Radioactive tracer techniques in the sequencing of glycoprotein oligosaccharides

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ABSTRACT **Complete sequencing of the oligosaccharide units of glycoproteins can be performed by conventional physical techniques if nanomole quantities of pure molecules are available. However, isolation of sufficient quantities of a glycoprotein may not be technically feasible (e.g., the analysis of biosynthetic intermediates, or rare molecules). Alternatively, partial structural analyses may answer the biological question at hand. In both instances, radioactive sugars can be used to metabolically label the oligosaccharide units of a glycoprotein, permitting substantial structural characterization. Several aspects of this approach are discussed in this overview, including selection of the labeled precursor, maximization of uptake and incorporation, determinants of the specificity of labeling, and general principles for the release and structural analysis of labeled oligosaccharides. Particular advantages include simplicity, ease of use without sophisticated instrumentation, and the fact that purification to radiometric homogeneity is sufficient. Radioactive tracer techniques cannot replace conventional approaches to sequencing oligosaccharides. However, they do provide a rapid, relatively simple approach to obtaining considerable information from limited amounts of material. For molecules such as short-lived biosynthetic intermediates, there is no substitute for these techniques. This approach has been responsible for the initial indentification and characterization of many novel oligosaccharides of biological interest.—Varki. A. Radioactive tracer techniques in the sequencing of glycoprotein oligosaccarides. *FASEB J.* 5: 226-235; 1991.**

Key Words: surface labeling • monosaccharide precursor • glycosylation • isotope • metabolic pathway • nucleotide • sequential chromatography

In recent years, there have been dramatic advances in understanding the structure and functions of the oligosaccharide units (sugar chains) of glycoconjugates (1). Shown in **Fig. 1** are schematic representations of the most common types of oligosaccharides found on vertebrate glycoconjugates. The complete sequence of many of these molecules has been proved by methods such as nuclear magnetic resonance (NMR)[1] spectroscopy, gas-liquid chromatography/mass spectrometry (GLC/MS), fast atom bombardment-mass spectrometry (FAB-MS), and more recently by anion-exchange HPLC with pulsed amperometric detection (HPLC-PAD) (2-7). In several cases identification and partial structural analysis of these oligosaccharides was accomplished first by metabolic labeling with radioactive monosaccharide precursors. In this review, I summarize the role that these radioactive tracer techniques can play in sequencing the N- and O-linked oligosaccharides of glycoproteins, some of the problems involved, and the prospects for the future. Many of the principles discussed are applicable to the study of other glycoconjugates, such as the glycolipids and proteoglycans.

WHY SEQUENCE OLIGOSACCHARIDES?

There are many examples in which oligosaccharides modulate or mediate important biological functions of glycoproteins. **Table 1** lists some representative situations involving N-linked oligosaccharides. In exploring the biological effects of such oligosaccharides, it usually is necessary to obtain information about their sequence and general branching patterns, and also about their biosynthetic precursors. When metabolic labeling of a glycoprotein is feasible, such information can be readily obtained by radioactive tracer techniques.

PRELIMINARY ANALYSIS OF THE OLIGOSACCHARIDES PRESENT ON A GLYCOPROTEIN

Using the methods outlined in **Table 2**, preliminary information about the sugar chains on a glycoprotein can be obtained if it can be labeled in the peptide moiety (e.g., with a radioactive amino acid or with surface labeling.) With some of these methods, a functional or immunological assay for an unlabeled protein can even be used. Important clues about the type, size, number, and charge of the oligosaccharides are obtained, helping to deterimine the approach to detailed structural analysis.

WAYS TO INTRODUCE RADIOACTIVITY INTO GLYCOPROTEIN OLIGOSACCHARIDES

One method is to use purified glycosyltransferases and labeled sugar nucleotides, transferring labeled monosaccha-

[1]Abbreviations: NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; FAB-MS, fast atom bombardment-mass spectrometry; anion-exchange HPLC with pulsed amperometric detection (HPLC-PAD); Neu5Ac, N-acetylneuraminic acid; $GlcNH_2$, glucosamine, GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; ManNAc, N-acetylmannosamine, Man, mannose; Fuc, fucose; Xyl, xylose, Gal, galactose; endoH, endo-β-N-acetyl-glucosaminidase H; PNGaseF, peptide:N-glycosidase F; concanavalin A, Con A; phytohemagglutinin E_4, E-PHA, phytohemagglutinin L_4, L-PHA; SDS, sodium dodecyl sulfate.

