O-linked chains (mucin-type glycans), which are protected from complete proteolysis by the high density of glycosylation, do not separate completely from the GAG chains. However, by eluting the DEAE-column in a step-wise fashion with 300 mM NaCl after the 100 mM NaCl step, it is possible to achieve substantial separation of these molecules from HS and CS chains (depending upon the degree of sulfation of the latter chains). Alternatively, β-elimination can release both types of chains before separation on the basis of size and/or charge. Finally, other molecules with extensive size/charge heterogeneity (i.e., HA, KS) are distributed amongst the DEAE fractions. However, this is actually an advantage, since such small GAG chains would be missed when using conventional strategies aimed at isolating "typical" molecules.

Contamination of the various fractions by other cellular constituents. Various low-molecular-weight components of cells (salts, nucleotides, amino acids, etc.) are recovered during the initial ultrafiltration/dialysis step and/or in the PLE fraction along with the monosaccharides, sugar nucleotides, and small free oligosaccharides. Very hydrophobic proteins (so-called "proteolipids") might be found in the PLE fraction. Except for regions carrying clustered O-linked chains, all remaining water-soluble polypeptides are completely digested by Proteinase K after boiling in SDS. Of course, small portions of peptide remain attached to the GPI-anchors and to the N- and O-glycans and to GAG chains. Most of the cellular phospholipids and neutral lipids are found in the initial organic extract, and the remainder are recovered in the second acidic organic extraction (as detected by HPTLC, data not shown). Fortuitously, all of the nucleic acids were recovered in the SLE (secondary lipid extract; see Figure 4), presumably because the acidification of the extraction mixture protonates the phosphodiester bonds in DNA and RNA. Regardless of the reason, the extraction of nucleic acids at this step assures that they do not contaminate the GAG chains, which are recovered later with a high salt elution from the DEAE column.

Options, variations and extensions of the approach. Depending on the question at hand, several options and variations of the Glycan Isolation Protocol can be used to achieve specific purposes (see Figure 3 and *Materials and methods*).

Profiling of released oligosaccharides. Various profiling methods were used to monitor the success of the protocol (see *Materials and methods*). In the remainder of this paper, we will focus mainly on profiling the N- and O-glycans of mouse tissues. Optimization and automation of hydrazinolysis has allowed uniform and simultaneous release of both N- and O-linked glycans in acceptable yield (Patel *et al.*, 1993). HPAEC is a suitable profiling strategy for such free N- and O-glycans derived from the low-charge (GP_{0-100}) glycopeptide fraction, since even isomeric forms differing in only one linkage can be resolved from each other (Hardy and Townsend, 1994). Indeed, as shown in Figure 5, the purity of the N- and O-glycans released from this fraction by automated hydrazinolysis is sufficient to obtain a clean HPAEC-PAD profile that is relatively reproducible from preparation to preparation. Also, different tissues give markedly different profiles of released oligosaccharides.

Some variations in the profiles between individual samples from the same tissue do occur (see Figures 5 and 6 for two separate examples). This is most likely explained by individual biological variation, e.g., recent data indicate that expression patterns of sialyl linkages in the rat brain change with aging (Sato and Endo, 1999). Another possibility is the relative amount of the hydroxylated Sia, N-glycolyl-neuraminic acid

Table III. Recovery of radioactivity from standard glycans added to mouse tissues prior to carrying out the optimized Glycan Isolation Protocol

^{3}H-Labeled standard added to each tissue	% of recovered radioactivity						
	PLE	PLMW10	SLMW3	SDS Pellet	SLE	$GP_{0–100}$	$GP_{100–1000}$
Brain							
Fetuin	1.7	3.6	**31.4**	0	0.2	**62.8**	0.4
G_{D1a}	**100**	0	0	0	0	0	0
Mucin	0	0	0	0	0	**70.7**	**29.3**
GAG	0	21.4	5.4	0	0	0	**73.2**
O-GlcNAc	0	0	**100**	0	0	0	0
SLac	51.2	**48.8**	0	0	0	0	0
GPI	0	0	0	0	**87.7**	0	12.3
GIPL	**100**	0	0	0	0	0	0
Kidney							
Fetuin	0	1.3	**46**	0	1.7	**50.5**	0.4
G_{D1a}	**100**	0	0	0	0	0	0
Mucin	0	0	0	0	0	**69.5**	**30.5**
GAG	0	17.6	0	0	0	0	**82.4**
O-GlcNAc	0	0	**100**	0	0	0	0
SLac	26.9	**69.7**	3.4	0	0	0	0
GPI	0	0	0	0	**89.5**	10.5	0
UDP-GalNAc	14.3	**85.7**	0	0	0	0	0

Aliquots (5000–10,000 c.p.m.) of ^{3}H-labeled glycan standards (see *Materials and methods*) were added into 50–100 mg samples of minced normal mouse brain or kidney. After 2–10 min, the mixtures were homogenized in the first organic solvent, and then subjected to the optimized Glycan Isolation Protocol (Figure 2), and aliquots of each final fraction analyzed for radioactivity. Overall recovery of the input radioactivity was >50% in all cases. The boldface numbers indicate the fractions where the radioactivity is predicted to be recovered, based upon known physical properties.

(Neu5Gc). Molecules containing this residue instead of N-acetyl-neuraminic acid (Neu5Ac) show markedly higher retention times on HPAEC-PAD. Thus, relatively small changes in the percentage of Neu5Gc could significantly change the profile. In this regard, we have noted that not all the Neu5Gc present in the original sample is transformed into Neu5Ac during the hydrazinolysis/re-N-acetylation procedure (data not shown). Thus, a complementary approach is the enzymatic release of the Sias followed by profiling of the underlying neutral oligosaccharides by HPAEC-PAD using a gradient designed to separate such chains.

Section 3. Application of the glycan isolation protocol to transgenic mice

Analysis of glycans from mice with a null mutation in the ST6Gal-I sialyltransferase. Following disruption of a gene encoding a specific glycosyltransferase in intact mice, the question arises if the predicted change in glycosylation does indeed occur in the genetically altered animal. The failure to find the predicted change in glycosylation in a particular tissue would indicate the presence of other enzymes capable of producing the same structure, while a complete loss of the pertinent structural element would prove that the gene deleted is the only one capable of biosynthesizing it in the tissue/cell type being examined. For example, the genetic disruption of the then only known β-galactosyltransferase (Asano *et al.*, 1997; Li *et al.*, 1997) did not result in elimination of all β-Gal residues (Kotani *et al.*, 1999), because of the existence of several additional isoforms of this enzyme (Lo *et al.*, 1998). Furthermore, since most major glycoconjugate classes are synthesized in the Golgi-ER pathway and compete for the same sugar nucleotide pools, the question arises if unexpected changes occur in other classes of glycans. The protocol described above is presented as a first step towards addressing these issues.

ST6Gal-I (CMP-Sia:Galβ1–4GlcNAc α2–6 sialyltransferase) is the only mammalian enzyme currently known to generate the Siaα2–6Gal linkage and its minimally required acceptor is Galβ1–4GlcNAcβ (Tsuji, 1996). Despite the widespread constitutive or regulated expression of ST6Gal-I in various cell types (O'Hanlon *et al.*, 1989; Wang *et al.*, 1989; Jamieson *et al.*, 1993; Lo and Lau, 1996) the phenotypic consequences of ST6Gal-I disruption in the mouse appeared to be limited to B cells, which also appeared to lose expression of the Siaα2–6Gal glycan structural element as determined by loss of binding by Sia specific lectins (Hennet *et al.*, 1998). It is conceptually possible that there are other ST6Gal enzymes in other tissues and cell types, transferases that could partly or fully compensate for the lack of ST6Gal-I expression. To ascertain the occurrence of glycans terminating in Siaα2–6Gal

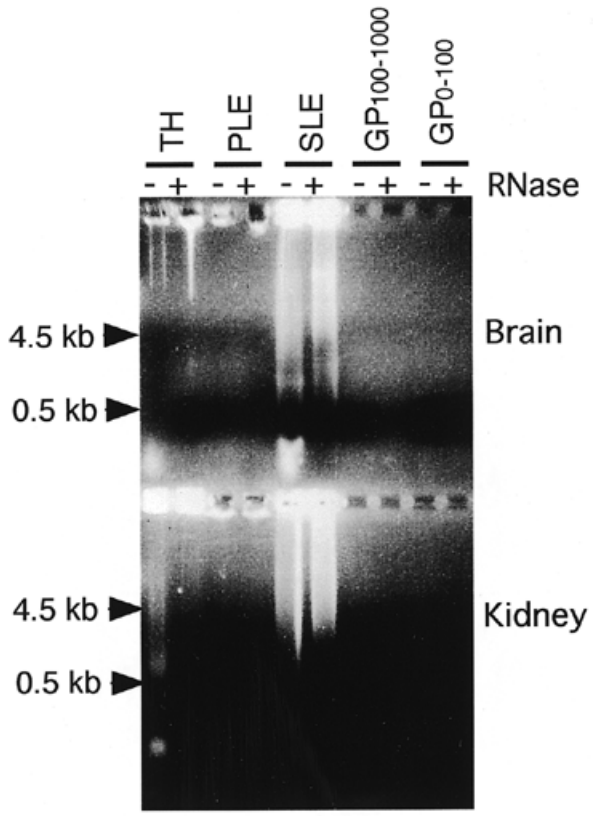

Fig. 4. Recovery of DNA and RNA in various fractions. Samples of mouse kidney (268 mg) and brain (379 mg) were subjected to the optimized protocol, and the recovery of DNA and RNA was monitored in 2.5% aliquots from each step by electrophoresis on 0.8% agarose gels, followed by ethidium bromide staining. Samples were analyzed with or without treatment with 1 µl (10 µg) of heat-treated RNase in a total volume of 5 µl at 37°C for 30 min. Loading buffer (1 µl, 6×) was added to each sample and the mixture loaded directly onto the agarose gel.

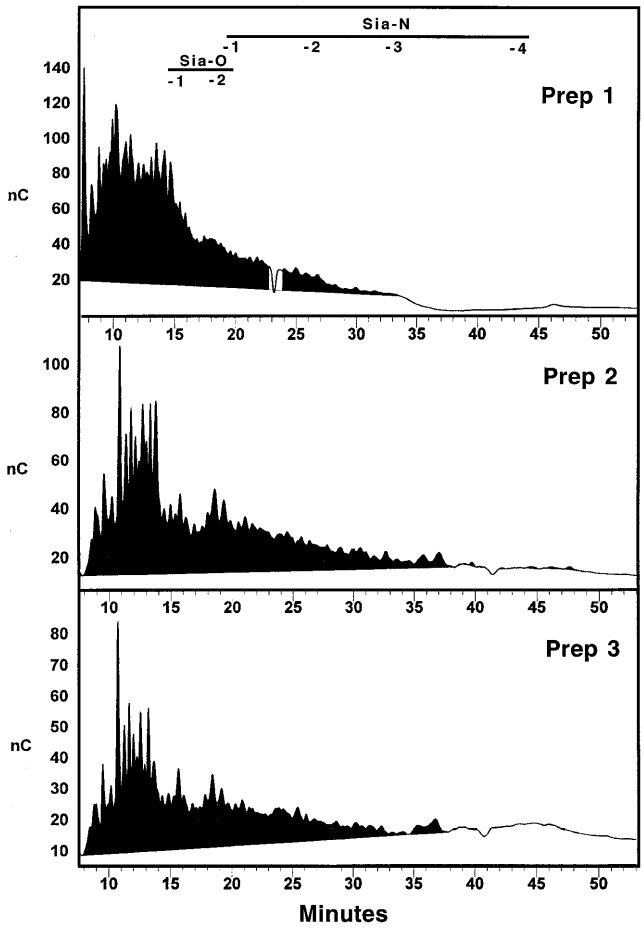

Fig. 5. Profiling of the N- and O-glycans recovered from mouse brain samples using the Glycan Isolation Protocol. N- and O-glycans were released by automated hydrazinolysis from $GP_{0–100}$ fractions derived from three independent samples of normal mouse brain subjected to the optimized protocol. Aliquots of the released glycans were profiled by HPAEC-PAD as described under *Materials and methods*. The markers indicate the expected elution positions of O-glycans with 1 or 2 sialic acids (Sia-O) or N-glycans with 1, 2, 3, or 4 sialic acids (Sia-N).

