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Glycoproteins: Rapid Sequencing Technology for N-linked and GPI Anchor Glycans

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Introduction

Recent advances in oligosaccharide sequencing technology have made routine glycan analysis of glycoproteins and the glycan moieties of glycosylphosphatidylinositol (GPI) anchors a real possibility for many laboratories. The strategies described here have been developed specifically to address the need for a rapid and robust method of glycan analysis which can be applied routinely to microgram levels of glycoproteins with the minimum requirement for specialised equipment or expertise. This strategy also allows the possibility of isolating individual sugars of particular interest for more detailed analysis. We discuss the N-glycosylation of CD48, IgG and IgA1, the Fab and Fc fragments of IgG and also the small (S) and middle (M) glycoproteins of the hepatitis B virus coat protein. We describe the analysis of the major O-glycans of neutrophil gelatinase B and also present a novel method for analysing the glycans

Abbreviations: 2AB: 2 aminobenzamide, AHM: anhydromannitol, CHO-K1: Chinese hamster ovary type K1, ES-MS: electrospray mass spectrometry, GPC: gel permeation chromatography, GPI: glycosylphosphatidylinositol, gu: glucose unit, HBV: hepatitis B virus, HPAEC: high performance anion exchange chromatography, HPLC: high performance liquid chromatography, MALDI-TOF MS: matrix assisted laser desorption ionisation time-of-flight mass spectrometry, NP: normal phase, PVDF: polyvinylidene difluoride, OGS: Oxford GlycoSciences, S, M and L: small, middle and large glycoproteins of HBV respectively, TIMP-1: tissue inhibitor metalloproteinase-1, VSG: variant surface glycoprotein.
Abbreviations used for enzymes: ABS: *Arthrobacter ureafaciens* sialidase; BTG: bovine testes β-galactosidase; BEF: bovine epididymis α-fucosidase; SPH: *Streptococcus pneumoniae* β-N-acetylhexosaminidase.
Abbreviations used for describing oligosaccharide structures: A(1–4) indicates the number of antennae linked to the trimannosyl core; G(0–4) indicates the number of terminal galactose residues in the structure; F: fucose; B: bisecting N-acetylglucosamine (GlcNAc); GalNAc: N-acetylgalactosamine; S: sialic acid; G, Gal: galactose; M, Man: mannose; H: hexose; N: N-acetylhexosamine.

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attached to the GPI anchor of a variant surface glycoprotein of *Trypanosoma brucei* directly from a Western blot. The underlying aim of these analytical strategies is to obtain oligosaccharide sequencing data which, in combination with oligosaccharide and protein structural data, can be visualised in a molecular model. In this way, oligosaccharides can be viewed in the context of the proteins to which they are attached, and some insight can be gained into the roles which sugars might play in the structure and function of the glycoproteins.

Glycoproteins generally exist as populations of glycosylated variants (glycoforms) of a single homogeneous polypeptide. Although the same glycosylation machinery is available to all proteins which enter the secretory pathway in a given cell, most glycoproteins emerge with characteristic glycosylation patterns and heterogeneous populations of glycans at each glycosylation site (Review: Rudd and Dwek, 1997). The composition of the glycoform populations and the role that heterogeneity plays in the function of glycoproteins are important questions for protein chemists and glycobiologists alike. It is only when glycoproteins are viewed in their entirety that the full significance of glycosylation for the function of the molecule can be appreciated. However, while peptide sequencing is routinely available to protein chemists, the robust, rapid and automated technology for oligosaccharide sequencing which we describe here has been developed only recently.

In this review we describe some of the recent advances in technology which enable N- and O-linked glycans and GPI anchor glycans to be analysed rapidly at the sub-picomole level. These include in-gel enzymatic release of N-linked sugars, release of GPI anchor glycans from Western blots, fluorescent labelling of glycan pools for high sensitivity and using a single HPLC run to make preliminary structural assignments of both neutral and charged sugars. We also show how enzyme arrays are used to analyse simultaneously the total sugars released from glycoproteins.

In the first instance the primary sequence of the protein determines whether or not it will be modified by the addition of N- or O-linked oligosaccharides or a GPI anchor. N-linked sugars are added to the amide side-chain of some Asn residues which form part of the triplet AsnXaaSer/Thr, while O-linked sugars can be added to the hydroxyl side-chain of some Ser, Thr or hydroxyproline residues. To receive a GPI anchor the protein must contain a signal sequence which commonly consists of 12–20 hydrophobic residues at the C-terminus of the primary translation product preceded by a polar region of amino acids (Ferguson, 1991; McConville and Ferguson, 1993). This signal sequence is cleaved and replaced by a pre-assembled GPI precursor. In the fully formed anchor the C-terminal cleavage amino acid (restricted to Cys, Asp, Asn, Gly, Arg or Ser) is linked via ethanolamine phosphate to a glycan with a conserved backbone sequence (Manα1→2Manα1→6Manα1→4GlcNH$_2$). Here we discuss some applications of the new analytical strategies for glycan synthesis (Rudd *et al.*, 1997) and show how they have been used to characterise the N-glycans of CD48, the S and M surface antigen glycoproteins of hepatitis B virus, and to compare the glycosylation of serum IgA1 with that of IgG and of IgG Fab with that of IgG Fc. We also discuss the analysis of the major O-glycans of neutrophil gelatinase B, and of the GPI anchor glycans of a variable surface glycoprotein (VSG) of *Trypanosoma brucei*. Importantly, we show how an analysis of the oligosaccharides combined with protein structural data can lead to insights into the structural roles and biological functions of the sugars within the context of the structure and function of the protein to which they are attached.

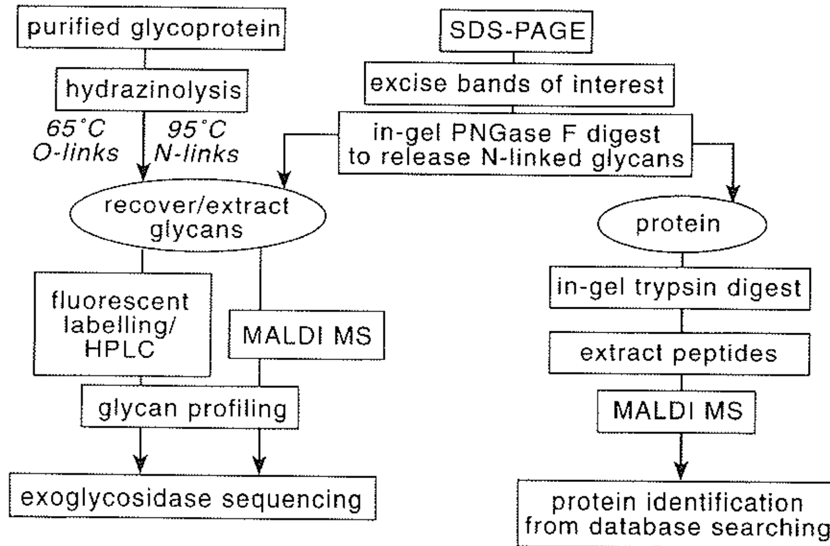

Figure 1. Strategies for glycan analysis and protein identification.

Strategies for analysing N- and O-linked oligosaccharides

Analysing the sugars attached to glycoproteins involves (i) releasing the sugars from the protein (ii) labelling the total glycan pool (iii) analysing the components of the pool by predictive HPLC or MALDI-TOF MS to obtain preliminary structural information (iv) confirming the preliminary assignments by simultaneous sequencing of the glycan pool with exoglycosidase arrays. A straightforward approach which is generally applicable to all glycoproteins is shown in *Figure 1* and discussed below.