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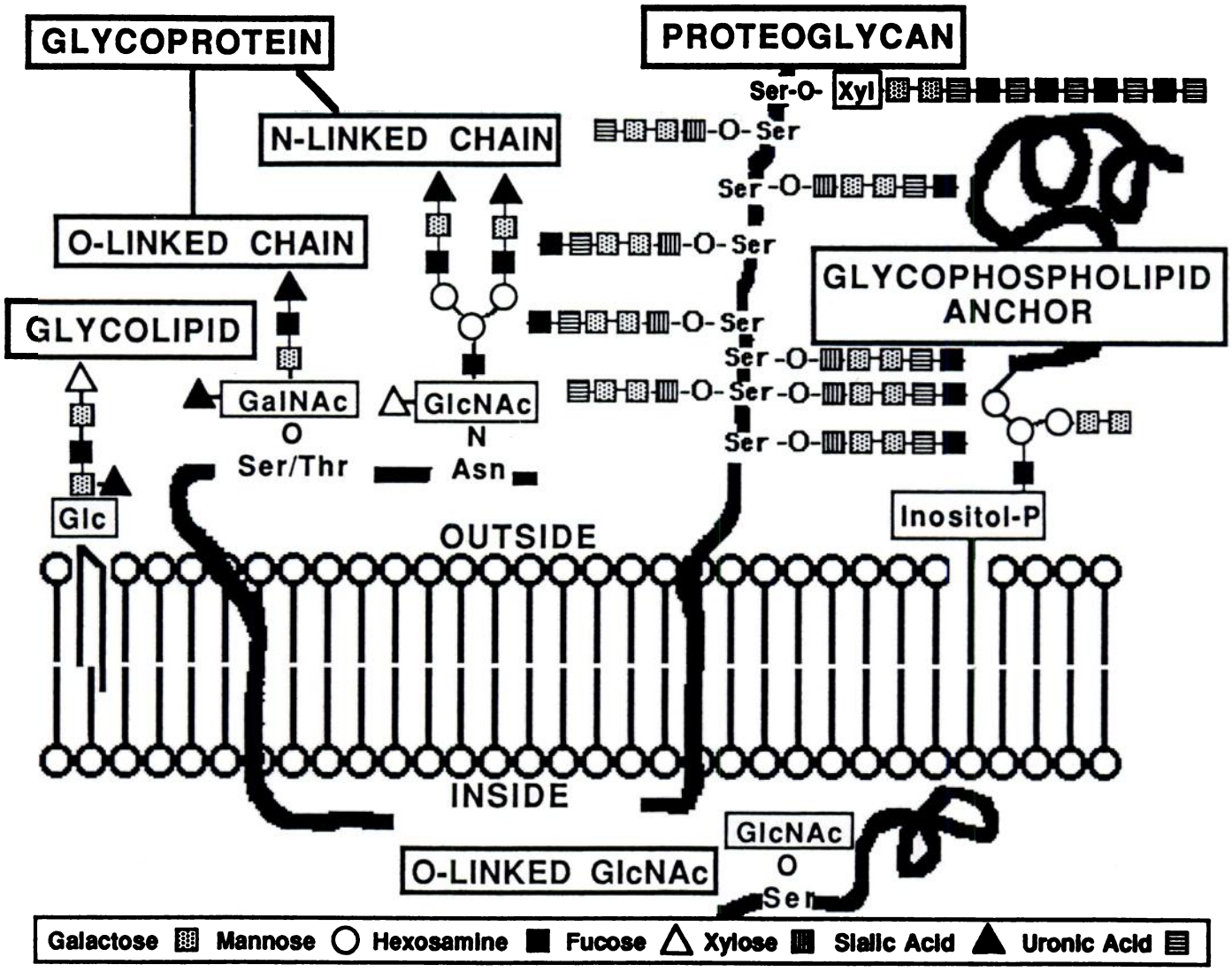

Figure 1. Common oligosaccharides found on vertebrate glycoconjugates. The most common linkage regions of oligosaccharides to proteins and lipids are shown in a schematic form, without emphasizing details of sugar chain structure or their variations. Note that mannose and fucose residues are found only on certain types of glycoconjugates. Thus, when labeling cells with the precursor [2-^{3}H]mannose (see Fig. 4), selective labeling of these sugar chains occurs.

rides to a terminal position on the glycoprotein oligosaccharides (16). However, because the label is on a terminal sugar, the extent of structural analysis possible is limited. A second general method is reduction with tritiated borohydride (2). On oligosaccharides released from glycoproteins by enzymatic or chemical means, this label enters the sugar at the reducing terminus (the innermost sugar residue). If the reduction is done after mild periodate oxidation or galactose oxidase treatment, the label enters the side chain of truncated sialic acid residues or the 6-position of terminal galactose/N-acetylgalactosamine residues, respectively (17). Another method is to chemically de-N-acetylate oligosaccharides and then to re-N-acetylate the resulting amino sugar residues with radioactive acetic anhydride (18). However,

TABLE 1. *Functional importance of the N-linked oligosaccharides of some glycoproteins*

Protein	Effect of lack/alteration of oligosaccharides	Selected references
AIDS virus glycoprotein (GP120)	Lowered fusion activity/decreased infectivity	8
Gonadotropic hormones	Agonist converted into antagonist	9
Granulocyte/macrophage colony-stimulating factor	Graded increase in activity with decrease in glycosylation	10
Viral coat proteins	Increased reactivity with polyclonal antisera	11
Lysosomal enzymes	Loss of targeting signal for delivery of enzymes to lysosomes	12
Erythropoietin	Failure of secretion/decrease in biological activity	13
Immunoglobulins	Alteration of some secondary effector functions of the Fc region	14
Many glycoprotein receptors	Failure of disulfide bond formation/activation	15

TABLE 2. *Preliminary analysis of the type of oligosaccharides present on a glycoprotein, labeled in the peptide moiety*

Method	Expected information
Cleavage with endoglycosidases and SDS-PAGE analysis	General types of oligosaccharides present Contribution of oligosaccharides to apparent mol wt of protein
Lectin binding	General types of oligosaccharides present Types of terminal sugars present
Pulse-chase analysis of biosynthesis/endoglycosidases	Time of addition and evidence for processing of oligosaccharide General types of oligosaccharides present Contribution of oligosaccharide to apparent mol wt of protein
Effects of glycosylation inhibitors of mol wt	Contribution of oligosaccharide to apparent mol wt of protein Confirmation of glycosylation type
Isoelectric focusing (effect of glycosidases)	Presence and type of charged groups; extent of heterogeneity

when using radioactive borohydride or acetic anhydride, the high background noise requires that a substantial mass of material be available for the labeling. Thus, molecules that can be cleanly labeled by such chemical means can usually also be sequenced by physical methods. Finally, radioactivity can be introduced into glycoprotein oligosaccharides by metabolic labeling with radioactive monosaccharide precursors (19, 20). This review focuses on the latter approach.