in tissues other than B-cells, we studied the $GP_{0–100}$ and $GP_{100–300}$ fractions derived from the liver, brain, and kidney of normal and ST6Gal-I null (homozygote and heterozygote) mice. Since these fractions contain many structurally distinct glycans, they were not amenable to fractionation and purification of each individual component. However, as shown in Figure 7, ^{1}H-NMR analysis of the glycopeptide mixtures present in the $GP_{0–100}$ fractions of normal and mutant mouse liver indicated that the ST6Gal-I deficient mouse tissue lacks any detectable α2–6 linked Sia, whereas α2–3 and α2–6 linked Sia in the corresponding glycans from control tissues occur in the ratio of approximately 1:1. Not only do these fractions represent mixtures of many glycans (more than 50, as seen by HPAEC-PAD of these fractions after hydrazinolysis), they contain the glycans in peptide-bound form. Despite the added complication posed by the presence of amino acid signals in the ^{1}H-NMR spectra, the relatively narrow line width and the characteristic patterns and well-defined positions of Sia H3ax and H3eq signals in the spectra enabled us to establish the presence of Siaα2–3Gal and Siaα2–6Gal units. Furthermore, it was possible to come to this conclusion with only ~200 µg of the glycopeptide mixtures. Indeed, removal of the peptide portions by automated hydrazinolysis and sample clean-up gave glycan mixtures that did not give better quality spectra by ^{1}H-NMR spectroscopy. Furthermore, the loss of glycan material during hydrazinolysis was ~50%, and the removal of ^{1}H containing contaminants was apparently incomplete. The NMR spectra of the liver $GP_{100–300}$ fractions (not shown) revealed the same trend as those of the $GP_{0–100}$ fractions: no Siaα2–6 was detectable in the material from ST6Gal-I null mice, whereas the spectrum of the wild-type material showed a minor proportion (~5%) in α2–6 linkage. The 500 MHz ^{1}H-NMR spectra of the $GP_{0–100}$ fractions from kidney and brain tissue (not shown) gave the same result as with liver. Taken together, these data indicate that ST6Gal-I is responsible for generating most or all of the Siaα2–6Gal residues on N- and O-glycans in these tissues of normal mice (the lower limit of sensitivity for detection of α2–6 linked Sia is 2–3% compared to the level of α2–3 linked Sia). Thus, direct ^{1}H-NMR analysis of complex mixtures of glycans can be an adequate tool to assess changes in the levels of specific Sia units in sialyltransferase-null

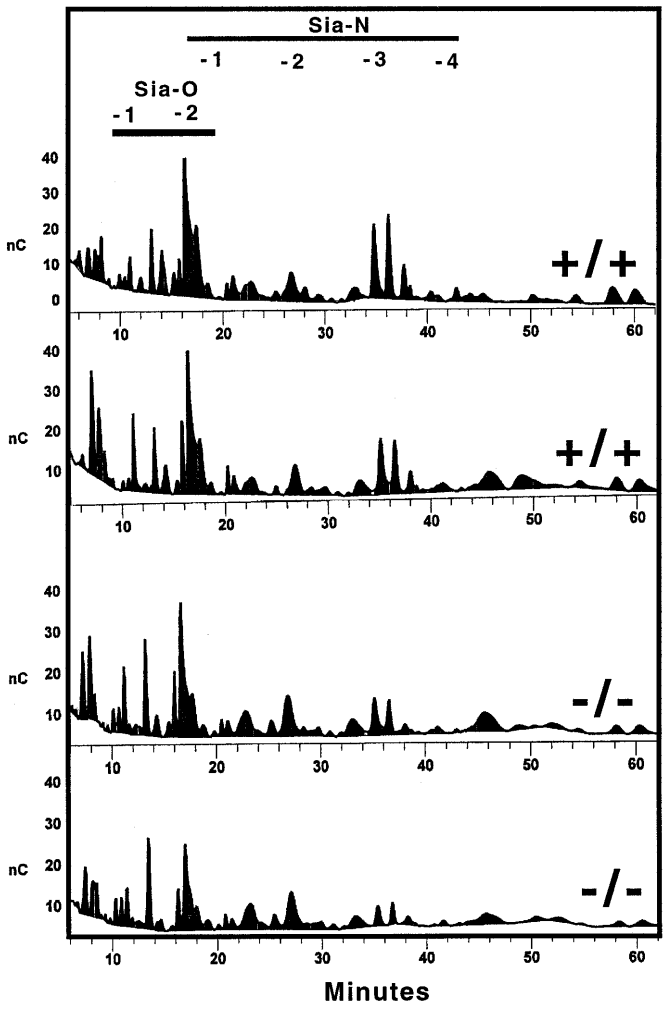

Fig. 6. N- and O-glycan profiles from thymocytes of normal and polypeptide GalNAcT-8 deficient mice. N- and O-glycans released by automated hydrazinolysis from the total glycopeptides (GP_U) obtained from mouse thymocytes were profiled by HPAEC-PAD as described under *Materials and methods*. Two examples each of the profiles covering the anionic glycan elution region are shown, from the mutant animals (–/–) and from wild-type littermates (+/+). The markers indicate the expected elution positions of O-glycans with 1 or 2 sialic acids (Sia-O) or N-glycans with 1, 2, 3, or 4 sialic acids (Sia-N).

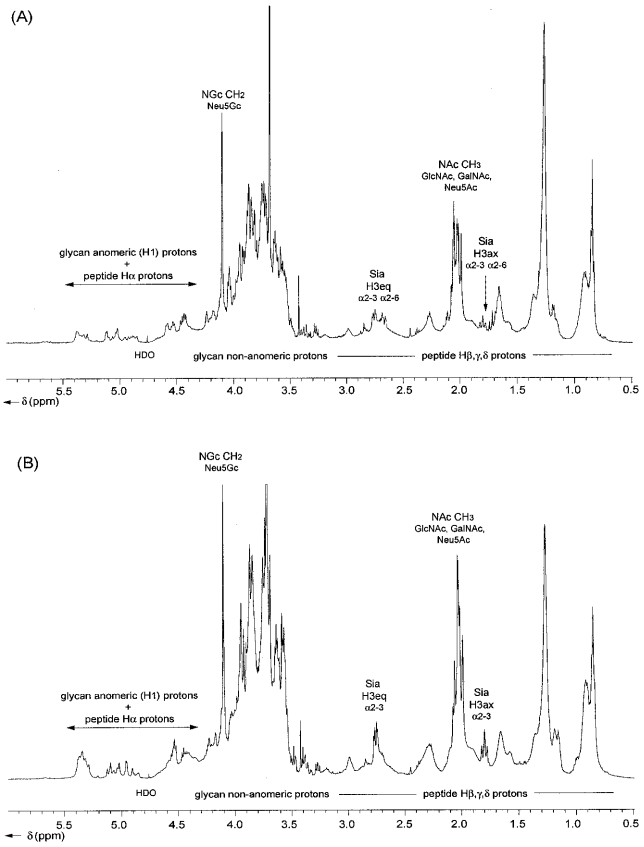

Fig. 7. ^{1}H-NMR spectra of "N- and O-glycopeptides" (fraction $GP_{0\text{-}100}$) obtained from liver of (**A**) control (+/+) and (B) ST6Gal-I null (–/–) mice. The spectra were recorded at 500 MHz and 27°C, in 1280 transients, using a ^{1}H-nanoprobe holding the sample in 40 µl D_2O (pD 6.5). The HDO signal (at δ 4.76) was selectively suppressed by presaturation. Each sample was obtained from 500 mg (wet weight) liver tissue; monosaccharide and Sia composition analyses indicated that each sample contained ~225 µg of glycan material. Assignments are given for Sia H3ax and H3eq signals, and for selected other structural reporter groups. The lack of the Siaα2–6Gal structural element in the $GP_{0\text{–}100}$ glycans from ST6Gal-I (–/–) liver is evident from the missing H3ax and H3eq signals at δ 1.72 and 2.67 in trace B.

mouse tissues, and to measure the relative ratios of sialic acids in different types of linkages.

The ST6Gal-I product is not detectable in normal mouse liver gangliosides. While the ST6Gal-I acceptor Galβ1–4GlcNAcβ1- is not found in mammalian brain GSLs, it is abundant in extraneural gangliosides (Stults *et al.*, 1989). However, the Siaα2–6Galβ1–4GlcNAcβ1- product seems to be generally rare in the GSLs of normal human tissues, and is reported primarily in fetal and malignant samples (Taki *et al.*, 1992a,b). Thus, it has been suggested that there may be a different enzyme responsible for generating this structure on GSLs (Taki *et al.*, 1992a). To address whether there was a change in relative abundance of Sia linkage types in GSL glycans between ST6Gal-I control and null mice, we enriched the PLE fractions from brain and liver in larger-chain GSLs by Folch partitioning. This step is justified since the minimal length of a lacto-type ganglioside structure potentially affected by the mutation is a pentaglycosyl ceramide, namely, Siaα2–6Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer; this component and all larger/branched lacto-type gangliosides end up in the PLE Folch upper phase. After release by endoglycoceramidase, the mixtures of larger-size GSL glycans were subjected to ^{1}H-NMR spectroscopy. The spectra shown in Figure 8 indicate that α2–6 linked sialic acids are hard to detect (i.e., they represent less than 2–3% of the α2–3 and α2–8 linked sialic acids combined) in the mixtures of GSL-derived oligosaccharides obtained from brain and liver PLE of control mice. While the absolute amounts of ganglioside oligosaccharides obtained per 100 mg of wet tissue from mouse brain far outweighed those from liver, the amount of GSL glycans obtained from 2 g of mouse liver was well within detection limits of nano-^{1}H-NMR spectroscopy. The apparent absence of Siaα2–6Gal containing gangliosides from liver in wild-type mice is remarkable, considering that ST6Gal-I expression in liver is very prominent, and glycoprotein-bound α2–6 linked sialic

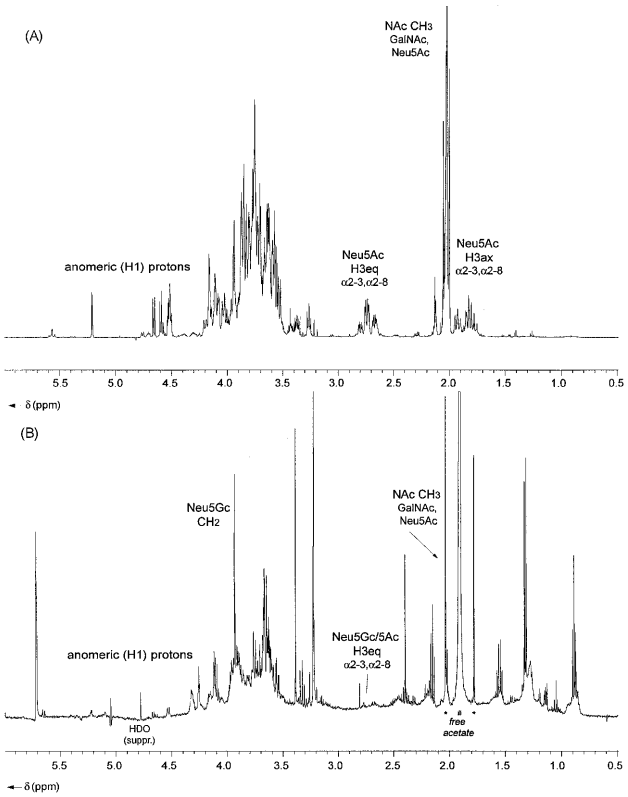

Fig. 8. ^{1}H-NMR spectra of "larger-size GSL glycans" obtained from (**A**) brain and (**B**) liver of ST6Gal-I control (+/+ and +/–) mice. The glycans were obtained by endoglycoceramidase digestion of Folch partitions of PLE fractions, as described in *Materials and methods*. The spectra were recorded at 500 MHz and 27°C, in 256 and 8000 transients, respectively, using a ^{1}H-nanoprobe holding the sample in 40 µl D_2O (pD 6.5). The samples were obtained from 100 mg (wet weight) brain tissue and 2080 mg (wet weight) liver tissue, respectively. The samples contained ~100 and ~40 nmol of total Sia, respectively. Assignments are given for Sia H3ax and H3eq signals, and for selected other structural reporter groups.

acids are quite common in liver (compare Figure 7). Thus, α2–6 linked sialic acids are very rare in the total GSLs of normal mouse brain and liver. We are currently searching for a normal mouse tissue wherein they are even detectable. Once we have found one or more examples of such a tissue, we can address whether the corresponding tissue(s) in ST6Gal-I null mice are deficient in ganglioside Siaα2–6Gal. This should allow us to establish if the same ST6Gal-I enzyme that adds α2–6 linked sialic acids to N-and O-glycans on proteins also synthesizes GSLs with this linkage.