RELEASE OF N- AND O-LINKED GLYCANS FROM GLYCOPROTEINS

(i) Enzymatic release and analysis of N-linked oligosaccharides from protein bands on SDS-PAGE gels. An 'in-gel' release method has been developed to release N-glycans from Coomassie blue stained protein bands on SDS-PAGE gels by peptide-N-glycosidase F (PNGase F) (Küster *et al.*, 1997). The released glycan pool can be analysed by MALDI-TOF MS and after fluorescent labelling, by HPLC. The individual sugars in the pool can be analysed simultaneously using enzyme arrays (Guile *et al.*, 1996) monitored either by MALDI-TOF MS or HPLC.

The development of this method has opened the way to analysing glycans from biologically important molecules which are difficult to purify or where amounts are limited. In many cases releasing sugars directly from SDS-PAGE gels eliminates the need for extensive protein purification. For example, when gelatinase B from some cell types is purified on gelatin-sepharose it is contaminated with its natural inhibitor, tissue inhibitor metalloproteinase-1 (TIMP-1) (Opdenakker *et al.*, 1991). Although this enzyme-inhibitor complex is stable, SDS-PAGE can be used to resolve the two proteins. N-linked oligosaccharides can then be released directly from the gel for analysis (Rudd *et al.*, 1997).

Another application of the technology is in situations where reducing gels allow the straightforward separation of proteins into their component subunits. Examples include the resolution of IgG into heavy and light chains and the surface coat proteins of hepatitis B virus or particles can be separated into three major glycoprotein components, small, middle and large (S, M and L, respectively) by SDS-PAGE analysis under reducing conditions (see the sections on 'In-gel glycan analysis . . .' and 'The detection of hyperglucosylated glycan . . .' below).

A third application for the 'in-gel' release method is to release the sugars from peptide fragments resulting from protease digests when these can be resolved by SDS-PAGE. In some cases this may enable a rapid glycosylation site analysis of glycoproteins.

An advantage of the in-gel release method is that the protein remains in the gel after the sugars have been removed. In-gel proteolysis of the protein, for example by trypsin, and analysis of the peptide fragments by nanospray MS, enables the protein to be identified. This is achieved by using a protein data base to compare the molecular weights of the tryptic fragments with those of the predicted sequences of fragments from tryptic digests of known proteins (Küster *et al.*, 1996).

(ii) Chemical release of N- and O-glycans from glycoproteins using anhydrous hydrazine. Hydrazinolysis is a general method for chemically and non-selectively releasing N- and O-glycans from glycoproteins (Patel and Parekh, 1994). The analysis of the N-glycans of IgA1 and IgG released by automated hydrazinolysis (GlycoPrep 1000 Oxford GlycoSciences (OGS)) is discussed in the section on 'IgA1 N-linked sugars are more processed . . .' below.

Hydrazine release of O-glycans is best achieved by manual hydrazinolysis since the conditions can be optimised to minimise the 'peeling' of oligosaccharides associated with the degradation of the terminal monosaccharide (N-acetyl galactosamine in the case of O-glycans) when it is substituted in the 3-position. The release and analysis of the O-glycans from human neutrophil gelatinase B is discussed in the section on 'Analysis of the O-glycans . . .' below. Base catalysed reductive β-elimination is also commonly used to release O-glycans. Although this minimises 'peeling' the reduction of the aldehyde group of the reducing monosaccharide to an alditol precludes subsequent derivitisation which involves a reductive amination step. The inability to label the oligosaccharides with fluorescent tags, such as 2-aminobenzamide (2AB) (see the following section) limits the sensitivity of detection and in many cases prevents the analyses of biologically relevant samples.

LABELLING THE GLYCAN POOL

Oligosaccharides released with hydrazine or PNGase F exist as an equilibrium between the cyclic (hydroxyl at C1) and opened ring form (aldehyde at C1) of the reducing GlcNAc residue (*Figure 2*). The opened ring form can be derivatised at C1 by fluorophores such as 2-aminobenzamide (2AB) via a reductive amination reaction which initially forms a Schiff's base with the sugar. The reaction is driven to completion by the reduction of the Schiff's base to an amine functionality, therefore favouring the forward direction of the tautomerisms.

Although mass spectromety can be applied to unlabelled sugars, in most other

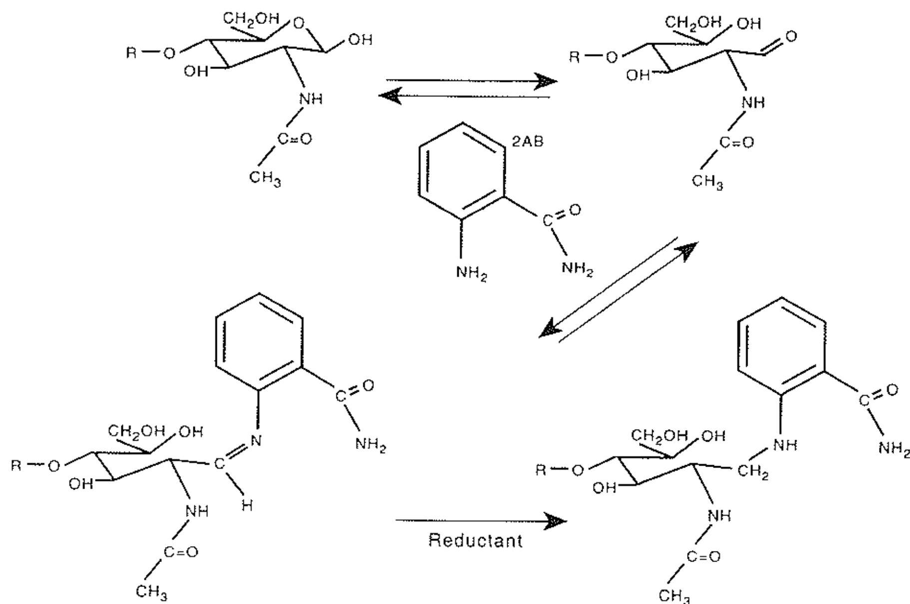

Figure 2. Mechanism for the reductive amination of an oligosaccharide with the fluorophore 2-aminobenzamide. The figure indicates that the reaction can only take place when the terminal GlcNAc residue in the chitobiose core is in the 'ring opened form'. R indicates the remainder of the oligosaccharide.

techniques the reducing termini of the sugars need to be tagged to allow sensitive detection. A range of fluorescent molecules is available which allow detection of sugars in the femtomole range. A suitable label must have a high molar labelling efficiency, and must also label the oligosaccharide components of a glycan pool non-selectively so that they are detected in their correct molar proportions. 2-amino-benzamide (2AB) fulfils these requirements and is compatible with a range of separation techniques including weak anion exchange and reverse phase HPLC, high performance anion exchange chromatography, MALDI-TOF MS and electrospray mass spectrometry (ES-MS) as well as BioGel P4 gel permeation chromatography (P4 GPC) (Bigge *et al.*, 1995) and normal phase (NP) HPLC.

RESOLUTION OF GLYCAN POOLS AND STRUCTURAL ASSIGNMENTS USING NP HPLC

NP HPLC is performed using a column with polar functional groups which interact with the hydroxyl groups on the sugars. Glycans are resolved on the basis of hydrophilicity. Sugars applied in high concentrations of organic mobile phase adsorb to the column surface and are eluted with an aqueous gradient. In general, larger oligosaccharides are more hydrophilic than small ones, and require higher concentrations of aqueous solvent to elute them.