WHEN ARE METABOLIC LABELING TECHNIQUES USEFUL IN THE STUDY OF GLYCOPROTEIN OLIGOSACCHARIDES?

When adequate amounts of material are available, complete and accurate sequencing of glycoprotein oligosaccharides is best accomplished by modern physical methods (see **Table 3** for a listing of the commonly used methods and their relative sensitivities). However, in several instances metabolic labeling techniques are useful or even required in sequencing glycoprotein oligosaccharides. These include the structural characterization of biosynthetic intermediates and short-lived glycoforms of various proteins; the identification and preliminary characterization of new structures; identification of minor components in complex mixtures; and the partial characterization of major components, when isolation of nmole amounts is not feasible, or when partial structural information is sufficient to answer the question at hand.

RATIONALE FOR THE USE OF MONOSACCHARIDE PRECURSORS IN METABOLIC LABELING

Although sugar nucleotides are the immediate donors for glycosylation reactions they cannot be taken up by cells. Hence, metabolic labeling is accomplished with radiolabeled monosaccharide precursors. These are taken up, activated to the labeled sugar nucleotides, and transported into the Golgi apparatus (21, 22) where luminally oriented transferases add the monosaccharides to luminally oriented acceptors (see **Fig. 2**). The few known exceptions to this topology have been reviewed by Hart (23).

Selecting a labeled monosaccharide precursor is based on several factors including the efficiency of uptake (see below) and the type of glycoconjugate to be labeled (the distribution of monosaccharides among different types of vertebrate oligosaccharides is nonrandom). The labeling can be long-term (equilibrium), or short-term for a pulse-chase analysis. The type of monosaccharide to be used and the period of labeling will determine whether normal or reduced glucose in the medium should be used (see below). The labeled glycoprotein of interest is isolated, and identification and

TABLE 3. *Comparison of different approaches to the sequencing of glycoprotein oligosaccharides*

Approach	Instrumentation required	Amounts required	Sample purity required	Analysis of mixtures
Nuclear magnetic resonance spectroscopy	500 Mhz NMR machine	10–100 nmol	Extreme	Fair
Fast atom bombardment/mass spectrometry	FAB-MS machine	1–10 nmol	Moderate	Good
Gas-liquid chromatography/mass spectrometry	GLC/MS machine	1–10 nmol	Moderate	Poor
Anion-exchange HPLC/pulsed amperometric detection	HPLC/PAD apparatus	0.1–1 nmol	Moderate	Fair
Metabolic labeling/radioactive tracer techniques	Scintillation counter	0.01–0.1 nmol[a]	Radiometric	Good

[a] For labeling with a ^{3}H-monosaccharide precursor of specific activity 10 Ci/mmol; assumes variable (10–100-fold) dilution by endogenous sugars. See text for discussion of some issues raised in this table.

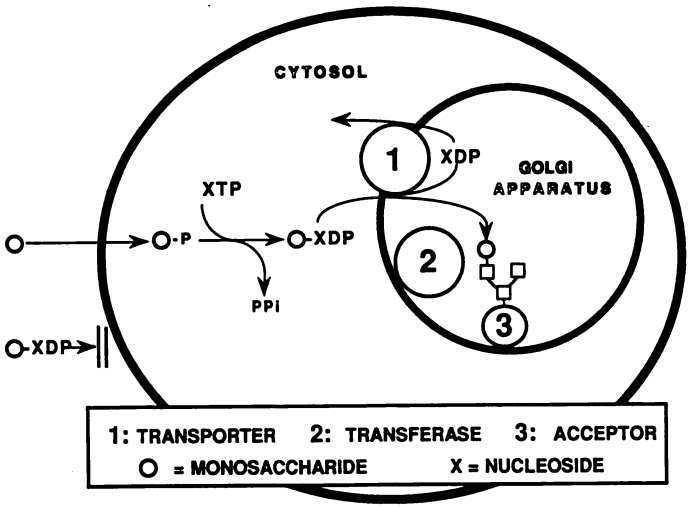

Figure 2. Utilization of labeled monosaccharide precursors by cells. Labeled monosaccharide precursors are taken up by cells and activated to the corresponding sugar nucleotides, which are used by the Golgi apparatus in the synthesis of oligosaccharide chains. Uptake into cells is either passive or active (see text for discussion).

separation of individual glycosylation sites may be necessary. Structural analyses of the labeled oligosaccharides are then performed.

THE TYPE OF MONOSACCHARIDE PRECURSOR AND THE POSITION OF THE ISOTOPE AFFECT SPECIFICITY AND FINAL DISTRIBUTION OF THE LABEL

The metabolic pathways for uptake, activation, utilization, and interconversion between the various monosaccharides and their nucleotide sugars have been well worked out (see **Fig. 3** and refs 19, 24–27 for examples). The final distribution and specific activity of a given label can be significantly affected by dilution from endogenous pathways (28, 29), the cell type under study, and the conditions of labeling (20, 28–30). The position of the label within the monosaccharide used can also affect the ultimate fate of the radioactivity. As shown in Fig. 3, label originating from the 6-position of [6-^{3}H]GlcNH$_2$, [6-^{3}H]GlcNAc, or [6-^{3}H]ManNAc is eventually found at either the 9-position of terminal sialic residues or at the 6-position of GlcNAc or GalNAc residues (30). It can also be seen from this figure why [6-^{3}H]ManNAc, which is touted as a specific precursor for the labeling of sialic acids, can eventually label other sugar residues (30).