Mice with a null mutation in the GalNAcT-8 transferase do not show a major loss of O-glycans. In the case of mice with deficiency of GalNAcT-8 (UDP-GalNAc:polypeptide O-Ser/Thr GalNAc transferase 8) (Hennet *et al.*, 1995), we analyzed thymocytes, as well as brain and kidney samples, for the profile of N- and O-linked chains derived from the total GP_U fraction. An example of the data obtained from the thymocytes is shown in Figure 6. In every case, we found that the region of the HPAEC-PAD profile expected to contain the O-glycans did not show any obvious change (although in some samples there appeared to be a decrease in the area of some of the O-glycan peaks). These data are best explained by the recent findings that there are several vertebrate polypeptide GalNAc-T enzymes capable of forming the GalNAcαO-Ser/Thr linkage (Bennett *et al.*, 1998; Hanisch *et al.*, 1999). These isozymes show some degree of tissue-specific expression. Thus, it is possible that if all the tissues of the mouse were studied in the aforementioned manner, one might find a major change in the amount of O-glycans in some location. This possibility has not yet been pursued. Note that we also did not examine the fraction SLMW3, that can contain some of the small, nonclustered O-glycan chains.

Conclusions and perspectives

We have demonstrated the value of applying NMR spectroscopy to whole mixtures of oligosaccharides or glycopeptides from mouse tissues. When coupled with the improved sensitivity of the nano-NMR probe, this approach allows the study of complex analytical situations such as those presented by glycosyltransferase-deficient mice. The advantages and limitations of this approach need further exploration. The application of this NMR technique to mouse tissues required the development of a novel Glycan Isolation Protocol. Most published protocols for extracting glycoconjugates from tissues and cells focus on recovering one specific class of molecules. There have been previous attempts to simultaneously extract more than one class, but none have taken the comprehensive approach presented here. Even if the initial goal is only to study one or two of the classes of glycans, the present approach leaves other purified fractions, in case a new question subsequently arises. We have also deliberately based our approach exclusively on the physicochemical properties of glycans, and avoided the use of tools such as lectins and enzymes. While the latter can show exquisite specificity, they can also give misleading results, e.g., some N-linked chains are resistant to PNGaseF, and some lectins will not bind their target structures when the latter are modified by anionic groups like sulfate. Using a primary organic extraction followed by solubilization of precipitated molecules in SDS, we also avoided most artifacts caused by degradative enzymes that might be released upon tissue homogenization (a small amount of GAG degradation was seen despite this). Another advantage of the protocol is that it uses relatively inexpensive materials and tools available to most laboratories doing conventional molecular biology or biochemistry. Furthermore, we have found the approach to be relatively reproducible when performed by four different operators following a standardized written set of instructions. Notably, although the method has not been optimized for this purpose, we were able to selectively extract the nucleic acids in one particular fraction (away from the GAGs), with the DNA being of relatively high molecular weight.

The approach has some minor disadvantages and limitations. First, the whole process takes about one week, and involves many steps. However, up to eight samples can be processed in parallel, and there are large time gaps between steps, allowing the operator to pursue other activities in parallel. Second, the glycoproteins and proteoglycans are recovered as an SDS extract, and are therefore not suitable for isoelectric focusing or functional studies. However, prior to proteolysis, the extract is suitable for analysis by SDS-PAGE, and for antibody or lectin blotting studies. Third, some highly acidic GSLs may be recovered in the fraction meant to contain only GPI-anchor

glycopeptides, and there is the potential for methyl esterification of sialic acids under these acidic conditions, in the presence of methanol. However, the fraction of such molecules is relatively small in most tissues, and other methods can be used to differentiate them from the GPI-anchors. Fourth, the use of exhaustive proteolysis limits the subsequent use of some N-glycanases which require some length of amino acids adjacent to the linkage region. Finally, the DEAE column gives incomplete separation of mucin glycopeptides from some GAG glycopeptides. However, as discussed earlier, a variety of nondegradative and degradative techniques can be used to achieve the desired separations.

Our immediate purpose in developing this approach was to study the outcome of genetic modulation of glycosyltransferases in mice. Given the rapid progress in identifying the genes encoding the glycosyltransferases, some might argue that it would be simpler to examine the expression pattern of their messenger RNAs as a means towards profiling glycosylation potential. However, there is now increasing evidence that expression of mRNA does not always correlate with the levels of a functional protein present in a given cell type (Page *et al.*, 1999). In the case of glycosylation, even a direct measurement of glycosyltransferase activity is not sufficient. Unlike the synthesis of RNA and proteins, glycosylation is not exclusively template driven, and one cannot accurately predict the glycosylation phenotype of a given tissue or cell type from patterns of glycosyltransferase activity expression. For example, even though GlcNAcT-IV activity is barely detectable in rat liver, many of the major serum glycoproteins produced by the rat hepatocyte have multiantennary N-linked chains that include the β1–4 branch synthesized by this enzyme (Schachter, 1991). Likewise, the ratio of α2–3 to α2–6 linked sialic acids was only moderately altered in cytokine-activated endothelial cells, despite a >10-fold increase in activity of the sialyltransferase ST6Gal-I that attaches α2–6 linked sialic acids (Hanasaki *et al.*, 1994). Thus, to accurately reveal the glycosylation potential of a given tissue or cell type, there appears no viable alternative to the extraction and direct glycoprofiling of the expressed glycans.

Studies of nucleic acids and proteins can now be done at a whole cell or whole organ level, with gene-chip arrays and 2-D gel based proteomics (Page *et al.*, 1999) providing a global overview of the expression of the genome and the status of the "proteome." Here we have made some early steps towards what might be eventually called "glycomics," i.e., elucidating the complete repertoire of glycan structures in a given cell-type or tissue. Obviously, much work is first needed to resolve the practical limitations in the protocol described here and in effectively profiling the resulting glycan fractions. It is possible that at least some of the steps could be automated. The approach might also be appropriately adapted or modified for other types of biological samples, e.g., insects, plants, and microbes. The potential applications of this approach are many, including the comparison of tissues between populations of a single species, between closely related species, between normal and disease states (e.g., cancer, inflammation), between cell lines with changes in glycosylation, in exploring genetic diseases of humans involving glycosylation and following genetic manipulation of glycosylation in intact cells or animals. We have demonstrated here two examples of the last situation. In each case, we were able to obtain the sought-after information simply by HPAEC-PAD profiling and/or nano-^{1}H-NMR spectroscopy of whole mixtures of N- and O-glycans. In the first case (ST6Gal-I deficiency) we show an essentially complete absence of the enzyme product in the N- and O-glycopeptides from several tissues. In the other case (polypeptide GalNAcT-8 deficiency), we saw little loss of expression of mucin-type O-glycans, corroborating that there are several other enzymes capable of synthesizing the GalNAc-O-Ser/Thr linkage. The clear biological phenotype of the ST6Gal-I null mouse (Hennet *et al.*, 1998), and the lack of an obvious phenotype in the other case (Hennet *et al.*, 1995) now make more sense.

Materials and methods

Materials

Most materials were obtained from Sigma Chemical Company. The following materials were obtained from the sources indicated: Sep-Pak C_{18} cartridges (#54955), Waters–Division of Millipore; SpectraPor membrane dialysis tubing, Spectrum; DEAE-Sephacel and Sephacryl S-200 High Resolution, Pharmacia; Bio-Gel P-2 and sodium dodecyl sulfate (SDS), Bio-Rad; Centricon and Centriprep concentrators, Amicon; ELISA plates (#25801; 96-well flat bottom), Corning; Silica-Gel 60 glass-backed HPTLC plates, Merck; agarose bound Jacalin and endoglycoceramidase, Vector Laboratories Inc.; [6-^{3}H]glucosamine hydrochloride (60 Ci/mmol), American Radiolabeled Chemicals; NaB[^{3}H]$_4$, New England Nuclear (NEN); carrier-free [^{35}S]sodium sulfate, ICN; orcinol, Eastman; ammonium acetate, Fisher; trifluoroacetic acid (TFA), Pierce; chondroitinase ABC, keratanase (Pseudomonas sp. Lyo), and keratanase II (Bacillus sp.), Seikagaku Corp.; and fungal Proteinase K, Gibco BRL. All other chemicals were of reagent grade or better, and were obtained from commercial sources.

Radiolabeled standards

Radiolabeled variant surface glycoprotein labeled in the GPI-anchors by [^{3}H]ethanolamine was a kind gift from Kojo Mensa-Wilmot (University of Georgia) (Mensa-Wilmot *et al.*, 1994). Synthetic (O-GlcNAc-)YSDSPSTST (Reason *et al.*, 1991) with a β-linked [6-^{3}H]Gal residue attached to GlcNAc was kindly provided by Bradley Hayes and Gerald Hart (Johns Hopkins University). CMP-[9-^{3}H]sialic acid and lactose were incubated with recombinant soluble α2–3 sialyltransferase (ST3Gal-III, kindly provided by Eric Sjoberg, Cytel Corp., San Diego) to enzymatically generate 3′[^{3}H]sialyllactose, and the product was isolated by gel filtration essentially as described previously (Beyer *et al.*, 1981).

Fetuin was radiolabeled in its Sia moieties by mild periodate oxidation followed by reduction with NaB[^{3}H]$_4$ (Van Lenten and Ashwell, 1971). Briefly, bovine fetuin (100 μg) was dissolved in a minimal volume of freshly made 2 mM sodium metaperiodate in phosphate-buffered saline (PBS) and allowed to react on ice, in the dark, for 15 min. A 100-fold molar excess of glycerol was added, and incubation continued for 2 h. The oxidized fetuin was dialyzed (MWCO 1000) against water, recovered by lyophilization, dissolved in 10–20 μl 10 mM NaOH and a 4-fold molar excess of NaB[^{3}H]$_4$ in dimethyl formamide (10 μl) was added. After 1–3 h at RT, reduction

was completed with a 20-fold molar excess of $NaBH_4$. Excess reducing agent was quenched with acetone (10-fold molar excess relative to $NaBH_4$) for 2 h at RT. The labeled fetuin was reisolated from the void volume of a Bio-Gel P-2 column eluted with 50 mM sodium acetate (pH 5.5), dialyzed (MWCO 1000) against 50 mM sodium acetate (pH 5.5) and then against distilled water. The sample was lyophilized and kept at –20°C until use. The uniform radiolabeling of all N- and O- sialyloligosaccharides was confirmed by releasing them via automated hydrazinolysis (see below), followed by HPAEC monitored by scintillation counting. The profile obtained was similar to that of larger amounts of unlabeled fetuin subjected to the same process and detected by PAD.

Disialoganglioside G_{D1a} (Matreya) was radiolabeled in its Sia moieties as described above for fetuin, except that purification from by-products was done by repeated evaporation with acidified methanol, followed by reverse-phase chromatography on a Sep-Pak C_{18} cartridge. The sample was loaded in water, the cartridge was washed with water and methanol, and [^{3}H]G_{D1a} eluted with chloroform. Further purification was achieved by HPLC on an Iatrobead 6RS 8010 (10 µm) column (4.6 × 500 mm), eluted with a linear gradient consisting of chloroform/methanol/water from 53:42:5 to 48:42:10 (v/v) over 60 min. The double peak (corresponding to two different acyl chain lengths of the sphingosine moiety) was pooled, dried, dissolved in water, dialyzed against water (MWCO 3500) at 4°C overnight, lyophilized, and kept at –20°C until use.