A sensitive and reproducible NP HPLC technology has been developed (Guile *et al.*, 1996) using the GlycoSep N column (OGS). This system is capable of resolving sub-picomole quantities of mixtures of fluorescently labelled neutral and acidic N- and O-glycans simultaneously and in their correct molar proportions. Elution posi-

tions are expressed as glucose units (gu) by comparison with the elution positions of glucose oligomers (dextran ladder). The contribution of individual monosaccharides to the overall gu value of a given glycan can be calculated and these incremental values are used to predict the structure of an unknown sugar from its gu value. The GlycoSep N column is able to resolve arm specific substitutions of galactose and linkage position can also have an effect on retention giving a further level of specificity (Guile *et al.*, 1996).

Both the increased resolving power of the predictive HPLC technology and the ability to analyse sialylated and neutral sugars in one run represent a considerable advance over the classical approach to glycan analysis which depends on P4 GPC. The HPLC strategy is now being extended to the analysis of O-linked glycans, such as those attached to human neutrophil gelatinase B (see the section on 'Analysis of the O-glucans . . .' below).

SIMULTANEOUS SEQUENCING OF OLIGOSACCHARIDES USING ENZYME ARRAYS

The preliminary assignment of structures from the initial HPLC run are confirmed rapidly by sequencing all of the oligosaccharides in a glycan pool simultaneously using enzyme arrays. Enzymatic analysis of oligosaccharides using highly specific exoglycosidases is a powerful means of determining the sequence and structure of glycan chains. However, until recently, it was necessary to isolate single sugars from the glycan pool for digestion with exoglycosidases, either sequentially or in arrays. The high resolving power of the NP HPLC system allowed a new approach to be developed (Guile *et al.*, 1996). This involves the simultaneous analysis of the total glycan pool by digesting aliquots with a set of enzyme arrays. After overnight incubation the products of each digestion are analysed by NP HPLC or by MALDI-TOF MS. On the HPLC system, structures are assigned to each peak from a knowledge of the specificity of the enzymes and the incremental values of individual monosaccharide residues. To illustrate this technique, the rapid profiling and simultaneous analysis of the major N-glycans attached to rat CD48 expressed in CHO cells is discussed in the section on 'Simultaneous analysis of the N-glycan pool . . .' below.

Applications of this technology

In the examples below we demonstrate how rapid oligosaccharide sequencing of fluorescently labelled sugars and the use of an oligosaccharide data base (which gives the sizes of the sugars) can be combined with protein structural data to give insights into roles for glycosylation in the function of individual proteins.

IgA1 N-LINKED SUGARS ARE MORE PROCESSED THAN THOSE OF IgG. THIS IMPLIES THAT THE IgA SUGARS CANNOT BE CONSTRAINED BETWEEN THE CH2 DOMAINS AS IS THE CASE FOR IgG

The annotated glycosylation profiles in *Figure 3a* indicate that, in contrast to IgG, most of the N-linked sugars attached to IgA1 are galactosylated and sialylated (Mattu *et al.,* 1998). This suggests that there are significant differences in the accessibility of

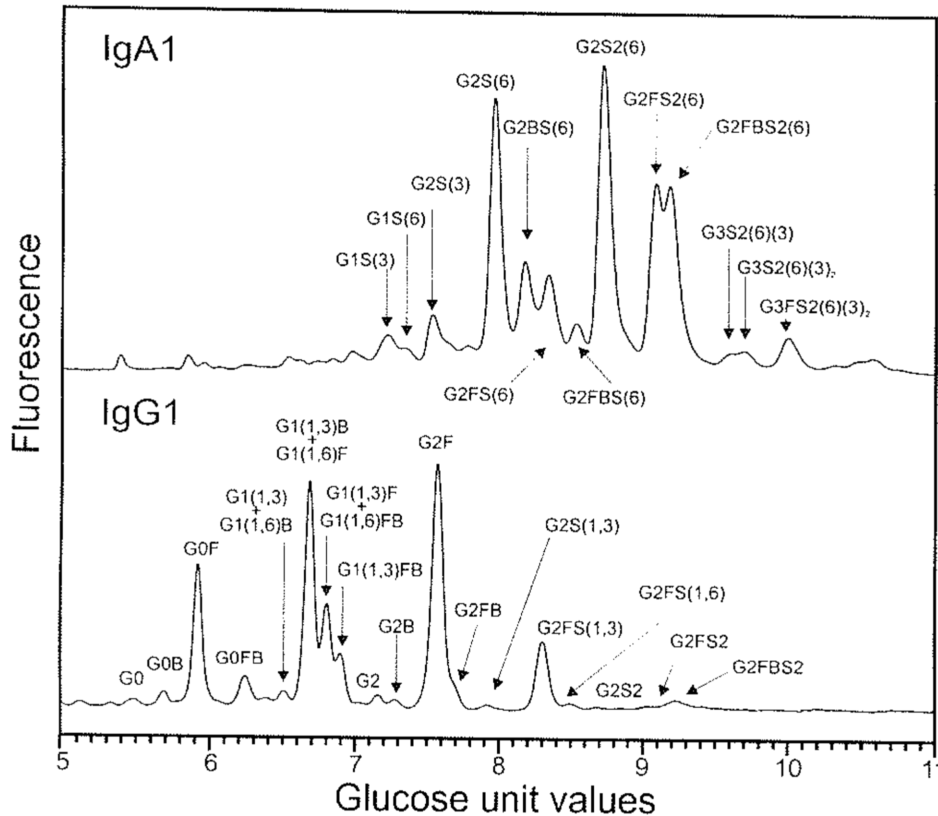

Figure 3a. The glycan analysis of the N-linked sugars from IgA1 and serum IgG. The hydrazine released 2AB labelled glycans from the IgA1 subclass purified from human serum (top) and total serum IgG (containing all subclasses) were resolved by normal phase HPLC. In contrast to IgG, the majority of IgA1 glycans are sialylated and fully galactosylated. The data have been plotted using a new computer program known as the Lineariser (E. Hart – unpublished data). This program automatically determines the gu values by comparison with a dextran standard, and can also transform a chromatogram plot to a linear gu scale. The use of an axis linear in gu values rather than elution times brings the advantage that a given gu increment, such as is produced by the action of a particular enzyme, is of constant length over the full range of the chromatogram. This allows for a more direct comparison of glycosylation profiles. G(0–3) indicates the number of terminal galactose residues in the structure; F: fucose; B: bisecting N-acetyl glucosamine; S: sialic acid.

the sugars to the glycosylating enzymes in the two immunoglobulins. The crystal structure of IgG Fc (Deisenhofer, 1981) indicates that the conserved sugars at Asn297SerThr are contained in the interstitial space between the CH2 domains (*Figure 3b*). The structure shows that the sugars are also involved in non-covalent interactions with the protein surface (Padlan, 1991) which further limits their accessibility to the glycosyltransferases. In order to compare the location of the IgG Fc oligosaccharides with those in IgA1 Fc, for which no protein structure is available, a molecular model was constructed using the primary sequence and disulphide bond pattern of IgA1, the crystal structure of IgG1 Fc and the glycan sequencing data (Mattu *et al.*, 1998). The model of IgA1 Fc shows that, as a consequence of the disulphide bonding arrangement, the amide side chains of Asn263LeuThr are pointing away from the protein and therefore the IgA1 Fc N-linked glycans cannot be