With most labeled monosaccharide precursors, eventual interconversion with glucose can be expected, thus spreading the label to nonoligosaccharide components of the cell. In contrast, extreme specificity can be obtained with [2-^{3}H]mannose. As shown in **Fig. 4**, cytosolic mannose is converted to mannose 6-phosphate, activated to labeled GDP-mannose and ultimately to GDP-fucose, thus entering various oligosaccharides. However, it can also be converted to glucose-6-phosphate and thereby enter other biochemical pathways. When the ^{3}H-label is at the 2-position of the monosaccharide precursor, any labeled molecule that is converted by phosphomannose isomerase to fructose-6-phosphate concomitantly loses the label from the 2-position as tritiated water, which is diluted into the pool of cellular water of very high molarity (31). Thus, in almost all cells studied, the label remains essentially confined to mannose and fucose residues, regardless of how long the labeling proceeds. Indeed, as depicted in Fig. 1, labeling of cells with [2-^{3}H]mannose results in highly selective labeling of certain structures, such as the N-linked oligosaccharides and glycophospholipid anchors.

However, a single exception has been reported in which ^{3}H-label from the 2-position of mannose was found in lactic acid in the low-molecular-weight pool (32). This indicates that as-yet-unknown mechanisms may exist for sugar interconversions in some cells, and suggests caution when dealing with previously unexplored pathways or cell types.

FACTORS AFFECTING UPTAKE AND INCORPORATION OF RADIOACTIVE MONOSACCHARIDE PRECURSORS

With radioactive amino acids, labeling to high specific activity can be obtained by omitting the unlabeled molecule from the medium. Labeling with radioactive sugars is not as efficient. Thus, it is necessary to consider several factors to optimize incorporation into the glycoconjugate of interest. The general factors are self-evident, including the amount of label, the concentration of label in the media, the number of cells, the duration of labeling, and the number of cell doublings that occur during the labeling. Some of these factors are at odds with one another, and the correct balance between them must be individualized to the particular cell type. One successful approach has been to expose a series of plates of cells sequentially to a small volume of media containing a high concentration of label. If the period of exposure is relatively short (e.g., a few hours), the labeling media can be reused sequentially for several plates of cells (33, 34). This approach can also be adapted to pulse-chase analyses (35, 36).

The most specific factor affecting the uptake and incorporation of a radioactive monosaccharide precursor is whether or not it competes with glucose for uptake. As glucose in normal tissue culture media is about 5 mM, monosaccharides that have to compete for active transport cannot be taken up any better than glucose itself. On the other hand, sugars that do not compete with glucose are taken up only by noncompetitive, passive, and therefore inefficient mechanisms. Although a comprehensive study of this issue has not been performed, past experience indicates that glucosamine,

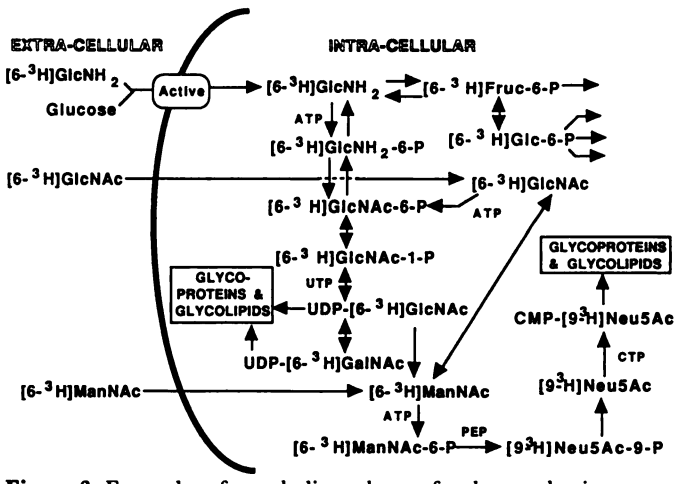

Figure 3. Examples of metabolic pathways for the uptake, interconversion, activation, and utilization of some labeled monosaccharide precursors. The primary abbreviations used for the sugars are in the list at the beginning of the article.

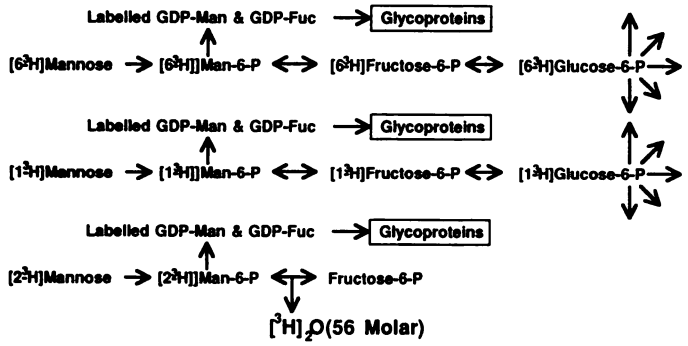

Figure 4. The ultimate fate of ^{3}H-labeled mannose depends on the position of the initial label in the monosaccharide. The primary abbreviations used for the sugars are in the list at the beginning of the article.