Tritium and ^{35}S-labeled HS- and CS-GAG glycopeptides were prepared by a modification of a previously described method (Bame and Esko, 1989). Briefly, CHO-K1 cell monolayers (4 P-100 plates) were metabolically radiolabeled with [6-^{3}H]GlcN (100 µCi/plate) in glucose-depleted media (glucose at 0.1 g/l final—10% of normal concentration) for 4 days, or with [^{35}S]sulfate (100 µCi/plate) in sulfate-depleted media with normal glucose concentration (1 g/l), 10 mg/l of cysteine (1/10 of normal concentration), and 3 mg/l methionine (1/5 of normal concentration) (Norgard-Sumnicht and Varki, 1995). Serum (10%) was present during the labeling (dialyzed in the case of the sulfate-depleted media). Labeled cells were washed three times with cold PBS, solubilized with 1 ml per plate of 100 mM NaOH at RT for 20 min, the extract was combined with the original labeling media, and the pH adjusted to 7.0. Proteinase K was added at a final concentration of 2 mg/ml and the mixture incubated at 50°C overnight. The samples were then diluted with water to a salt concentration of 100 mM or lower (checked with a Vapor Pressure Osmometer, 5500XR, Wescor, calibrated against standard solutions). The sample was applied to a 2 ml DEAE-Sephacel column and washed with 30 column volumes of 100 mM NaCl, 20 mM Tris–HCl, pH 7.4. The HS- and CS-GAGs were eluted with 4 column volumes of 1.0 M NaCl, 20 mM Tris–HCl, pH 7.4, and dialyzed against water in MWCO 3500 dialysis tubing.

Tritiated mucin was prepared from one P-100 plate of LS180 human colon carcinoma cells metabolically radiolabeled in Dulbecco's Modified Eagle's Medium with 20% fetal calf serum for 3 days with 1 mCi of [6-^{3}H]GlcN. The media containing shed mucins and GAGs was treated with a mixture of chondroitinase ABC (200 mU) and heparinase II (1.25 U) in 50 mM Tris acetate, 2.5 mM $CaCl_2$, pH 6.9 overnight at 37°C. The sample was adjusted to 100 mM NaCl and then loaded onto a 2 ml Jacalin agarose column at 4°C, and the flow was stopped overnight. The column was then washed with 20 ml of 10 mM Tris–HCl, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ (pH 7.5), and bound O-glycosylproteins eluted with 4 column volumes of 100 mM melibiose in the same buffer. The eluate was repeatedly dialyzed against water (MWCO 3500) at 4°C, lyophilized, dissolved in 100 µl of 100 mM Tris–HCl buffer (pH 7.4) and treated with Proteinase K (final concentration 2 mg/ml) overnight at 50°C. After boiling for 10 min, mucin-type glycopeptides were fractionated on a Sephacryl S-200 column (10 ml) in 10 mM ammonium acetate. The largest mucin glycopeptides running in the void volume were pooled and used as a mucin standard.

Non-protein-bound GPI (GIPL) from *Trypanosoma cruzi* (Y-strain) (Carreira *et al.*, 1996) was a kind gift from Lucia Mendonca-Previato (Universidade Federal do Rio de Janeiro, Brazil). The GIPL (200 µg) was treated with 20 mM metaperiodate in 100 mM sodium acetate buffer (pH 5.5) for 10 min on ice to oxidize between C-5 and C-6 of the galactofuranosyl moiety. The excess reagent was consumed by adding ethylene glycol. After 30 min, the reaction mixture was desalted by microdialysis (MWCO 1000) and dried before reducing with $NaB[^3H]_4$ (25 mCi) in 10 mM NaOH. The reaction was terminated with a 10-fold excess of unlabeled $NaBH_4$, the excess reagent was consumed with glacial acetic acid, and methyl borate eliminated by repeated evaporation with acidified methanol. The radiolabeled GIPL was recovered by binding to a Sep-Pak cartridge and elution with chloroform/methanol.

Concurrent isolation of different classes of glycans from cells and tissues

A detailed Glycan Isolation Protocol for preparation of different classes of glycans from cells or tissues is described below. Samples are used fresh or immediately frozen upon collection and stored at –80°C until processed. As described in the companion paper (Norgard-Sumnicht *et al.*, 2000) the protocol is also applicable to cultured cells, which are first pelleted and washed three times with ice-cold PBS to remove any serum components from the culture media. Body fluids (e.g., plasma), can also be processed according to the protocol, after partial concentration by lyophilization to obtain a protein concentration of up to 200 mg/ml. For convenience, the individual steps of the protocol are presented in sequential days. All procedures are carried out in glass tubes, or in the presence of detergent, until the samples are free of any lipid material. Lipid-containing fractions are stored in glass tubes, and other samples may be stored in plastic vials. Figure 2 outlines the overall approach, and also presents the nomenclature and abbreviations used to identify the individual fractions.

Day 1

After thawing and removal of loosely attached connective tissue, the weight of the tissue sample is determined (or the volume of the cell pellet is measured). Optimal starting amounts are ~250–500 µg/ul. The tissue is finely minced, and the sample is transferred into a conical glass tube of appropriate volume.

Extraction of lipids. Lipids are extracted at RT in six sequential steps, with 20 volumes of chloroform/methanol (2:1, 1:1 and 1:2, v/v, each done twice), using a Polytron homogenizer

(Brinkmann Instruments, Westbury, NY), applying 3–5 bursts of 5–15 s each, at 40–50% of the maximum power, to ensure a uniform suspension at each step. After the first homogenization in chloroform/methanol 2:1 (v/v), an aliquot (2% of the total volume) of the "Total Homogenate" (TH) suspension may be set aside for protein, neutral/amino sugar and Sia analyses, or β-counting (as appropriate), and stored at –20°C until analyzed (see "Options" section in *Results and discussion*). After each extraction step, the organic supernatant containing the lipid material is recovered by centrifugation at 1500 r.p.m. for 10 min and transferred with a glass (Pasteur) pipette into a 15-ml glass tube with a Teflon-lined screw cap. To remove any residual chloroform/methanol from the pellet (containing proteins, including glycoproteins and proteoglycans, and also nucleic acids), a final extraction step is done with 5 volumes of 95% ethanol. After removal of the ethanol, the final pellet is not allowed to dry out, but immediately hydrated in 0.5 ml of 1% (w/w) SDS in 100 mM Tris–HCl (pH 7.4), and kept at –20°C overnight (except when the appearance of a non-pelletable, gel-like substance is observed; see "Options" section in *Results and discussion* for degradation of this gel by DNase-I). If cultured cells grown in a monolayer are being studied (see Norgard-Sumnicht *et al.*, 2000, the following article), the extracellular matrix may be extracted from the culture plate with 1% SDS in 100 mM Tris–HCl (pH 7.4), and added to the pellet obtained after the primary lipid extraction. Meanwhile, the organic extracts are combined and evaporated to dryness, either under vacuum or under a stream of nitrogen gas. The residue is dissolved in chloroform/methanol 2:1 (v/v) (~2 ml/100 mg of initial wet tissue or 2 ml/100 µl cell pellet) and the solution is stirred at 4°C overnight.

Day 2

Recovery of coextracted glycoproteins from the organic extract. After stirring overnight, the organic extract is centrifuged at 1500 r.p.m. for 10 min, and the supernatant is transferred into a 15 ml glass tube with a Teflon-lined screw cap. The residue is washed with 95% ethanol, centrifuged once again, and the supernatant is pooled with the previous one. The combined organic extracts constitute the "Primary Lipid Extract" (PLE); after evaporation of the organic solvents, the PLE is stored in dry state at –20°C until further use (see "Options" section below).

Solubilization of glycoproteins and proteoglycans. The pellet left behind in the "recovery" step is promptly hydrated in a small volume of 1% SDS in 100 mM Tris–HCl (pH 7.4), and combined with the main glycoprotein/proteoglycan containing fraction obtained on Day 1. The volume of the suspension is increased by addition of 1% SDS in 100 mM Tris–HCl (pH 7.4), to 2 ml/100 mg of starting wet tissue. The sample is then boiled at 100°C for 15–20 min to dissolve as much of the residue as possible. The tube is vortexed vigorously before and after heating, to ensure that no denatured (glyco-)protein material remains attached to the wall of the tube. The pH of the solution is verified to remain in the 7.0–7.5 range. The resulting denatured material is labeled the "Total Protein" (TP) fraction (see Figure 2). An aliquot (2%) of TP may be set aside for protein and carbohydrate analyses (see "Options" section).

Removal of small molecules. The denatured protein sample is centrifuged at 1500 r.p.m. for 15 min, to precipitate any insoluble material. The supernatant is removed for filtration, while the precipitate is washed with a small volume of 100 mM Tris–HCl (pH 7.4), recentrifuged, with the secondary supernatant being used in the later stages of the filtration (the precipitate is saved for a following step). The supernatant is transferred into a 10,000 MWCO Centricon 10 or Centriprep 10 filtration unit (Amicon, Beverly, MA), chosen according to the wet weight of the starting tissue material (i.e., under 200 mg, and between 200 and 500 mg, respectively). For a typical TP solution with a volume of 15 ml, centrifugation is done at just under $3000 \times g$, in three steps of 40, 10 and 5 min, with collection of the filtrate (containing various types of low-molecular weight material) after each step. To maximize the efficiency of removal of the small molecules, the concentrated retentate is then diluted with 100 mM Tris–HCl (pH 7.4) (and/or the secondary supernatant from the recentrifugation of the pellet; see above) to the maximum volume that the unit will hold, and subjected to repeated centrifugation. All filtrates are collected and pooled. The combined Centricon/Centriprep 10 run-through fraction (called PLMW10) contains sugar nucleotides, free monosaccharides, small oligosaccharides, and other cellular small molecules. PLMW10 is lyophilized and stored at –20°C. If the low-molecular-weight compounds are not of interest, a 12,000 MWCO dialysis tubing can be used as an alternative to the Centricon or Centriprep unit; dialysis against 400 volumes of 100 mM Tris–HCl (pH 7.4) at 4°C overnight (followed by one additional dialysis for 4 h under the same conditions) will retain the glycoproteins/proteoglycans, free of small saccharides and sugar nucleotides.

Preparation of glycopeptides. The retentate in the Centricon/Centriprep (or the solution inside the dialysis tubing) is mixed with the precipitate left behind in the above centrifugation step of the TP solution in SDS and Tris buffer, and the total volume is brought back up with 100 mM Tris–HCl (pH 7.4) to 2 ml/100 mg of wet tissue (since all of the originally added SDS is retained during ultrafiltration, this maintains the concentration of SDS at or below 1%); when using dialysis instead of a filtration unit, the volume typically recovered is a bit larger than 2 ml/100 mg wet tissue. An aliquot of the total protein extract may be saved at this point if it is desirable to study the intact glycoproteins/proteoglycans, or to use PNGases or endoglycosidases to specifically release N-glycans (see "Options" section below). The bulk of the solution is subjected to proteolytic digestion by adding 1/10th volume of Proteinase K (Gibco BRL, Gaithersburg, MD, 20 mg/ml in 50 mM Tris–HCl, 2 mM calcium acetate, pH 8), to obtain a final concentration of ~2.0 mg/ml. Incubation is at 50°C overnight.

Day 3

A second aliquot of Proteinase K (at a final concentration of 2 mg/ml) is added in the morning of Day 3, and incubation continues for 8 h. The sample is then heated at 100°C for 15 min to inactivate Proteinase K, and subsequently cooled and centrifuged at 1500 r.p.m. for 10 min. If any insoluble material is left at this stage, the pellet may be saved and subjected to carbohydrate analysis (minor amounts of residue were obtained when extracting tissues with high matrix content such

as the kidney, and compositional analysis revealed only traces of carbohydrate).

Day 4

Separation of O-GlcNAc-Ser/Thr and/or other small Ser/Thr-linked O-linked glycans from larger glycopeptides. The Proteinase K-digest supernatant is transferred into a 3000 MWCO Centriprep 3 or Centricon 3 unit and centrifuged at just under $3000 \times g$, in three steps (of 95, 35, and 10 min, respectively, for a volume of 15 ml) with removal of the filtrate after each step. The final retentate is diluted once more by adjusting the volume with 100 mM Tris–HCl buffer (pH 7.4) to the original volume, and recentrifuged. The combined filtrates containing O-GlcNAc-Ser/Thr and/or other short Ser/Thr-linked oligosaccharide chains are lyophilized and stored at –20°C until analysis or further fractionation. This secondary low-molecular-weight fraction (SLMW3) is expected to also contain all released amino acids, including any modified amino acids such as tyrosine sulfate, and any small peptides having molecular weights of less than 3000. If none of the small glycopeptides are of interest, a 3000 MWCO dialysis tubing can be used as an alternative to the Centricon or Centriprep unit, dialyzing against 400 volumes of 100 mM Tris–HCl (pH 7.4) at 4°C for at least 72 h.