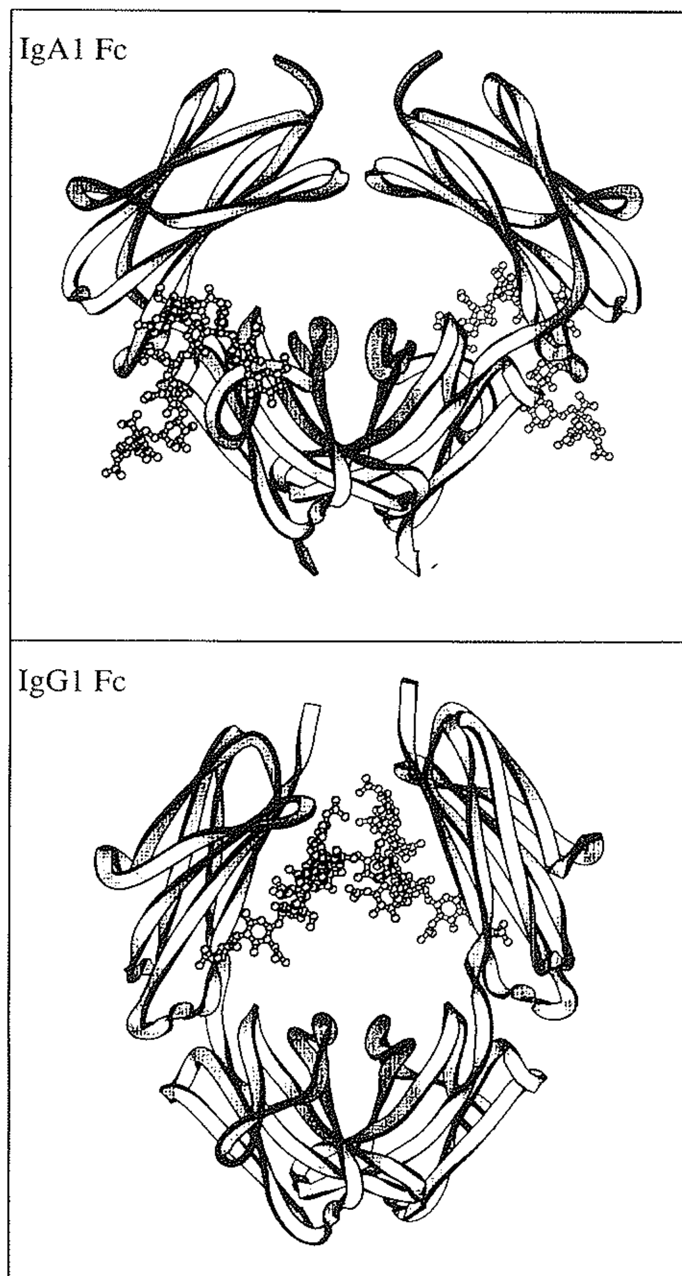

Figure 3b. Comparison of the molecular models of IgA1Fc and IgG1Fc. In contrast to the IgGFc glycans, which are contained in the interstitial space between the CH2 domains (Deisenhofer, 1981), the N-glycans attached to IgA1 are exposed on the outside of the CH2 domains (Mattu *et al.*, 1998). This is the result of the different location of the glycosylation sites in the CH2 domains and the altered quaternary structure of IgA1 Fc. The IgG molecule modelled here is of the subclass type 1.

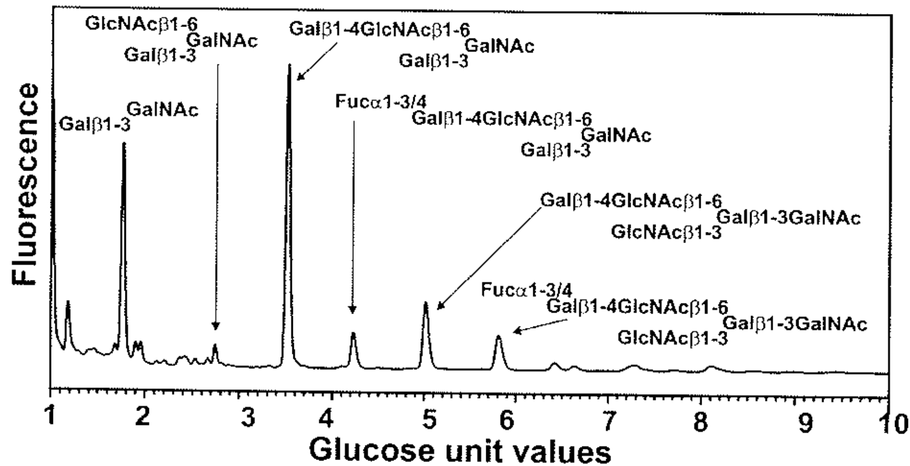

Figure 4. The normal phase HPLC profile of the desialylated O-glycan pool released from human neutrophil gelatinase B.

contained in the interstitial space between the CH2 domains (*Figure 3b*). They are therefore more accessible to the glycosylating enzymes, in particular the galactosyl and sialyl transferases, than those on IgG Fc. The analysis of the IgA sugars (*Figure 3a*) confirms this and molecular modelling (*Figure 3b*) indicates that the IgA1 Fc glycans are too large to fit into the predicted space between the two CH2 domains.

The differences in the structure of the Fc regions in IgA1 and IgG have implications for the functions of the sugars. In IgG Fc the protein-oligosaccharide interactions play a role in maintaining the relative geometry of the domains (Rudd *et al.*, 1991). This is consistent with the finding that, as a result of perturbation of a region proximal to the receptor binding sites (Burton and Woof, 1992), both non-glycosylated and degalactosylated IgG bind less efficiently to the Fcγ receptors (Nose and Wigzell, 1983; Leatherbarrow *et al.*, 1985; Tsuchiya *et al.*, 1989). In contrast, for IgA1, no reduction in binding to its Fc receptor was detected in CHO-K1 mutants in which the N-linked sugars sites in the CH2 domains had been deleted (Mattu *et al.*, 1998). This suggests that in IgA1 the sugars in the CH2 domains do not play a role in maintaining domain structure. However, just as the multiple O-glycans in the hinge have been shown to protect the hinge region of IgA1 against non-specific proteolysis (Mestecky and Kilian, 1985), so the N-glycans are expected to shield large areas of the Fc region from proteases.

ANALYSIS OF THE O-GLYCANS FROM HUMAN NEUTROPHIL GELATINASE B SUGGESTS THAT THEY MAY PRODUCE AN EXTENDED AND RIGID REGION OF THE PEPTIDE

Gelatinase B is a multidomain metalloproteinase (MMP 9) which cleaves extracellular matrix substrates such as denatured collagens (gelatins) after these have been clipped by collagenases, stromelysin or other metalloproteinases. Gelatinase B contains seven protein domains, three potential N-glycosylation sites, a Pro/Ser/Thr rich region and a number of isolated serine and threonine residues. The