galactosamine, galactose, and mannose compete with glucose for uptake into most cells, whereas N-acetylglucosamine, N-acetylmannosamine, mannosamine, fucose, and xylose do not (20). Thus in the former case, more radioactivity can be incorporated into cells if glucose concentration is reduced (the addition of pyruvate can help to increase the tolerance of cells to glucose-poor media). On the other hand, manipulation of the glucose concentration has little effect on the relatively poor uptake of the latter group of sugars. However, it has recently been recognized that glucose transporters are a family of different gene products that are tissue-specific in their expression (37). Thus, it may be necessary to try each monosaccharide with the specific cell type under study to determine which competes with glucose. It should also be kept in mind that the lipid-linked oligosaccharide precursor can become altered when glucose-free media is used in some cell types (38).

Other factors affecting incorporation are the dilution of label by endogenously synthesized monosaccharides, the pool size of the individual monosaccharides and nucleotides, and the flux rates between interconverting pathways mentioned earlier. The investigator has relatively little control over these factors, but must take them into account in designing the final labeling protocol. The ultimate goal is to obtain a sufficient amount of label in the glycoprotein of interest to permit the studies planned, without significantly altering the metabolic state of the cell.

ISOLATION OF THE LABELED GLYCOCONJUGATES BEFORE RELEASE AND ANALYSIS OF OLIGOSACCHARIDES OR GLYCOPEPTIDES

After metabolic labeling, the goal is to achieve purification of the glycoprotein of interest to radiometric homogeneity, e.g., a single band on an autoradiogram of a gel. Because glycoproteins are a minority of the total proteins in a typical cell, purification to such radiometric homogeneity is far less difficult when the protein is labeled in the sugar rather than in the peptide. This is one of the major advantages of this approach over conventional purification of unlabeled glycoproteins for structural analysis. The glycoprotein is solubilized if necessary, conventional purification steps are carried out when needed, and selective isolation is done by steps such as immunoaffinity chromatography with an antibody against a peptide epitope, or electroelution from a gel slice. In many cases, such purification to radiometric homogeneity can be achieved with a single affinity step. Lectin affinity chromatography is better avoided at this stage because of the danger of selective losses of certain glycoforms of the protein.

ANALYSIS OF SITE-SPECIFIC GLYCOSYLATION OF A LABELED PROTEIN

Oligosaccharides can be attached to glycoproteins at more than one site. If it is necessary to analyze glycosylation in a site-specific manner, the labeled glycoprotein is isolated, selectively digested with enzymes such as trypsin and chymotrypsin, and the labeled glycopeptides are separated by reverse-phase high-pressure liquid chromatography (HPLC). In this type of separation, the hydrophobicity of the peptide is the major determinant of fractionation, with relatively little contribution to chromatography from the oligosaccharide itself. Thus, individual radioactive peaks can be pooled as individual glycosylation sites (as the label is only in sugars, it is not necessary to monitor the elution profile of the unlabeled peptides). It may be necessary to digest with additional proteases to rule out multiple glycosylation sites in each peak. Finally, the isolated glycopeptides are digested further with Pronase (or proteinase K); alternatively, the oligosaccharides can be released with a specific endoglycosidase for analysis. In this manner, structural analysis of the oligosaccharides at individual glycosylation sites can be achieved (see refs 35, 39–41 for examples).

ENDOGLYCOSIDASE-RELEASED OLIGOSACCHARIDES VS. PROTEASE-GENERATED GLYCOPEPTIDES: ADVANTAGES AND DISADVANTAGES

The traditional approach has been to study Pronase-generated glycopeptides in which one or very few amino acids remain attached to the oligosaccharide. The advantages of this approach are that it is traditional, well established, reliable, and rapid, and that the reagents are cheap. However, there are several disadvantages. First, one cannot be certain that each peptide has only one glycosylation site. Second, the charge of the peptide can affect subsequent chromatographic analyses. Finally, the generation of glycopeptides does not assure removal of any nonsaccharide, protease-insensitive material that might be carrying radioactivity.

The study of free, released oligosaccharides is much more specific. As the endoglycosidases used for release have well-known specificities (42, 43), the release itself provides significant structural information. If endoglycosidases are used sequentially (e.g., endoH followed by PNGaseF), partial fractionation of distinct classes of oligosaccharides can also be achieved. The disadvantages of this approach are that the endoglycosidases are relatively expensive and the methods of analysis of released oligosaccharides are not as well established as those for the glycopeptides. Also, all of the possible endoglycosidases required to release all the currently known oligosaccharides are not yet available.

SEPARATION OF RELEASED OLIGOSACCHARIDES FROM UNRELEASED RADIOACTIVITY

After digestion with endoglycosidases, it is necessary to separate and isolate the released oligosaccharides from the undigested label associated with the protein. As subsequent analyses proceed on the assumption that all of the label is in free oligosaccharides, it is vital that such separation be com-

plete. It is also important that nonspecific proteolysis not occur during the release reaction or proteolytic fragments could be mistaken for released sugar chains. We have taken advantage of the fact that several endoglycosidases such as endoH and PNGaseF can work in the presence of sodium dodecyl sulfate (SDS), if final concentrations and additional buffer components are appropriately adjusted. Labeled material to be examined is first boiled in SDS to inactivate all endogenous enzymes, and then isolated from the void volume region of an S-200 column run in an SDS-containing buffer (see **Fig. 5**). Proteins of all sizes bind SDS and tend to cluster at or near the void volume region under these conditions, separating them from low-molecular-weight contaminants. The macromolecules are then concentrated by acetone precipitation, redissolved in SDS, and the conditions adjusted for treatment with a specific endoglycosidase. After incubation with the enzyme, the material is reapplied to the same column. A buffer control is also similarly incubated and studied on the same column. If the buffer control shows no significant included radioactivity, the radioactivity released by the enzyme treatment can be confidently identified and pooled, free of any undigested material. The released oligosaccharides can be freed of SDS using potassium precipitation and then desalted before analysis (see Fig. 5 for general rationale and refs 34, 44, 45 for examples and experimental details).