Precipitation of SDS. The final retentate from the Centriprep/Centricon unit (or the dialysis bag) is transferred into a glass tube capable of withstanding centrifugation forces greater than 12,000*g* (e.g., a Corex No. 8441 tube). It is diluted with 100 mM Tris–HCl (pH 7.4) to a volume of 2–3 ml. A saturated KCl solution (1% of the original volume of the SDS solution used for denaturation at the beginning of Day 2) is added and the solution is kept at 4°C overnight.

Day 5

The insoluble K-SDS is pelleted in the Corex tube by centrifugation at 10,000 r.p.m. for 20 min (~$12,000 \times g$), and the supernatant is transferred into a clean glass tube.

Separation of GPI anchors. GPI-anchored glycopeptides and any remaining GSLs which failed to enter the PLE fraction (i.e., highly charged GSLs or GSLs originally trapped in the tissue matrix) are extracted from the aforementioned supernatant with chloroform/methanol/1 M HCl (65:35:1, v/v) using a volume equal to that of the sample. The phases are separated by centrifugation (at 1500 r.p.m. for 15 min at RT). The lower (organic) phase contains GPI-anchored glycopeptides and any remaining GSLs, and the upper (aqueous) phase contains all other glycopeptides. Depending on the absolute volume(s) of aqueous (and organic) solvent(s) used, i.e., on the salt concentration of the starting supernatant, an inter-phase emulsion appears more or less clearly observable during the extraction process; it is taken together with the upper phase and treated as part of the main aqueous phase. The pH of both phases is checked with pH paper, and adjusted to 7.4 if necessary (with 10–20 µl aliquots of 1 M NaOH). The aqueous phase is transferred into another glass tube, and the organic extraction is repeated. The two organic phases are then pooled, constituting the Secondary Lipid Extract (SLE); it is dried and kept at –20°C until further analysis. It is noteworthy that this SLE fraction contains the nucleic acids in addition to GPI-anchored peptides (see below).

Days 5–6

Separation of high-charge GAG and mucin-type glycopeptides from N- and O-type glycopeptides. The aqueous phases from the previous step are pooled (the GP_U fraction, Figure 2), diluted with an equal volume of water (to decrease the salt concentration to less than 100 mM), and then applied to a DEAE-Sephacel column equilibrated in 100 mM NaCl in 20 mM Tris–HCl (pH 7.4) (1 ml of column volume per 100 mg wet weight of starting tissue or 100 µl cell pellet) (Bame and Esko, 1989). The initial run-through is reapplied to the column, which is then eluted with 30 column volumes of the equilibration buffer. The total 100 mM NaCl eluate is collected; this fraction ($GP_{0–100}$) contains primarily the low-charge N- and O-type glycopeptides. Elution of the high-charge glycopeptides (mostly GAGs and clustered O-linked glycans from mucin regions resistant to the protease) is accomplished with 10 column volumes of 1 M NaCl in 20 mM Tris–HCl (pH 7.4), resulting in the $GP_{100–1000}$ fraction. As discussed in the "Options" section (see *Results and discussion*), the latter step may be split into two stages: elution with 10 column volumes of 300 mM NaCl in 20 mM Tris–HCl (pH 7.4) to selectively elute most of the mucin glycopeptides in $GP_{100–300}$, followed by a 1 M NaCl elution (as mentioned above) for collection of the GAG-type glycans in $GP_{300–1000}$.

Days 6–9

Desalting of glycopeptides. Salts are removed from the glycopeptides in fractions $GP_{0–100}$ and $GP_{100–1000}$ (c.q. $GP_{100–300}$ and $GP_{300–1000}$) by extensive dialysis against water at 4°C using 1000 MWCO tubing. If hydrazinolysis is planned, frequent changes of the water are required to achieve a final salt concentration of less than 5 nM. Samples are lyophilized and stored at –20°C until analysis.

Monitoring the fractionation process

Qualitative and quantitative recoveries for each step in the Glycan Isolation Protocol were monitored by analyzing aliquots for monosaccharide composition (by acid hydrolysis, followed by HPAEC-PAD), total Sia content (by the thiobarbituric acid colorimetric assay and/or by weak acid hydrolysis followed by DMB derivatization and HPLC analysis with fluorescence detection), total neutral sugar content (by the phenol/sulfuric acid assay), total protein content (by the Bio-Rad assay), the presence of peptides (by amino acid analysis), and (when applicable) radioactivity arising from internal standards. For the thiobarbituric acid, phenol/sulfuric acid and Bio-Rad assays, conventional spectrophotometric absorbance readings were replaced by analyzing 200 µl of the reaction mixture in a 96-well quartz plate, using a plate reader.

Monosaccharide composition analysis by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Aliquots of Glycan Isolation Protocol fractions were hydrolyzed with 2 M TFA (sequencing grade) at 100°C for 4 h, cooled down, dried in a Speed-Vac (Savant Instruments,

Holbrook, NY), dissolved in water, and transferred into autosampler vials for injection onto a CarboPac PA-1 column (4 × 250 mm) (Dionex Corporation, Sunnyvale, CA). Elution was performed with sodium hydroxide (16 mM, isocratically) for neutral and amino monosaccharide composition analysis (or 2 mM sodium hydroxide, isocratically, for the specific purpose of separating xylose and mannose). For uronic acid analysis, samples were hydrolyzed for 6 h, under otherwise identical conditions. Elution of a uronic acid containing hydrolyzate on CarboPac PA-1 utilized 16 mM sodium hydroxide, isocratically for 24 min (as for neutral and amino monosaccharide analysis), followed by 100 mM sodium hydroxide with a gradient of sodium acetate, from 20 to 200 mM over 45 min, allowing the separation of the uronic acids. Calibration of retention times on the CarboPac PA-1 column and of PAD molar response factors was based on analysis of mixtures of monosaccharide standards exposed to the hydrolysis conditions detailed above.

Determination of total Sia content

Sias were released by hydrolysis after saponification of O-acetyl esters, derivatized with DMB and analyzed by reverse-phase HPLC on a TSK-Gel ODS-120T 5 μm column (4.6 mm × 25 cm) (Tosohaas, Montgomeryville, PA) as described (Manzi *et al.*, 1990). DMB derivatives were eluted with acetonitrile/methanol/water (either isocratically, 9:7:84, v/v (Diaz *et al.*, 1989), or with a linear gradient from 7:7:86 to 11:7:82, v/v), at a flow rate of 0.9 ml/min for 40 min.

Release of N- and O-glycans from glycopeptides by automated hydrazinolysis

Since many inorganic anions and cations can interfere with hydrazinolysis, glycopeptide samples were extensively dialyzed (see Days 6–9) against water at 4°C using 1000 MWCO tubing. Salt-free samples (GP_U, $GP_{0–100}$, $GP_{100–300}$) were transferred into clean glass reactor vials and lyophilized for at least 24 h at <50 millitorrs. The vials from the lyophilizer were immediately placed on the ports of an automated hydrazinolysis instrument (GlycoPrep 1000, Oxford GlycoSciences, U.K., up to 2 mg of starting material per vial). Operating conditions were selected for release of N- and O-glycans (hydrazine reaction at 95°C for 4 h) or only O-glycans (hydrazine reaction at 65°C for 5 h), as required. After the hydrazinolysis reaction and built-in purification (~16–24 h), the released glycans were filtered through 0.5 μm PTFE filters (Oxford GlycoSciences) to remove silica gel particles, and lyophilized. The products were used as such for HPAEC-PAD glycan profiling (see below). However, prior to ^{1}H NMR spectroscopy, further clean-up over an activated charcoal filter was necessary (see below).

HPTLC profiling of neutral GSLs and gangliosides

The PLE fraction (obtained in dry state at the end of Glycan Isolation Protocol Day 1) was dissolved in a minimal volume of chloroform/methanol 2:1 (v/v); methanol and water were then added to attain a final ratio chloroform/methanol/water of 2:43:55 (v/v). Any insoluble material was precipitated by centrifugation. The solution was loaded three times onto a prewashed Sep-Pak C_{18} cartridge, which was then washed with water (10 ml), and eluted with methanol (2 ml), chloroform/methanol 2:1 (v/v, 5 ml), and chloroform (5 ml) (Schnaar, 1994). The methanol and chloroform/methanol eluates were pooled and dried under a stream of nitrogen gas. Under these conditions, the methanol and chloroform/methanol eluates contained all GSLs, as judged from Sia and neutral/amino monosaccharide analyses. After evaporation of the solvents, the GSLs were profiled by HPTLC, as follows. Glass-coated Silica Gel-60 HPTLC plates (10 × 10 cm) (Merck; Darmstadt, Germany) were activated for 20 min at 110°C. An aliquot of the GSL sample (corresponding to 2–5 nmol Sia) was spotted on two different plates and developed in chloroform/methanol/0.02% aqueous $CaCl_2$ (60:40:9, v/v, for optimal separation of gangliosides) and chloroform/methanol/water (10:10:3, v/v for neutral GSLs) (Schnaar and Needham, 1994). Neutral and anionic GSLs were detected by spraying the plate with orcinol-sulfuric acid, while gangliosides were detected with resorcinol-HCl-Cu^{2+} (Schnaar and Needham, 1994). Radiolabeled GSLs were detected by fluorography after spraying the HPTLC plate with En^{3}Hance (DuPont-New England Nuclear, Boston, MA).

Release of glycans from GSLs by endoglycoceramidase

Prior to enzymatic release of the oligosaccharides, the PLE fraction was enriched in GSLs, either by chloroform/methanol extractions as described above (see HPLTC profiling), or by Folch partitioning (Smith, 1996). The latter procedure involved dissolving the PLE in chloroform/methanol 2:1 (v/v) (0.5 ml/100 mg tissue) in a glass tube, adding 0.1 M aqueous KCl (20% of the total volume), vortexing and centrifuging at 1500 r.p.m. for 15 min. The resulting upper (aqueous) phase was transferred into another glass tube, and the lower (organic) phase was extracted twice more with methanol/0.1 M aqueous KCl (1:1, v/v), under centrifugation at 1500 r.p.m. The upper phases, containing the GSLs with glycans consisting of 4 or more monosaccharide units, were pooled, washed with chloroform/methanol 2:1 (v/v), and eventually diluted with methanol/water (1:1, v/v). The compositions of the upper and lower phases were monitored by HPTLC and DMB Sia analysis (see above). After desalting over a prewashed Sep-Pak C_{18} cartridge (with salts being eluted by water, GSLs by chloroform/methanol 1:1 followed by chloroform/methanol/water 4:8:3), the GSL-containing solution was evaporated to dryness.

The GSLs, dissolved in 50 mM sodium acetate buffer (pH 5.0), were subjected to endoglycoceramidase digestion, in the presence of sodium cholate (at a final concentration of 0.75 mg/ml, thoroughly pre-mixed with the sodium acetate buffer), at 37°C for 48 h under toluene atmosphere (Ito and Yamagata, 1989; Miller-Podraza *et al.*, 1993). The progress of the digestion was monitored by HPTLC. The reaction mixture was extracted with 5 volumes of chloroform/methanol 2:1 (v/v). The phases were separated by centrifugation at 1500 r.p.m. for 15 min. The organic phase was washed twice with 3 volumes of water, and all aqueous phases were pooled. After washing once more with chloroform/methanol 2:1 (v/v), the aqueous phase was lyophilized. Desalting and removal of other noncarbohydrate contaminants was achieved by applying the sample (in 1–2 ml of water) to a GlycoClean H charcoal cartridge (Oxford GlycoSciences) (for details, see section on "NMR spectroscopy"). Essentially, after elution of non-carbohydrate contaminants by 5% acetonitrile/water, the glycans were eluted quantitatively by 50% acetonitrile/water and recovered in dry state by evaporation. The mixture of GSL-derived

oligosaccharides was analyzed by ^{1}H NMR spectroscopy and HPAEC-PAD profiling.