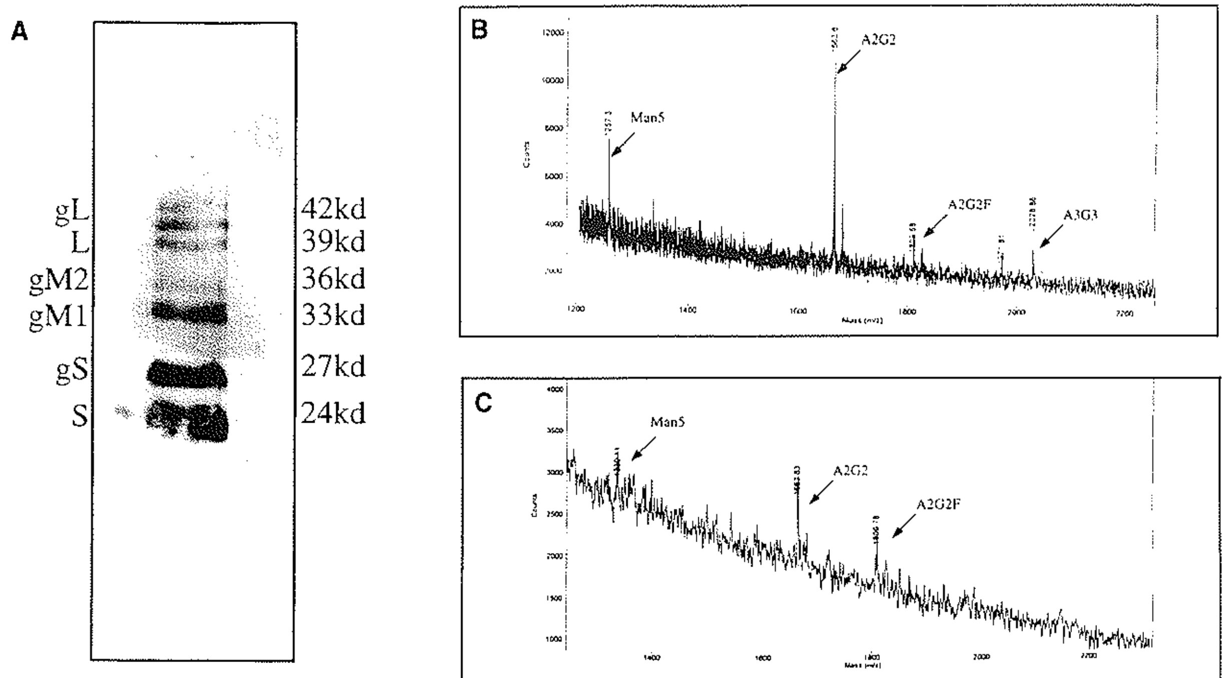

Figure 5A–C. Analysis of the glycosylation of the individual HBV envelope proteins by in-gel glycan analysis. **A)** The HBV envelope glycoproteins as resolved by a 12.5% SDS-PAGE gel and stained with Coomassie brilliant blue (see text). The proteins as indicated are from top to bottom: gL, glycosylated HBV L protein; L, unglycosylated HBV L protein, gM2, doubly glycosylated HBV M protein; gM1, singly glycosylated HBV M protein; gS, glycosylated HBV S protein; S, unglycosylated HBV S protein. **B)** The glycans associated with band gS (the 27kDa S protein) as determined by the in-gel release method. Arrows indicate the glycans: Man5, high mannose type glycan with five mannose residues; A2G2, biantennary complex type glycan; A2G2F, fucosylated biantennary complex type glycan; A3G3, triantennary complex type glycan. **C)** The glycans associated with gM1 (the 33 kDa M protein) as determined by the in-gel release method. Arrows indicate the glycans: Man5, high mannose type glycan with five mannose residues; A2G2, biantennary complex type glycan; A2G2F, fucosylated biantennary complex type glycan. Note that the signal intensity is much reduced compared with 5B but that this reduction correlates with the band intensity seen in *Figure 5A*. Also, while the gS band consists of predominantly the biantennary complex type glycan, the gM1 band contains much a greater amount of the fucosylated biantennary complex type glycan.

O-glycans, which are expected to be located mainly in the Ser/Thr/Pro rich domain, were released by manual hydrazinolysis, profiled (*Figure 4*) and analysed using enzyme arrays (Rudd, P.M., Mattu, T.S., Opdenakker, G. and Dwek, R.A. Manuscript in preparation). *Figure 4* shows that the glycans in the major peaks contained core types 1, 2 and 6. The attachment of these glycans to the Ser/Thr/Pro rich domain may produce an extended and rigid region of the peptide. Electron microscopic studies indicate that the extension contributed per residue in an O-glycosylated peptide varies from 0.2–0.25nm in CD43 (Cyster *et al.*, 1991) and mucins (Shogren *et al.*, 1989; Jentoft *et al.*, 1990).

IN-GEL GLYCAN ANALYSIS OF THE HEPATITIS B VIRUS (HBV) SUB-VIRAL PARTICLES LOCALISES FUCOSYLATED GLYCANS TO THE HBV M PROTEIN

HBV, which is a major etiological agent of liver disease and hepatocellular carcinoma (Sherker *et al.*, 1990), encodes for three envelope glycoproteins proteins: large (L), middle (M) and small (S). These are derived from a single open reading frame through the utilization of alternative translational start sites (Heerman *et al.*, 1992). All three glycoproteins contain a common site of N-glycan attachment at Asn146 of the S domain with the M protein containing an additional site at Asn4 of the pre-S2 domain. In addition to being the major component of the viral envelope, these glycoproteins are secreted in the form of smaller non-infectious sub-viral particles. These particles, which lack DNA, are secreted in vast excess compared to the viral particle.

The glycosylation of the individual HBV glycoproteins can be studied by the in-gel release method described (Küster *et al.*, 1997). Briefly, the envelope glycoproteins associated with subviral particles from Hep G2.2.2.15 cells were separated on an SDS polyacrylamide gel and the glycans removed *in situ* with PNGase F. The glycans were subsequently desalted and analyzed by mass spectrometry. *Figure 5* shows the major glycan structures found on the HBV S (gp27) and M (gp33) proteins respectively from Hep G2.2.15 cells (*Figure 5A*). The major glycan structures found on the S protein are biantennary, fucosylated biantennary, and triantennary glycan structures (*Figure 5B*). Although the glycan structures found on the M protein are similar (*Figure 5C*), the proportions are different. The M protein appears to contain much more of the fucosylated biantennary glycan. As these are the same glycans found on sub-viral particles from Hep G2.2.15 cells (Mehta *et al.*, 1997), this provides evidence that the fucosylated biantennary glycan is found predominantly on the HBV M protein. *Figure 5* also shows the sensitivity of this technique. The mass spectrum seen in *Figure 5C* was determined from less than 1μg of protein. Therefore this technique can be used to examine the glycosylation of individual constituents of large oligomeric structures and from small quantities of starting material.