STRUCTURAL ANALYSIS OF THE OLIGOSACCHARIDES (OR GLYCOPEPTIDES)

Unlike the linear sequences of RNA, DNA, and proteins, oligosaccharides show complexity in branching and in having α- or β-anomeric linkages. Thus, accurate sequencing of oligosaccharides requires multiple overlapping and iterative techniques. Space does not permit a detailed discussion of such techniques here: an excellent review of the subject has appeared recently (46). An overall approach to the structural analysis of labeled oligosaccharides or glycopeptides is outlined in **Fig. 6**. The initial goal is to obtain a composition of labeled monosaccharides (i.e., define which monosaccharides carry the label) and then to fractionate the molecules according to negative charge. The latter is necessary because anionic groups (e.g., sialic acids, sulfate, and phosphate esters)

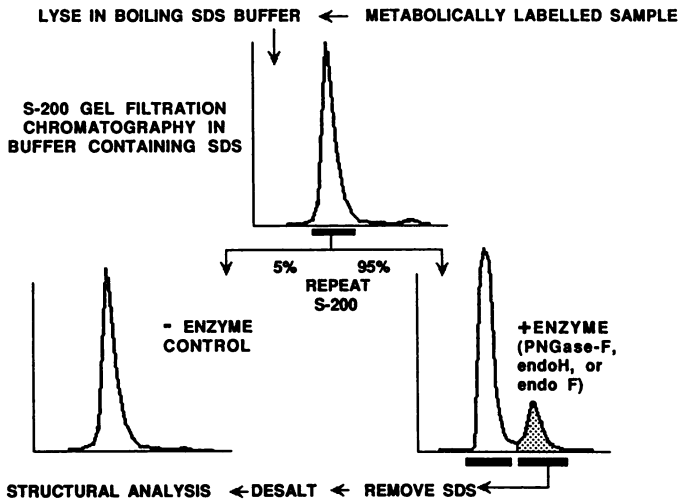

Figure 5. An approach to the release and isolation of labeled oligosaccharides from macromolecules.

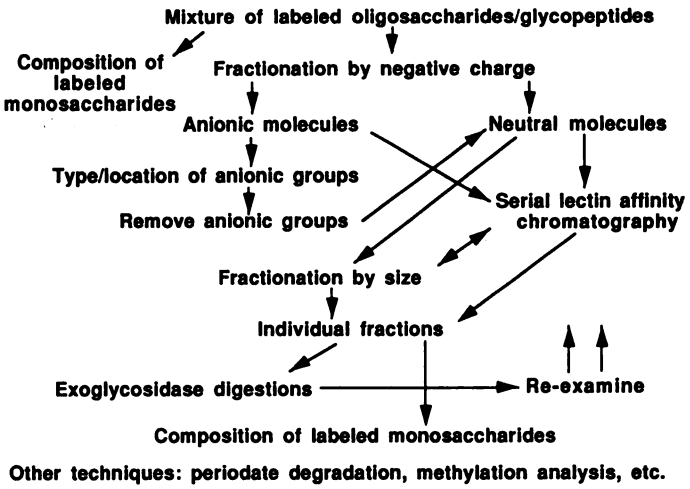

Figure 6. General approach to the structural analysis of labeled oligosaccharides of glycopeptides.

interfere with some methods used for structural analysis. After identification and removal of the anionic groups, the neutral and neutralized molecules can be fractionated by size, and individual fractions can be examined by serial digestions with exoglycosidases. Both anionic and neutral molecules can also be studied by serial lectin affinity chromatography (see below). Several other specialized techniques have been adapted to the study of radiolabeled oligosaccharides, e.g., gas-liquid chromatography (30, 47), periodate degradation (30), acetolysis (44, 47, 48) and methylation analysis (47, 49). Many excellent examples of such detailed structural analyses can be found in the cited literature (see below, and **Table 4**).

SERIAL LECTIN AFFINITY CHROMATOGRAPHY OF GLYCOPEPTIDES OR OLIGOSACCHARIDES

Special mention must be made of serial lectin affinity chromatography, initially suggested by Cummings and Kornfeld (50) and expanded on by several groups (51–54). In this approach, labeled glycopeptides or oligosaccharides are analyzed by sequential chromatography on a variety of different immobilized lectin columns. The unique specificity of binding of immobilized lectins to individual oligosaccharide sequences is well understood. Thus, substantial structural information can be inferred solely from patterns of interaction. For example, [2-^{3}H]mannose-labeled glycopeptides that fail to bind to immobilized concanavalin A and E-PHA, but bind to immobilized Pea-lectin and L-PHA, contain "tri or tetra-antennary complex-type N-linked oligosaccharides with fucosylation in the chitobiose core" (50). This technique was originally devised for glycopeptides. The interaction of free oligosaccharides with lectin columns is similar but not necessarily identical, and can also be affected by reducing the oligosaccharide. Detailed rules for these interactions are under continual development and improvement (46, 51). Thus, considerable structural information concerning a mixture of labeled glycopeptides or oligosaccharides can be obtained simply by sequential chromatography on a series of small and inexpensive lectin columns. The recent adaption of this method to HPLC (55) promises to further strengthen its utility and reproducibility.