Oligosaccharide mapping by HPAEC-PAD

Aliquots of free oligosaccharides produced by hydrazinolysis or endoglycoceramidase digestion were analyzed by HPAEC-PAD (Hardy and Townsend, 1994) on a CarboPac PA-1 column (4 × 250 mm). A Dionex DX500 HPLC system equipped with UV and PAD detectors and an AS3500 ThermoSeparations autosampler were used; data acquisition and processing was controlled by Dionex PeakNet software (version 5.1, running under Windows 95 on an Intel Pentium MMX PC). Elution of glycan mixtures with 1 to 4 or 5 negative charges per molecule (i.e., typical anionic N- and O-glycans from glycoproteins/glycopeptides and oligosaccharides from gangliosides) was achieved using a 60 min linear gradient of sodium acetate (20–250 mM from 5 to 65 min) with a constant concentration of sodium hydroxide (100 mM), and maintaining the 250 mM concentration of acetate for another 25 min. For separation of neutral oligosaccharides, a shallower linear gradient was employed (2–40 mM sodium acetate in 100 mM sodium hydroxide for 40 min). The column was calibrated with glycoprotein and glycolipid derived standards of known structure, run under the same conditions.

Analysis of GAG constituents among the high-charge glycopeptides

The presence and type of GAGs in the highest-charge DEAE fractions ($GP_{100–1000}$ or $GP_{300–1000}$) were assessed using a cocktail of GAG-degrading enzymes. Samples were dissolved in 50 mM Tris-acetate, 100 mM NaCl, and 2.5 mM $CaCl_2$ (pH 6.9), incubated with keratanase II (100 mU), chondroitinase ABC (50 mU), chondroitinase AC (25 mU), and heparin lyase II (156 mU) at 37°C for 3–4 h, and the reaction mixture boiled for 15 min to inactivate the enzymes. A Superose-12 HR 10/30 FPLC column (Pharmacia, Uppsala, Sweden) was used to size both undigested and digested products. The column was run isocratically in 100 mM NaCl/20 mM MOPS (pH 7.4), containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.02% sodium azide. A Pharmacia FPLC system (P-LKB-pump P-500; P-LKB-controller LCC-500 Plus) was used to elute the column at 0.4 ml/min flow rate. Fractions were collected directly into scintillation vials; 4 ml of scintillation fluid was added, and the radioactivity was determined in a β-counter.

NMR spectroscopy of glycan mixtures

Oligosaccharide mixtures released from peptides by automated hydrazinolysis or from sphingolipids by endoglycoceramidase digestion (see above) were further purified over GlycoClean H charcoal cartridges (Oxford GlycoSciences). Briefly, GlycoClean H cartridges were prewashed with 1 M sodium hydroxide, water, 30% acetic acid and water (3 ml each), and then conditioned with 50% acetonitrile/water/0.1% TFA (3 ml) and 5% acetonitrile/water/0.1% TFA (6 ml). The glycan sample was applied (in less than 1 ml of aqueous solution), and salts and other hydrophilic contaminants washed off the cartridge with water (3 ml), followed by 5% acetonitrile/water/0.1% TFA (3 ml). Glycans were eluted with 50% acetonitrile/water/0.1% TFA (4 portions of 0.5 ml each) and recovered by evaporation, while contaminants remained bound to the charcoal. Specifically, this procedure effectively removed contaminants that would be detrimental to ^{1}H-NMR analysis, namely, di-N-acetylhydrazine, a by-product of hydrazinolysis that is not removed by the GlycoPrep built-in automated clean-up procedure, and also sodium cholate and sodium acetate from endoglycoceramidase digests. Glycan samples were repeatedly exchanged in D_2O (99.96%, Sigma/Aldrich, St. Louis, MO) with intermediate lyophilization, finally dissolved in 40 µl of 99.996% D_2O (Cambridge Isotope Laboratories, Andover, MA), and transferred into a 40 µl nano-cell (Varian, Palo Alto, CA). ^{1}H-NMR spectra were recorded on a Varian Unity Inova 500 MHz spectrometer, equipped with a Varian ^{1}H nano-NMR probe and controlled by a Sun Microsystems Sparc 5 computer, running VNMR software (version 5.3 B). NMR spectra were obtained in 1000–10,000 scans using presaturation of the HDO resonance by a low-power transmitter pulse, at spin rates of 1.5–2.0 kHz. Line broadening (by 0.2–0.5 Hz) of the spectra was applied in data processing before Fourier transformation. Chemical shifts are given relative to 4,4-dimethyl-4-silapentane-1-sulfonate (DSS); they were typically measured relative to the residual free acetate peak (δ 1.908 p.p.m. at 22–30°C and pD 6–8).

Options, variations, and extensions of the glycan isolation protocol (see also Figure 3)

1. Early removal of DNA. Tissues (and cells) rich in DNA can pose the following problem. At high concentrations, DNA presents itself as a gel which would not solubilize when boiling the residue from the organic extract in 1% SDS (day 2). This gel also interferes with the subsequent ultrafiltration step(s). The problem can be circumvented using DNase-I to degrade the DNA gel-like material before hydrating the protein pellet in SDS in Tris–HCl at the end of Day 1. Optimization of this step is currently being pursued.

2. Subfractionation of GSLs. The GSLs in the PLE can be further purified by using various previously described methods (Hakomori and Siddiqui, 1974), including Folch partitioning, fractionation into gangliosides, GIPLs and neutral lipids by DEAE chromatography, etc. Note that the PLE also contains the bulk of the cellular cholesterol, sphingomyelin and phospholipids. If labile O-acyl groups and GIPLs are not of interest, the phospholipids can be destroyed by base hydrolysis (Hakomori and Siddiqui, 1974).

3. Release of glycans from GSLs by endoglycoceramidase. GSL-specific endoglycoceramidases (Ito and Yamagata, 1989; Miller-Podraza *et al.*, 1993) can be used to release the oligosaccharide portion from GSLs (see *Materials and methods*). Once recovered, these glycans can be profiled or fractionated (with or without tagging), and structurally characterized, as indicated below for the N- and O-glycans.

4. Release of N-glycans from denatured glycoproteins by endoglycosidases. N-glycans in the delipidated SDS-solubilized extract can be released from proteins by a variety of endoglycosidases (endoH, endoD, endo$F_{1–3}$) or peptide:N-glycosidases (PNGaseF or PNGaseA) (Maley *et al.*, 1989; Plummer and Tarentino, 1991). Most of these enzymes can be used despite the presence of SDS, provided that the SDS concentration is appropriately adjusted and/or an appropriate

ratio of non-ionic detergent is added (Maley *et al.*, 1989; Plummer and Tarentino, 1991). The released oligosaccharides can be recovered by gel filtration or other methods.

5. Subfractionation of free oligosaccharides and sugar nucleotides. These molecules found in the first ultrafiltration (PLMW10) can be further fractionated by various techniques (Maley *et al.*, 1989; Plummer and Tarentino, 1991; Strecker *et al.*, 1992; Chaturvedi *et al.*, 1997). If quantitative recovery is important, it should be noted that a portion of these molecules can enter the PLE fraction, and need to be recovered by ultrafiltration or dialysis.

6. Recovery and analysis of small O-type glycopeptides. The O-linked GlcNAc and other small glycopeptides (including a portion of the mono- and di-sialylated glycopeptides) are recovered in the second ultrafiltration (SLMW3) and can be studied by methods such as galactosyltransferase-catalyzed labeling with UDP-[^{3}H]Gal (Greis *et al.*, 1996) followed by β-elimination, or by mass spectrometry after separation from free amino acids and other proteolysis products.

7. Release of glycans from GPI-anchors. The SLE contains the bulk of the GPI anchors, with short residual peptides attached to the ethanolamine residue in their core structure. The GPI-anchors can be cleaved by GPI-specific phospholipases or the glycans can be released by de-acylation, nitrous acid deamination, and HF treatment (Norgard-Sumnicht *et al.*, 2000, following article). The released glycans can be profiled, fractionated, or analyzed as needed (Ferguson, 1992).

8. Subfractionation of DEAE-bound glycopeptides and oligosaccharides by charge characteristics. The high-charge fraction from the DEAE column ($GP_{100–1000}$) comprises a mixture of HA, mucin fragments resistant to further proteolysis, and KS, CS and HS glycopeptides. These components can be partially separated by selective salt elutions and/or pH shift elutions from DEAE (e.g., a 300 mM NaCl elution prior to the 1 M NaCl step will recover most mucin-type glycopeptides). If there is great complexity, gradient elution from a DEAE-HPLC column and/or electrophoretic techniques can be used to fractionate them on the basis of differing charge density.

9. Subfractionation of DEAE-bound glycopeptides or free oligosaccharides by lectin affinity chromatography. Affinity chromatography methods (e.g., jacalin sepharose for binding mucins, columns of heparin-binding proteins for certain fractions of HS) can also be useful to achieve further separations.

10. Degradative subfractionation of DEAE-bound glycopeptides and oligosaccharides. Each component within the $GP_{100–1000}$ DEAE fraction could also be recovered by an approach in which the other components are destroyed by specific enzymes. Most mucins could be eliminated by treating with sialidases, and then re-running the DEAE column (this might not work if the mucin is heavily sulfated). Alternatively, the mucins could be recovered intact by treating this fraction with a mixture of broad-spectrum GAG-degrading enzymes. If these degradative procedures are carried out in a sequential manner, the resulting GAG fragments could be isolated by gel filtration and analyzed to ascertain indirectly the general structure of the parent fractions (i.e., percentage of each type of GAG), by identifying the specific disaccharides contained in these fractions.

11. Release and fractionation of N- and O-glycans in the 100 mM NaCl DEAE eluate. The oligosaccharides can be released by manual or automated hydrazinolysis, and then radiolabeled or fluorescently tagged. Subsequent fractionation may employ a variety of techniques, including different types of HPLC and serial lectin affinity chromatography (Varki *et al.*, 1996).

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Abbreviations

Cer, ceramide; CHO, Chinese hamster ovary; CS, chondroitin sulfate; DEAE, diethyl aminoethyl; DMB, 1,2-diamino-4,5-methylenedioxybenzene; DS, dermatan sulfate; GAG, glycosaminoglycan; GIPL, glycosylinositol phospholipid; GPI, glycosylphosphatidylinositol; GSL, glycosphingolipid; HA, hyaluronan (hyaluronic acid); HPAEC-PAD, high-pH anion-exchange chromatography with pulsed amperometric detection; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin layer chromatography; HS, heparan sulfate, KS, keratan sulfate; LacNAc, N-acetyllactosamine (Galβ1–4GlcNAc); MWCO, molecular weight cut-off; nC, nano-Coulomb; PBS, phosphate-buffered saline; PNGase, peptide:N-glycosidase; RT, room temperature; SLac, 3′-sialyllactose, and TFA, trifluoroacetic acid. The abbreviations used for the fractions obtained in the Glycan Isolation Protocol are defined in the Figure 2 caption.

References

Asano,M., Furukawa,K., Kido,M., Matsumoto,S., Umesaki,Y., Kochibe,N. and Iwakura,Y. (1997) Growth retardation and early death of β-1,4-galactosyltransferase knockout mice with augmented proliferation and abnormal differentiation of epithelial cells. *EMBO J.*, **16**, 1850–1857.

Baenziger,J.U. (1996) Glycosylation: to what end for the glycoprotein hormones? *Endocrinology*, **137**, 1520–1522.

Baenziger,J.U. and Fiete,D. (1979) Structure of the complex oligosaccharides of fetuin. *J. Biol. Chem.*, **254**, 789–795.

Bai,X.M., Bame,K.J., Habuchi,H., Kimata,K. and Esko,J.D. (1997) Turnover of heparan sulfate depends on 2-*O*-sulfation of uronic acids. *J. Biol. Chem.*, **272**, 23172–23179.

Bame,K.J. and Esko,J.D. (1989) Undersulfated heparan sulfate in a Chinese hamster ovary cell mutant defective in heparan sulfate *N*-sulfotransferase. *J. Biol. Chem.*, **264**, 8059–8065.

Bennett,E.P., Weghuis,D.O., Merkx,G., Van Kessel,A.G., Eiberg,H. and Clausen,H. (1998) Genomic organization and chromosomal localization of three members of the UDP-N-acetylgalactosamine: polypeptide N-acetylgalactosaminyltransferase family. *Glycobiology*, **8**, 547–555.

Bernard,N., Lombart,C., Strecker,G., Montreuil,J., van Halbeek,H. and Vliegenthart,J.F.G. (1983) Structural studies of glycans isolated from rat plasma hemopexin. *Biochimie,* **65**, 185–192.

Beyer,T.A., Sadler,J.E., Rearick,J.I., Paulson,J.C. and Hill,R.L. (1981) Glycosyltransferases and their use in assessing oligosaccharide structure and structure-function relationships. *Adv. Enzymol.*, **52**, 23–175.