THE DETECTION OF HYPERGLUCOSYLATED GLYCAN IN THE SERUM OF WOODCHUCKS TREATED WITH THE GLUCOSIDASE INHIBITOR N-NONYL-DNJ ACTS AS A SURROGATE MARKER FOR DRUG EFFICACY

The high sensitivity of glycan analysis using fluorescently labelled sugars and NP HPLC (femtomole range) is demonstrated in this study in which hyperglucosylated

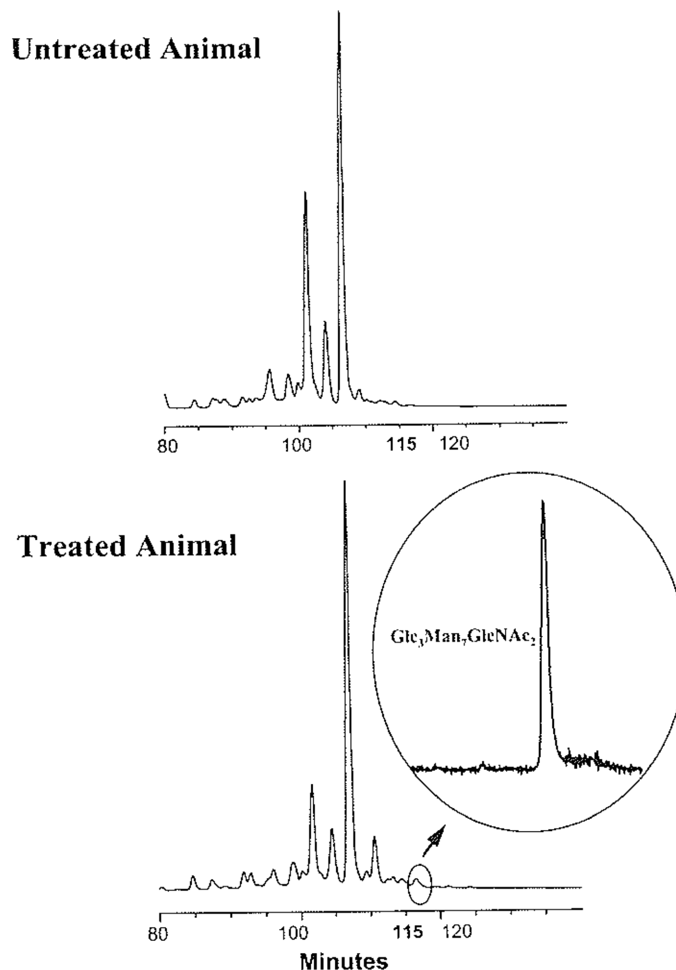

Figure 5D. Serum glycan profiles from untreated woodchuck (top) and treated woodchuck 4 weeks post treatment. NP HPLC profile of serum glycans from untreated animal and treated animal 4 weeks after treatment with the α-glucosidase inhibitor N-nonyl-deoxynorjirimycin. The X-axis shows the retention time of the glycans on the HPLC column. Note that while after 115 minutes, there are no peaks in the untreated animal, the treated animal has a small peak at approximately 116.5 minutes. This peak, which has been shown by oligosaccharide sequencing to be the $Glc_3Man_7GlcNAc_2$ structure is indicated. Glucosidase inhibition does not lead a 100% production of the $Glc_3Man_7GlcNAc_2$ structure due to the presence of a shunt pathway (Moore *et al.*, 1990).

glycans were detected in serum from woodchucks that had been treated with the glucosidase inhibitor N-nonyl-deoxynojirimycin (Block *et al.*, 1998). The total glycans associated with the glycoproteins from 15 μl of woodchuck serum were released with hydrazine using a Glycoprep 1000 machine (OGS). Released glycans were fluorescently labelled at their reducing end with 2AB using the Signal Labelling Kit (OGS) and subsequently analyzed by NP HPLC. The glycan profiles from the serum of the untreated and treated animal are compared in *Figure 5D*. The result of glucosidase inhibition is that treated animals contain small amounts of the

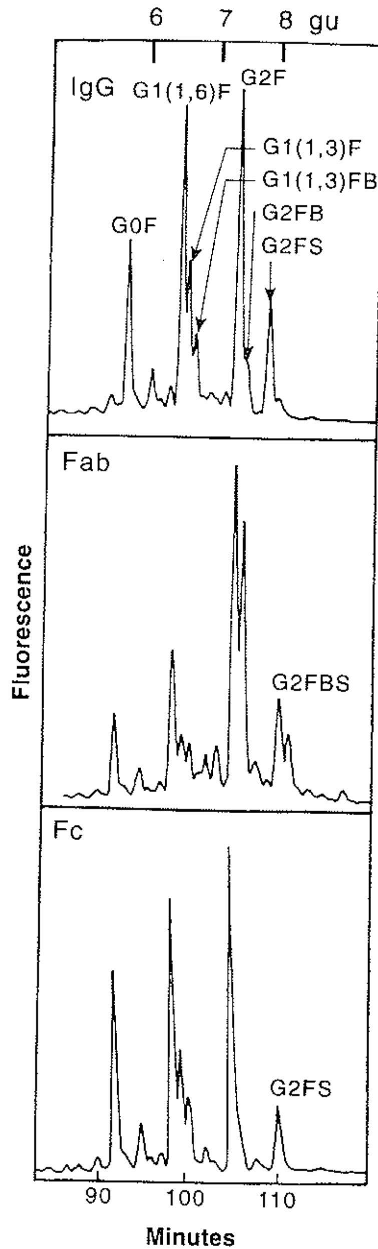

Figure 6. Analysis of oligosaccharides released from IgG, Fab and Fc. Panels a-c show the NP HPLC profiles of the 2AB labelled oligosaccharides released from normal serum IgG, Fab and Fc, respectively.

$Glc_3Man_7GlcNAc_2$ structure which represents less than 2% of the total serum glycan pool. The levels of $Glc_3Man_7GlcNAc_2$ correlated with the reduction in viremia in individual animals treated with the inhibitor (Block *et al.*, 1998). Thus, in this model, the high sensitivity of this technique allows the detection of $Glc_3Man_7GlcNAc_2$ structures to act as a surrogate marker for drug efficacy.

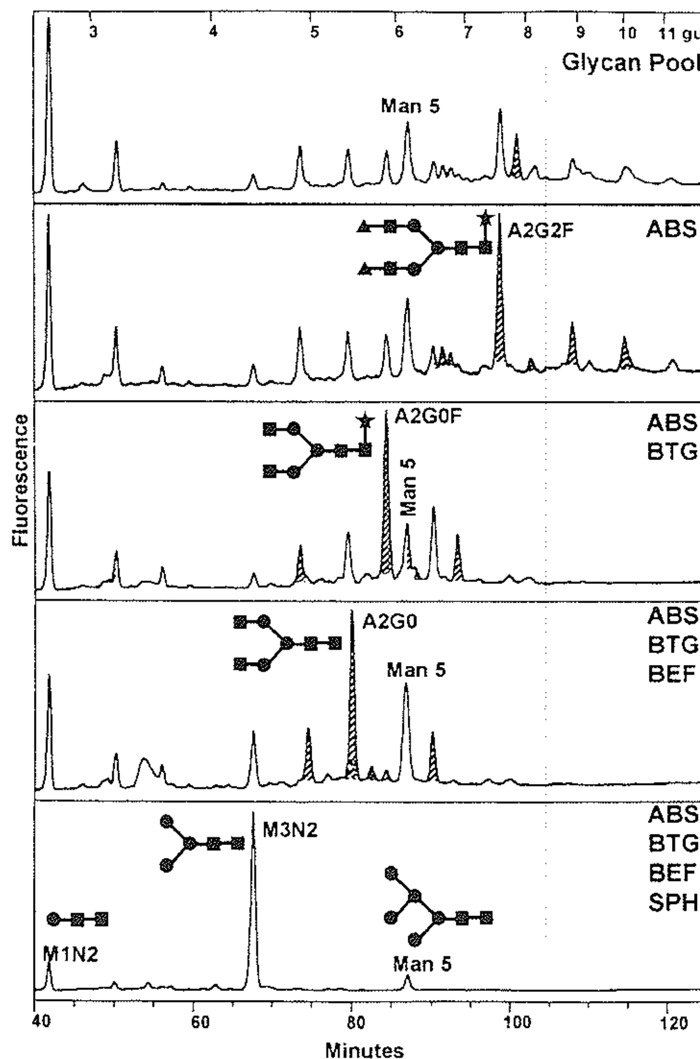

Figure 7. Simultaneous analysis of N-glycans released from rat CD48 (expressed in CHO cells) using enzyme arrays. The figure shows the HPLC analysis of the total glycan pool and the products resulting from the digestion of four aliquots of the total CD48 glycan pool with a series of enzyme arrays. The particular enzyme array which produced each profile is shown on the appropriate panel. The shaded areas define the peaks which contain glycans which were subsequently digested by the additional enzyme present in the next array. The gu value of each peak was calculated by comparison with the dextran hydrolysate ladder shown at the top of the figure. Structures were assigned from the gu values, previously determined incremental values for monosaccharide residues (Guile *et al.*, 1996) and the known specificity of the exoglycosidase enzymes. The structures of the most abundant glycan populations are shown. ABS: *Arthrobacter ureafaciens* sialidase; BTG: bovine testes β-galactosidase; BEF: bovine epididymus α-fucosidase; SPH: *Streptococcus pneumoniae* β-N-acetylhexosaminidase.