The exquisitely specific rules for interaction of lectins with isolated oligosaccharides or glycopeptides do not necessarily apply when an intact protein is studied. The effects of mul-

TABLE 4. *Examples of the structural analysis of metabolically labeled glycoprotein oligosaccharides*

Oligosaccharide structures(s) sequenced from metabolically labeled cells	References (for examples)
Lipid-linked oligosaccharide precursor and processing pathway intermediates of N-linked oligosaccharides	47, 56-60
Phosphomannosyl recognition marker of lysosomal enzymes	12, 33, 35, 44, 61-64
Phosphorylated complex-type N-linked oligosaccharides from Varicella-Zoster virus	65
O-linked oligosaccharides of rat mammary adenocarcinoma mucin	66
N-and O-linked oligosaccharides of the LDL receptor	67
N-linked oligosaccharides of the EGF receptor	68
Sulfated N-linked oligosaccharides from endothelial cells	34
Sulfated N-linked oligosaccharides from bovine lutropin	69
Sulfated N-linked oligosaccharides from LFA-I	70
N-linked oligosaccharides at individual glycosylation sites of mouse H2K (MHC) proteins	41
N-linked oligosaccharides of mouse I-A^k α and β chains	39
O-linked oligosaccharides on proteoglycans	28, 71, 72
Novel N-linked oligosaccharides of *D. discoideum* glycoproteins	45, 73
Novel N-linked oligosaccharides of *Schistosoma mansoni* glycoproteins	74
N-linked oligosaccharides at individual glycosylation sites of Sindbis virus glycoproteins	40
N-linked oligosaccharides of the HIV (AIDS) virus glycoprotein gp120	75

tivalency, position of glycosylation, and steric hindrance by the peptide can cause significant deviations from the rules predicted from isolated sugar chains. For this reason, the interaction of intact glycoproteins with lectin columns can be used only to obtain a rough prediction of the type of oligosaccharides present.

USE OF THE POSITION OF THE LABEL OF STUDY DETAILED STRUCTURE

As mentioned earlier, the position of the label in a given monosaccharide can be predicted from its original position in the precursor used. We have used this positional information to analyze in detail the structure of an individual monosaccharide within an oligosaccharide. For example, label from [6-^{3}H]N-acetyl-mannosamine is eventually found at the 9-position of N-acetylneuraminic acid (sialic acid) residues (see Fig. 3). Mild periodate treatment of such sialic acid molecules releases the label as [^{3}H]formaldehyde, which can be specifically identified. When the sialic acid is substituted at the 9-position, this reaction does not proceed, permitting identification of the position of substitution. If the substitution is alkali labile (for example, an O-acetyl-ester), its removal then regenerates sensitivity to periodate (30). Similar principles could theoretically be used to study a variety of detailed features of a labeled oligosaccharide.

METABOLIC LABELING OF MODIFICATIONS ON OLIGOSACCHARIDES

Modifications of oligosaccharides such as acetylation, sulfation, and phosphorylation can have significant effects on their behavior, both in biological systems and during analysis. Metabolic labeling with the appropriate precursors such as [^{3}H]acetate, $^{35}SO_4$, and ^{32}Pi can be used to label these modified oligosaccharides. As such precursors are expected to enter into a wide variety of other cellular macromolecules, release and isolation of the labeled oligosaccharides with specific endoglycosidases is necessary before analysis. In this regard, double-labeling of the modification and the underlying sugar chain (e.g., with a ^{14}C-labeled monosaccharide precursor) can be useful in monitoring the purificaiton and in the subsequent structural analysis (34).

EXAMPLES OF STRUCTURAL ANALYSIS OF OLIGOSACCHARIDES USING TRACER TECHNIQUES

There are many instances in which detailed, nearly complete structures of oligosaccharides of biological interest have been derived exclusively from the study of radioactively labeled molecules (see Table 4 for examples). The classic instance is the determination of the structure of lipid-linked oligosaccharide precursor of N-linked oligosaccharides. In this case, a nearly complete structural analysis of this important dolichol-oligosaccharide was accomplished using material derived exclusively from [2-^{3}H]mannose-labeled cells. Likewise, the structural intermediates in the processing pathway of N-linked oligosaccharides were worked out by several groups using the approach of mannose labeling. In some of these studies, relatively detailed structural information regarding the biosynthetic intermediates was obtained by structural analysis of the labelled molecules. In exploring the pathway for generation of the phosphomannosyl recognition marker of lysosomal enzymes, the structures of the anionic N-linked oligosaccharides were initially worked out using a metabolic labeling approach (see below). Metabolic labeling techniques have also been used for extensive structural analysis of oligosaccharides from a variety of specific proteins including sugar chains of both the N- and O-linked variety (see Table 4). Again, substantial information, including anomeric linkages and sequences, was obtained from metabolically labeled materials. Although not the immediate subject of this review, such techniques have also been used to analyze the biosynthetic precursors of the glycophospholipid anchor, in the structural analysis of the glycosaminoglycan chains and in the identification of novel ganglioside structures. In many instances, the initial identification and structural characterization of biologically important oligosaccharides was carried out using the labeling approach, and their complete structure was only later confirmed by physical methods. Conventional sequencing approaches have rarely uncovered errors in previous work done with labeled molecules.