Burda,P. and Aebi,M. (1999) The dolichol pathway of *N*-linked glycosylation. *Biochim. Biophys. Acta Gen. Subj.*, **1426**, 239–257.

Carreira,J.C., Jones,C., Wait,R., Previato,J.O. and Mendonca-Previato,L. (1996) Structural variation in the glycoinositolphospholipids of different strains of *Trypanosoma cruzi*. *Glycoconjugate J.*, **13**, 955–966.

Chan,A.L., Morris,H.R., Panico,M., Etienne,A.T., Rogers,M.E., Gaffney,P., Creighton-Kempsford,L. and Dell,A. (1991) A novel sialylated *N*-acetylgalactosamine-containing oligosaccharide is the major complex-type structure present in Bowes melanoma tissue plasminogen activator. *Glycobiology*, **1**, 173–186.

Chaturvedi,P., Warren,C.D., Ruiz-Palacios,G.M., Pickering,L.K. and Newburg,D.S. (1997) Milk oligosaccharide profiles by reversed-phase HPLC of their perbenzoylated derivatives. *Anal. Biochem.*, **251**, 89–97.

Chen,F.T.A., Dobashi,T.S. and Evangelista,R.A. (1998) Quantitative analysis of sugar constituents of glycoproteins by capillary electrophoresis. *Glycobiology*, **8**, 1045–1052.

Crocker,P.R. and Feizi,T. (1996) Carbohydrate recognition systems: Functional triads in cell–cell interactions. *Curr. Opin. Struct. Biol.*, **6**, 679–691.

Cumming,D.A., Hellerqvist,C.G., Harris-Brandts,M., Michnick,S.W., Carver,J.P. and Bendiak,B. (1989) Structures of asparagine-linked oligosaccharides of the glycoprotein fetuin having sialic acid linked to *N*-acetylglucosamine. *Biochemistry*, **28**, 6500–6512.

Dennis,J.W., Granovsky,M. and Warren,C.E. (1999) Protein glycosylation in development and disease. *Bioessays*, **21**, 412–421.

Devine,P.L. and McKenzie,I.F.C. (1992) Mucins: structure, function and associations with malignancy. *Bioessays*, **14**, 619–625.

Diaz,S., Higa,H.H., Hayes,B.K. and Varki,A. (1989) *O*-acetylation and de-*O*-acetylation of sialic acids. 7- and 9-*O*-acetylation of α2,6-linked sialic acids on endogenous *N*-linked glycans in rat liver Golgi vesicles. *J. Biol. Chem.*, **264**, 19416–19426.

Esko,J.D. (1991) Genetic analysis of proteoglycan structure, function and metabolism. *Curr. Opin. Cell Biol.*, **3**, 805–816.

Esko,J.D. and Zhang,L.J. (1996) Influence of core protein sequence on glycosaminoglycan assembly. *Curr. Opin. Struct. Biol.*, **6**, 663–670.

Ferguson,M.A.J. (1992) Lipid anchors on membrane proteins. *Curr. Opin. Struct. Biol.*, **1**, 522–529.

Fujimoto,I., Menon,K.K., Otake,Y., Tanaka,F., Wada,H., Takahashi,H., Tsuji,S., Natsuka,S., Nakakita,S., Hase,S. and Ikenaka,K. (1999) Systematic analysis of *N*-linked sugar chains from whole tissue employing partial automation. *Anal. Biochem.*, **267**, 336–343.

Furukawa,K. and Kobata,A. (1992) Protein glycosylation. *Curr. Opin. Biotechnol.*, **3**, 554–559.

Gahmberg,C.G. and Tolvanen,M. (1996) Why mammalian cell surface proteins are glycoproteins. *Trends Biochem. Sci. Sci.*, **21**, 308–311.

Geyer,H. and Geyer,R. (1998) Strategies for glycoconjugate analysis. *Acta Anat. (Basel)*, **161**, 18–35.

Green,E.D., Adelt,G., Baenziger,J.U., Wilson,S. and van Halbeek,H. (1988) The asparagine-linked oligosaccharides on bovine fetuin. Structural analysis of *N*-glycanase-released oligosaccharides by 500-megahertz ^{1}H NMR spectroscopy. *J. Biol. Chem.*, **263**, 18253–18268.

Greis,K.D., Hayes,B.K., Comer,F.I., Kirk,M., Barnes,S., Lowary,T.L. and Hart,G.W. (1996) Selective detection and site-analysis of O-GlcNAc-modified glycopeptides by β-elimination and tandem electrospray mass spectrometry. *Anal. Biochem.*, **234**, 38–49.

Hakomori,S. (1990) Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions. *J. Biol. Chem.*, **265**, 18713–18716.

Hakomori,S.I. and Siddiqui,B. (1974) Isolation and characterization of glycosphingolipid from animal cells and their membranes. *Methods Enzymol.*, **32**, 345–367.

Hanasaki,K., Varki,A., Stamenkovic,I. and Bevilacqua,M.P. (1994) Cytokine-induced β-galactoside α-2,6-sialyltransferase in human endothelial cells mediates α2,6-sialylation of adhesion molecules and CD22 ligands. *J. Biol. Chem.*, **269**, 10637–10643.

Hanisch,F.G., Müller,S., Hassan,H., Clausen,H., Zachara,N., Gooley,A.A., Paulsen,H., Alving,K. and Peter-Katalinic,J. (1999) Dynamic epigenetic regulation of initial *O*-glycosylation by UDP-*N*-acetylgalactosamine:peptide *N*-acetylgalactosaminyltransferases—site-specific glycosylation of MUC1 repeat peptide influences the substrate qualities at adjacent or distant Ser/Thr positions. *J. Biol. Chem.*, **274**, 9946–9954.

Hardy,M.R. and Townsend,R.R. (1988) Separation of positional isomers of oligosaccharides and glycopeptides by high-performance anion-exchange chromatography with pulsed amperometric detection. *Proc. Natl. Acad. Sci. USA*, **85**, 3289–3293.

Hardy,M.R. and Townsend,R.R. (1994) High-pH anion-exchange chromatography of glycoprotein-derived carbohydrates. *Methods Enzymol.*, **230**, 208–225.

Hart,G.W. (1992) Glycosylation. *Curr. Opin. Cell Biol.*, **4**, 1017–1023.

Hart,G.W., Haltiwanger,R.S., Holt,G.D. and Kelly,W.G. (1989) Glycosylation in the nucleus and cytoplasm. *Annu. Rev. Biochem.*, **58**, 841–874.

Hascall,V.C., Calabro,A., Midura,R.J. and Yanagishita,M. (1994) Isolation and characterization of proteoglycans. *Methods Enzymol.*, **230**, 390–417.

Hennet,T., Chui,D., Paulson,J.C. and Marth,J.D. (1998) Immune regulation by the ST6Gal sialyltransferase. *Proc. Natl Acad. Sci. USA*, **95**, 4504–4509.

Hennet,T., Hagen,F.K., Tabak,L.A. and Marth,J.D. (1995) T-cell-specific deletion of a polypeptide *N*-acetylgalactosaminyltransferase gene by site-directed recombination. *Proc. Natl. Acad. Sci. USA*, **92**, 12070–12074.

Herlant-Peers,M.C., Montreuil,J., Strecker,G., Dorland,L., van Halbeek,H., Veldink,G.A. and Vliegenthart,J.F.G. (1981) Structures of fifteen oligosaccharides isolated from new-born meconium. *Eur. J. Biochem.*, **117**, 291–300.

Hermentin,P., Doenges,R., Witzel,R., Hokke,C.H., Vliegenthart,J.F.G., Kamerling,J.P., Conradt,H.S., Nimtz,M. and Brazel,D. (1994) A strategy for the mapping of *N*-glycans by high-performance capillary electrophoresis. *Anal. Biochem.*, **221**, 29–41.

Holt,G.D., Snow,C.M., Senior,A., Haltiwanger,R.S., Gerace,L. and Hart,G.W. (1987) Nuclear pore complex glycoproteins contain cytoplasmically disposed O-linked N-acetylglucosamine. *J. Cell Biol.*, **104**, 1157–1164.

Hooper,L.V., Manzella,S.M. and Baenziger,J.U. (1996) From legumes to leukocytes: Biological roles for sulfated carbohydrates. *FASEB J.*, **10**, 1137–1146.

Iozzo,R.V. (1998) Matrix proteoglycans: from molecular design to cellular function. *Annu. Rev. Biochem.*, **67**, 609–652.

Ishii-Karakasa,I., Iwase,H. and Hotta,K. (1997) Structural determination of the O-linked sialyl oligosaccharides liberated from fetuin with endo-α-*N*-acetylgalactosaminidase-S by HPLC analysis and 600-MHz ^{1}H-NMR spectroscopy. *Eur. J. Biochem.*, **247**, 709–715.

Ito,M. and Yamagata,T. (1989) Endoglycoceramidase from *Rhodococcus* species G-74–2. *Methods Enzymol.*, **179**, 488–495.

Jamieson,J.C., McCaffrey,G. and Harder,P.G. (1993) Sialyltransferase: a novel acute-phase reactant. *Comp. Biochem. Physiol. [B]*, **105B**, 29–33.

Jungalwala,F.B. (1994) Expression and biological functions of sulfoglucuronyl glycolipids (SGGLs) in the nervous system—a review. *Neurochem. Res.*, **19**, 945–957.

Karlsson,K.A. (1995) Microbial recognition of target-cell glycoconjugates. *Curr. Opin. Struct. Biol.*, **5**, 622–635.

Kelm,S. and Schauer,R. (1997) Sialic acids in molecular and cellular interactions. *Int. Rev. Cytol.*, **175**, 137–240.

Kobata,A. and Furukawa,K. (1992) Methods of carbohydrate analysis and structural determination: chemical and enzymatic methods. In Allen,H.J. and Kisailus,E.C. (eds.), *Glycoconjugates: Composition*, *Structure and Function*. Marcel Dekker, New York, pp. 33–70.

Kotani,N. and Takasaki,S. (1997) Analysis of O-linked oligosaccharide alditols by high-pH anion-exchange chromatography with pulsed amperometric detection. *Anal. Biochem.*, **252**, 40–47.

Kotani,N., Asano,M., Iwakura,Y. and Takasaki,S. (1999) Impaired galactosylation of core 2 O-glycans in erythrocytes of β1,4-galactosyltransferase knockout mice. *Biochem. Biophys. Res. Commun.*, **260**, 94–98.

Kunz,C. and Rudloff,S. (1993) Biological functions of oligosaccharides in human milk. *Acta Paediatr.*, **82**, 903–912.

Laurent,T.C. and Fraser,J.R.E. (1992) Hyaluronan. *FASEB J.*, **6**, 2397–2404.

Lecat,D., Lemonnier,M., Derappe,C., Lhermitte,M., van Halbeek,H., Dorland,L. and Vliegenthart,J.F.G. (1984) The structure of sialyl-glycopeptides of the O-glycosidic type, isolated from sialidosis (mucolipidosis I) urine. *Eur. J. Biochem.*, **140**, 415–420.

Li,Q.X., Hasty,P. and Shur,B.D. (1997) Targeted mutation in β1,4-galactosyltransferase leads to pituitary insufficiency and neonatal lethality. *Dev. Biol.*, **181**, 257–267.

Lis,H. and Sharon,N. (1993) Protein glycosylation—structural and functional aspects. *Eur. J. Biochem.*, **218**, 1–27.

Lo,N.W. and Lau,J.T.Y. (1996) Transcription of the β-galactoside α2,6-sialyltransferase gene in B lymphocytes is directed by a separate and distinct promoter. *Glycobiology*, **6**, 271–279.

Lo,N.W., Shaper,J.H., Pevsner,J. and Shaper,N.L. (1998) The expanding β4-galactosyltransferase gene family: messages from the databanks. *Glycobiology*, **8**, 517–526.

Maley,F., Trimble,R.B., Tarentino,A.L. and Plummer,T.H.,Jr. (1989) Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. *Anal. Biochem.*, **180**, 195–204.

Manzi,A.E., Diaz,S. and Varki,A. (1990) High-pressure liquid chromatography of sialic acids on a pellicular resin anion-exchange column with pulsed amperometric detection: A comparison with six other systems. *Anal. Biochem.*, **188**, 20–32.