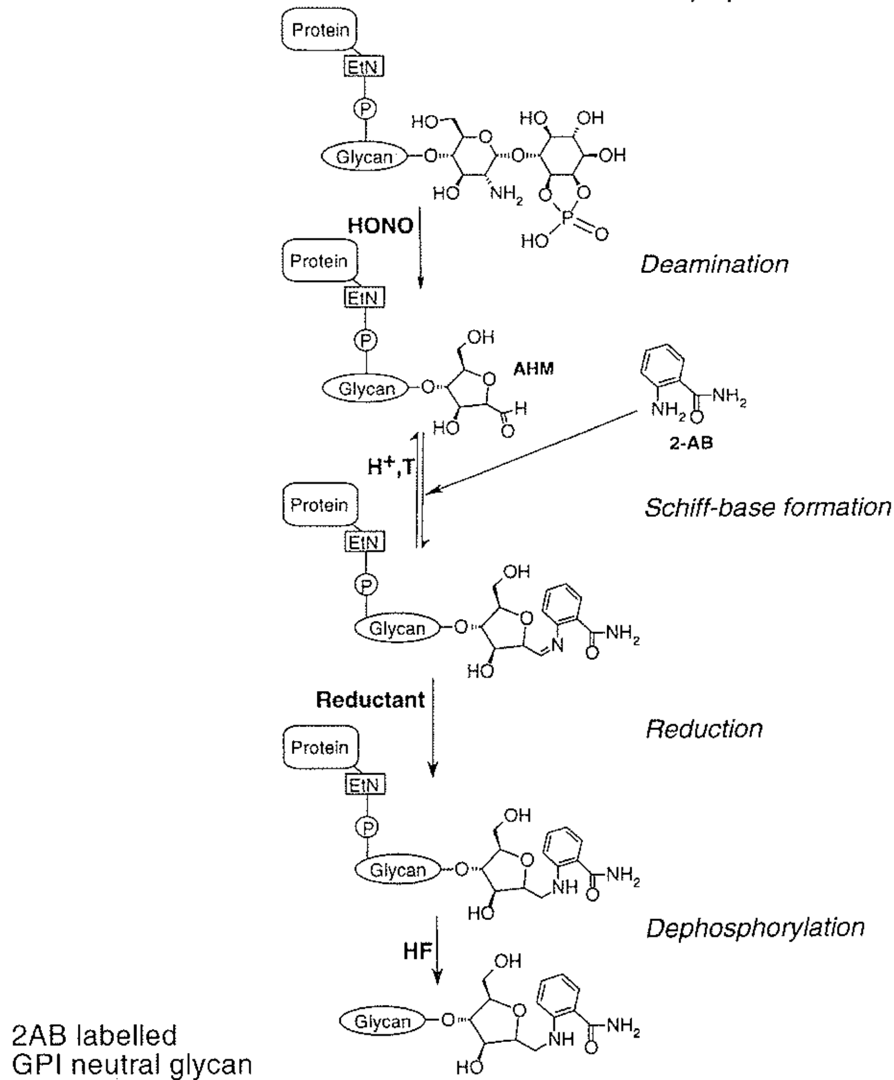

Figure 8A. General scheme for preparing 2AB labelled GPI neutral glycans 'on the blot'. The GPI-anchored protein is run on an SDS-PAGE gel and blotted onto a PVDF membrane. Nitrous acid (HONO) deamination converts the GlN residue to 2,5-anhydromannose (AHM) which is reacted with 2AB by reductive amination. These steps are performed while the protein is still attached to the PVDF membrane. The 2AB labelled glycan is subsequently released by dephosphorylation with cold aqueous hydrofluoric acid (HF).

COMPARISON OF THE N-GLYCOSYLATION PROFILES OF IgG FAB AND FC INDICATE THAT THE FAB GLYCANS ARE MORE ACCESSIBLE TO SOME GLYCOSYLATING ENZYMES

A comparison of the high resolution HPLC profiles of oligosaccharides released from glycoproteins gives a rapid insight into alterations of glycosylation with disease and in some cases into pathogenesis. In particular, decreases in the galactosylation of

serum IgG in rheumatoid arthritis, which have been shown to correlate with disease severity and activity, have been monitored using this technique (Routier *et al.*, 1998; Wormald *et al.*, 1997). The alterations in glycosylation are mainly confined to the Fc region (Youings *et al.*, 1996). Comparison of the sugars attached to normal IgG Fab and Fc, indicates that there is 'site' specific glycosylation in IgG (*Figure 6*). In normal serum IgG approximately 60% of the Fab sugars are of the digalactosylated complex type, while 68% of the sugars on the Fc are mono- or non-galactosylated. In the Fab there is an increase in glycoforms containing sialylated sugars and in those containing bisecting GlcNAc. These data indicate that the Fab glycans are more accessible to the GlcNAcV, galactosyl and sialyl transferases which attach bisecting GlcNAc, galactose and silica acid residues, respectively, to the glycan chain. In contrast, the Fc oligosaccharides are partially protected by the protein structure at the stages of the biosynthetic pathway when these enzymes act.

SIMULTANEOUS ANALYSIS OF THE N-GLYCAN POOL FROM RAT CD48 EXPRESSED IN CHINESE HAMSTER OVARY (CHO) CELLS REVEALS EXTENSIVE HETEROGENEITY

Soluble rat CD48, containing 5 N-linked glycosylation sites, was expressed in CHO cells. The oligosaccharides in the glycan pool were analysed simultaneously using

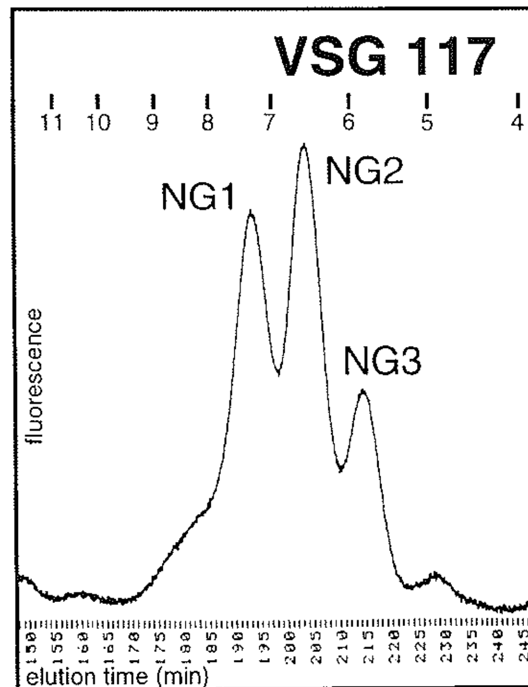

Figure 8B. Bio-Gel P4 gel filtration chromatography and identification of 2AB labelled GPI neutral glycans from purified *T. brucei* VSG 117, prepared 'on the blot'. An electroblot band of 5 μg (100 pmol) VSG 117 was treated as described in the legend to *Figure 8A* and the resulting 2AB labelled GPI neutral glycans were analysed by Bio-Gel P4 gel filtration chromatography. The structures represented by peaks NG1, NG2 and NG3 are shown in *Figure 8C*. The small numbers at the top indicate the glucose units (gu).