Space does not permit adequate discussion of the many excellent studies mentioned in Table 4; the reader is referred to the original citations. Only one example is considered below in some detail.

PHOSPHORYLATED N-LINKED OLIGOSACCHARIDES OF LYSOSOMAL ENZYMES: AN EXAMPLE OF THE USE OF RADIOACTIVE TRACER TECHNIQUES TO SOLVE A BIOLOGICAL PROBLEM

These novel N-linked sugar chains contain mannose-phosphate esters, that play a critical role in targeting newly synthesized lysosomal enzymes to lysosomes in many cell types (reviewed in refs 12 and 64). However, they are generally transient biosynthetic intermediates that last for only a few hours in the lifetime of the enzymes. Thus, the first elucidation of their structure was achieved almost exclusively by using a combination of metabolic labeling with [2-^{3}H]mannose and release with endoH. Studies of individual enzymes and whole-cell oligosaccharides were carried out after both equilibrium labeling and pulse-chase labeling with [2-^{3}H]mannose. The relevant oligosaccharides were specifically released with endoH, isolated by gel filtration, and fractionated by anion exchange chromatography. The types of negatively charged groups were then analyzed by treatments with mild acid, alkaline phosphatase, and sialidase. The resulting neutralized oligosaccharides could then be studied further by digestions with exoglycosidases, HPLC analyses, and other specialized techniques such as acetolysis. These studies resulted in the detailed structural elucidation of a novel family of phosphorylated N-linked oligosaccharides and their biosynethetic relationships to one another. This in turn permitted identification and characterization of the enzymes and receptors involved in the pathway, and the eventual elucidation of the defect in the human genetic disorders, I-cell disease, and pseudo-Hurler polydystrophy.

PITFALLS IN METABOLIC LABELING FOR THE STUDY OF GLYCOPROTEIN OLIGOSACCHARIDES

Although metabolic labeling can provide much useful information regarding glycoprotein oligosaccharides, there are limitations to its use. First, it is difficult to determine when true equilibrium labeling of a cell occurs (three or four doublings are usually assumed to be sufficient). Thus, unless the specific activity of individual monosaccharide pools are carefully determined, the numerical ratio between various labeled glycoconjugates can be misleading. Second, individual precursors have greatly differing uptake and incorporation in different types of cells. Third, almost all the labeled precursors are only partially specific for certain monosaccharides, and the degree of this specificity can be quite variable, depending on the cell type. With prolonged labeling, some of the radioactivity can enter molecules other than oligosaccharides. Fourth, although monosaccharides that compete with glucose for uptake show improved incorporation when glucose concentrations are lowered, a low supply of glucose can have direct effects on the oligosaccharide precursors in some cell types. Finally, the glycosylation patterns seen on a glycoprotein synthesized by an immortalized or transformed cell line may not accurately reflect those present on the normal counterpart in vivo.

SUMMARY: METABOLIC LABELING IN THE STRUCTURAL ANALYSIS OF GLYCOCONJUGATES

Metabolic labeling can be used to identify the types of oligosaccharides and monosaccharides present on a given glycoconjugate if it can be purified to radiometric homogeneity from the cells synthesizing it. The approach can provide partial structural information regarding the sugar chains, and can be used to identify new types of oligosaccharides in glycoconjugates. It is of particular value in identifying and characterizing biosynthetic intermediates and short-lived forms of oligosaccharide chains.

Metabolic labeling cannot be used in systems where adequate uptake and incorporation of the appropriate monosaccharide precursor does not occur. It usually does not provide complete structural information regarding the sequence of the oligosaccharide. It also cannot be used to obtain ratios of glycoconjugates to one another unless specific activities of individual monosaccharide pools are carefully determined.

FUTURE DIRECTIONS

It is necessary to devise methods to improve the uptake of the labeled monosaccharide precursors. Consideration could be given to O-acylation of hydroxyl groups to improve hydrophobicity and uptake. Subsequently, cytosolic nonspecific esterases might remove such acyl groups and restore the free monosaccharide within the cytosol. It would be valuable to improve the specificity of labeling by designing monosaccharide precursors with the ^{3}H-labeled isotope in desirable positions (e.g., we are currently studying [2-^{3}H]ManNAc as a specific precursor for sialic acids and [4-^{3}H]Gal as a specific precursor for labeling Gal residues).

It is also necessary to improve methods for determining the specific activity of labeled monosaccharide pools from tissue culture cells. New HPLC methodologies capable of detecting monosaccharides in the picomole range (28, 76) now make this easier. Improvements in the specific and diagnostic release of oligosaccharide types could be achieved by discovering endoglycosidases with both broader and narrower substrate specificity. In this regard, the recent description of several such new endoglycosidases (77–79) is encouraging. The powerful technique of serial lectin affinity chromatography needs to be expanded, improved, and even automated. Adaptation of this method to HPLC (53) should improve speed and reproducibility. Finally, it is necessary to improve and further adapt chemical and enzymatic methods for the analysis of monosaccharides and linkages when they are available only in radioactive tracer amounts. In this regard, we have recently adapted a variety of techniques for the conventional analysis of sialic acids to the study of radioactively labeled gangliosides (80). FJ

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