Manzi,A.E., Salimath,P.V., Spiro,R.C., Keifer,P.A. and Freeze,H.H. (1995) Identification of a novel glycosaminoglycan core-like molecule I. 500 MHz ^{1}H NMR analysis using a nano-NMR probe indicates the presence of a terminal α-GalNAc residue capping 4-methylumbelliferyl-β-D-xylosides. *J. Biol. Chem.*, **270**, 9154–9163.

Margolis,R.K. and Margolis,R.U. (1993) Nervous tissue proteoglycans. *Experientia*, **49**, 429–446.

Marth,J.D. (1994) Will the transgenic mouse serve as a Rosetta Stone to glycoconjugate function? *Glycoconjugate J.*, **11**, 3–8

Mechref,Y. and Novotny,M.V. (1998) Mass spectrometric mapping and sequencing of N-linked oligosaccharides derived from submicrogram amounts of glycoproteins. *Anal. Chem.,* **70**, 455–463.

Mensa-Wilmot,K., LeBowitz,J.H., Chang,K.-P., Al-Qahtani,A., McGwire, B.S., Tucker,S. and Morris,J.C. (1994) A glycosylphosphatidylinositol (GPI)-negative phenotype produced in *Leishmania major* by GPI phospholipase C from *Trypanosoma brucei*: topography of two GPI pathways. *J. Cell Biol.*, **124**, 935–947.

Miller-Podraza,H. Andersson,C. and Karlsson,K.-A. (1993) New method for the isolation of polyglycosylceramides from human erythrocyte membranes. *Biochim. Biophys. Acta Lipids Lipid Metab.*, **1168**, 330–339.

Nilsson,B., Norden,N.E. and Svensson,S. (1979) Structural studies on the carbohydrate portion of fetuin. *J. Biol. Chem.*, **254**, 4545–4553.

Norgard-Sumnicht,K.E. and Varki,A. (1995) Endothelial heparan sulfate proteoglycans that bind to L-selectin have glucosamine residues with unsubstituted amino groups. *J. Biol. Chem.*, **270**, 12012–12024.

Norgard-Sumnicht,K.E., Roux,L., Toomre,D.K., Manzi,A.E., Freeze,H.H. and Varki,A. (1995) Unusual anionic N-linked oligosaccharides from bovine lung. *J. Biol. Chem.*, **270**, 27634–27645.

Norgard-Sumnicht,K.E., Bai,X., Esko,J.D., Varki,A. and Manzi,A.E. (2000) Exploring the outcome of genetic modifications of glycosylation in cultured cell lines by concurrent isolation of the major classes of vertebrate glycans. *Glycobiology*, **10**, 691–700.

O'Hanlon,T.P., Lau,K.M., Wang,X.C. and Lau,J.T.Y. (1989) Tissue-specific expression of β-galactoside α-2,6-sialyltransferase. Transcript heterogeneity predicts a divergent polypeptide. *J. Biol. Chem.*, **264**, 17389–17394.

Page,M.J., Amess,B., Rohlff,C., Stubberfield,C. and Parekh,R. (1999) Proteomics: a major new technology for the drug discovery process. *DDT*, **4**, 55–62.

Patel,T., Bruce,J., Merry,A., Bigge,C., Wormald,M., Jaques,A. and Parekh,R. (1993) Use of hydrazine to release in intact and unreduced form both N- and O-linked oligosaccharides from glycoproteins. *Biochemistry*, **32**, 679–693.

Plummer,T.H.,Jr. and Tarentino,A.L. (1991) Purification of the oligosaccharide-cleaving enzymes of Flavobacterium meningosepticum. *Glycobiology*, **1**, 257–263.

Rademacher,T.W., Parekh,R.B. and Dwek,R.A. (1988) *Glycobiology*. *Annu. Rev. Biochem.*, **57**, 785–838.

Reason,A.J., Blench,I.P., Haltiwanger,R.S., Hart,G.W., Morris,H.R., Panico,M. and Dell,A. (1991) High-sensitivity FAB-MS strategies for O-GlcNAc characterization. *Glycobiology*, **1**, 585–594.

Reuter,G. and Gabius,H.J. (1999) Eukaryotic glycosylation: whim of nature or multipurpose tool? *Cell. Mol. Life Sci.*, **55**, 368–422.

Rose,M.C. (1992) Mucins: structure, function and role in pulmonary diseases. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **263**, L413–L429.

Rudd,P.M. and Dwek,R.A. (1997) Rapid, sensitive sequencing of oligosaccharides from glycoproteins. *Curr. Opin. Biotechnol.*, **8**, 488–497.

Rutishauser,U. (1996) Polysialic acid and the regulation of cell interactions. *Curr. Opin. Cell Biol.*, **8**, 679–684.

Salmivirta,M., Lidholt,K. and Lindahl,U. (1996) Heparan sulfate: a piece of information. *FASEB J.*, **10**, 1270–1279.

Sandhoff,K. and Klein,A. (1994) Intracellular trafficking of glycosphingolipids: Role of sphingolipid activator proteins in the topology of endocytosis and lysosomal digestion. *FEBS Lett.*, **346**, 103–107.

Sato,Y. and Endo,T. (1999) Differential expression of sialoglycoproteins in the rat hippocampus and its changes during aging. *Neurosci. Lett.*, **262**, 49–52.

Schachter,H. and Brockhausen,I. (1992) The biosynthesis of serine (threonine)-N-acetylgalactosamine-linked carbohydrate moieties. In Allen,H.J. and Kisailus,E.C. (eds.), *Glycoconjugates: Composition*, *Structure and Function*. pp. 263–**332**, Marcel Dekker, New York.

Schachter,H. (1991) The "yellow brick road" to branched complex N-glycans. *Glycobiology*, **1**, 453–462.

Schnaar,R.L. (1994) Isolation of glycosphingolipids. *Methods Enzymol.*, **230**, 348–370.

Schnaar,R.L. and Needham,L.K. (1994) Thin-layer chromatography of glycosphingolipids. *Methods Enzymol.*, **230**, 371–389.

Smith,D.F. (1996) Special considerations for glycolipids and their purification. In Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A., Struhl,K., Albright,L.M., Coen,D.M. and Varki,A. (eds.), *Current Protocols in Molecular Biology*. John Wiley & Sons, New York, unit 17–3.

Smith,P.L., Skelton,T.P., Fiete,D., Dharmesh,S.M., Beranek,M.C., MacPhail,L., Broze,G.J.,Jr. and Baenziger,J.U. (1992) The asparagine-linked oligosaccharides on tissue factor pathway inhibitor terminate with SO_4-4GalNAcβ1,4GlcNAcβ1,2Manα. *J. Biol. Chem.*, **267**, 19140–19146.

Spellman,M.W., Basa,L.J., Leonard,C.K., Chakel,J.A., O'Connor,J.V., Wilson,S. and van Halbeek,H. (1989) Carbohydrate structures of human tissue plasminogen activator expressed in Chinese hamster ovary cells. *J. Biol. Chem.*, **264**, 14100–14111.

Spellman,M.W., Leonard,C.K., Basa,L.J., Gelineo,I. and van Halbeek,H. (1991) Carbohydrate structures of recombinant soluble human CD4 expressed in Chinese hamster ovary cells. *Biochemistry*, **30**, 2395–2406.

Stanley,P. (1992) Glycosylation engineering. *Glycobiology*, **2**, 99–107.

Stanley,P. and Ioffe,E. (1995) Glycosyltransferase mutants: Key to new insights in glycobiology. *FASEB J.*, **9**, 1436–1444.

Strecker,G., Fièvre,S., Wieruszeski,J.-M., Michalski,J.-C. and Montreuil,J. (1992) Primary structure of four human milk octa-, nona- and undeca-saccharides established by ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy. *Carbohydr. Res.*, **226**, 1–14.

Strous,G.J. and Dekker,J. (1992) Mucin-type glycoproteins. *CRC Crit. Rev. Biochem. Mol. Biol.*, **27**, 57–92.

Stults,C.L.M., Sweeley,C.C. and Macher,B.A. (1989) Glycosphingolipids: Structure, biological source and properties. *Methods Enzymol.*, **179**, 167–214.

Takasaki,S. and Kobata,A. (1986) Asparagine-linked sugar chains of fetuin: occurrence of tetrasialyl triantennary sugar chains containing the Gal beta 1–3GlcNAc sequence. *Biochemistry*, **25**, 5709–5715.

Taki,T., Rokukawa,C., Kasama,T. and Handa,S. (1992a) Human hepatoma gangliosides: occurrence of a novel I-type glycolipid with NeuAcα2–6Gal structure. *Cancer Res.*, **52**, 4805–4811.

Taki,T., Rokukawa,C., Kasama,T., Kon,K. Ando,S., Abe,T. and Handa,S. (1992b) Human meconium gangliosides. Characterization of a novel I-type ganglioside with the NeuAcα2–6Gal structure. *J. Biol. Chem.*, **267**, 11811–11817.

Troy,F.A., II (1992) Polysialylation: from bacteria to brains. *Glycobiology*, **2**, 5–23.

Tsuji,S. (1996) Molecular cloning and functional analysis of sialyltransferases. *J. Biochem. (Tokyo),* **120**, 1–13.

Van Halbeek,H. (1993). Structural characterization of the carbohydrate moieties of glycoproteins by high-resolution ^{1}H-NMR spectroscopy. *Methods Mol. Biol.*, **17**:115–148.

Van Halbeek,H. (1994) ^{1}H nuclear magnetic resonance spectroscopy of carbohydrate chains of glycoproteins. *Methods Enzymol.*, **230**, 132–168.

Van Lenten,L. and Ashwell,G. (1971) Studies on the chemical and enzymatic modification of glycoproteins A general method for the tritiation of sialic acid-containing glycoproteins. *J. Biol. Chem.*, **246**, 1889–1894.

Varki,A. and Diaz,S. (1984) The release and purification of sialic acids from glycoconjugates: methods to minimize the loss and migration of O-acetyl groups. *Anal. Biochem.,* **137**, 236–247.

Varki,A. (1992) Diversity in the sialic acids. *Glycobiology*, **2**, 25–40.

Varki,A. (1993) Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology*, **3**, 97–130.

Varki,A. and Freeze,H.H. (1994) The major glycosylation pathways of mammalian membranes; a summary. In Maddy,A.H. and Harris,J.R. (eds.), *Subcellular Biochemistry*. Plenum Press, New York, pp. 71–100.

Varki,A., Manzi,A.E. and Freeze,H.H. (1996) Introduction: preparation and analysis of glycoconjugates. In Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A., Struhl,K., Albright,L.M., Coen,D.M. and Varki,A. (eds.), *Current Protocols in Molecular Biology*. John Wiley & Sons, New York, pp. Unit 17.0.

Verma,M. and Davidson,E.A. (1994) Mucin genes: structure, expression and regulation. *Glycoconjugate J.*, **11**, 172–179.

Vliegenthart,J.F. G., Dorland,L., van Halbeek,H. and Haverkamp,J. (1982) NMR spectroscopy of sialic acids. In Schauer,R. (ed.), *Sialic Acids: Chemistry*, *Metabolism and Function*, *Cell Biology Monographs*, Volume 10. Springer Verlag, Vienna/New York, pp. 127–172.

Vliegenthart,J.F.G., Dorland,L. and van Halbeek,H. (1983) High resolution proton nuclear magnetic resonance spectroscopy as a tool in the structural analysis of carbohydrates related to glycoproteins. *Adv. Carbohydr. Chem. Biochem.*, **41**, 209–378.

Wang,X., O'Hanlon,T.P. and Lau,J.T.Y. (1989) Regulation of β-galactoside α2,6-sialyltransferase gene expression by dexamethasone. *J. Biol. Chem.*, **264**, 1854–1859.

Watson,E., Bhide,A. and van Halbeek,H. (1994) Structure determination of the intact major sialylated oligosaccharide chains of recombinant human erythropoietin expressed in Chinese hamster ovary cells. *Glycobiology*, **4**, 227–237.

Wing,D.R., Rademacher,T.W., Field,M.C., Dwek,R.A., Schmitz,B., Thor,G. and Schachner,M. (1992) Use of large-scale hydrazinolysis in the preparation of *N*-linked oligosaccharide libraries: Application to brain tissue. *Glycoconjugate J.*, **9**, 293–301.

Zeller,C.B. and Marchase,R.B. (1992) Gangliosides as modulators of cell function. *Am. J. Physiol. Cell Physiol.*, **262**, C1341–C1355.