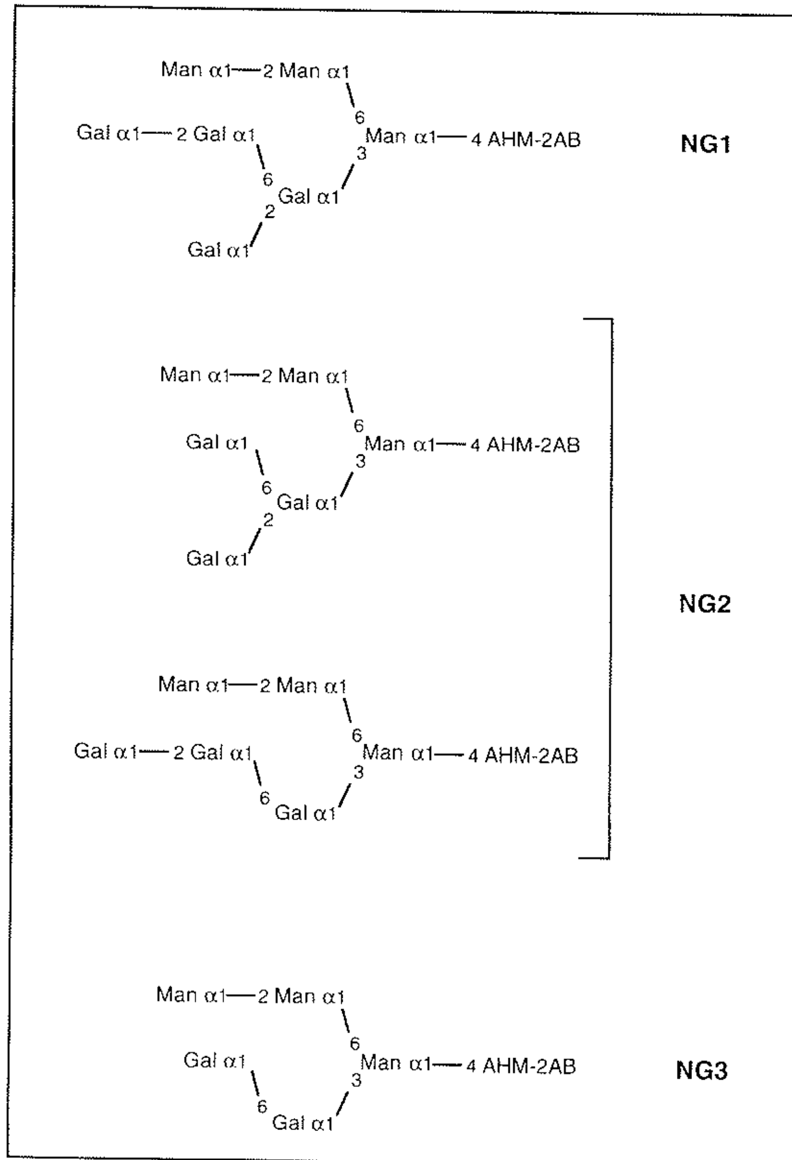

Figure 8C. Structures of the VSG 117 neutral glycans (NGs). The structures shown were determined by Ferguson (1988).

enzyme arrays (*Figure 7*) (Rudd *et al.*, submitted). In molecular modelling studies the range of complex, hybrid and oligomannose structures were found to shield large areas of the two IgSF domains of which CD48 is composed (Rudd *et al.*, submitted). CD48 is the major ligand for CD2 and this cell adhesion pair mediates the precise alignment of the cell surfaces of cytolytic T-lymphocytes carrying the TCR complex with those of target cells carrying loaded HLA class I molecules (Dustin *et al.*, 1996). The glycosylation analysis suggests that the sugars protect CD48 against proteases and play

a role in the packing of CD48 on the cell surface. In addition the sugars at the sites close to the membrane may serve to orientate the face of the protein which contains the binding sites.

A NOVEL STRATEGY FOR ANALYSING GPI ANCHOR GLYCANS FROM MICROGRAM QUANTITIES OF PROTEIN ON ELECTROBLOTS

A novel strategy for the analysis of 2AB labelled GPI anchor glycans directly from electroblots, known as the 'on the blot' method (Zitzmannn and Ferguson, 1999) has recently been developed (*Figure 8A*).

ES MS methods to characterise the phosphatidylinositol components (Treumann *et al.*, 1997) and chromatographic methods to characterise the glycan components of GPI anchors (Treumann *et al.*, 1997; Ferguson, 1992; Schneider and Ferguson, 1995) have been described previously. However, the methods for glycan characterisation relied on introducing a tritium radiolabel into the GPI glycan with all of the associated complications of handling NaB3H4 reductant. This disadvantage led to the development of a new method for labelling GPI anchor glycans using the fluorophore 2AB that can be used both on purified glycoproteins and on microgram quantities of glycoproteins immobilised on polyvinylidene difluoride (PVDF) membranes after electroblotting (Zitzmannn, N. PhD Thesis, 1997; Zitzmannn and Ferguson, 1999).

The 2AB labelling procedure depends upon the presence of non-N-acetylated glucosamine (GlcN) in all GPI structures. The free amino group of the GlcN residue can be exploited by the nitrous acid deamination reaction that converts the GlcN to 2,5-anhydromannose (AHM) and simultaneously releases the inositol residue (*Figure 8A*). The aldehyde group of the AHM residue is then coupled to 2AB by reductive amination to give a stable covalent linkage between the GPI glycan and the fluorophore. The protein and ethanolamine substituents are subsequently removed by aqueous HF dephosphorylation to yield 2AB labelled GPI neutral glycans that can be characterised by liquid chromatography.

THE ANALYSIS OF THE GPI ANCHOR GLYCANS OF THE VARIANT SURFACE GLYCOPROTEIN (VSG) FROM *T. BRUCEI*: THE α-GALACTOSE SIDEBRANCH HAS BEEN IMPLICATED IN THE PACKING OF VSG ON THE CELL SURFACE

Results obtained using 5 μg (100 picomoles) of VSG from *Trypanosoma brucei* (Ferguson *et al.*, 1988) following the 'on the blot' labelling method described in the previous section are shown in *Figure 8B*. The structures represented by peaks NG1, NG2 and NG3 are shown in *Figure 8C*. Their identity was confirmed by electrospray mass spectrometry analysis (*Figure 8D*).

GPI anchors afford a stable means of attachment of proteins to the membrane and can be viewed as an alternative mechanism to a single-pass hydrophobic transmembrane peptide domain. Although functions for the different glycan sidechains of GPI anchors are not generally known, the α-galactose sidebranch of the VSG GPI anchor has been implicated in the dense packing of the surface coat which acts as a barrier protecting the parasite from complement-mediated lysis in the host blood stream (Homans *et al.*, 1989).

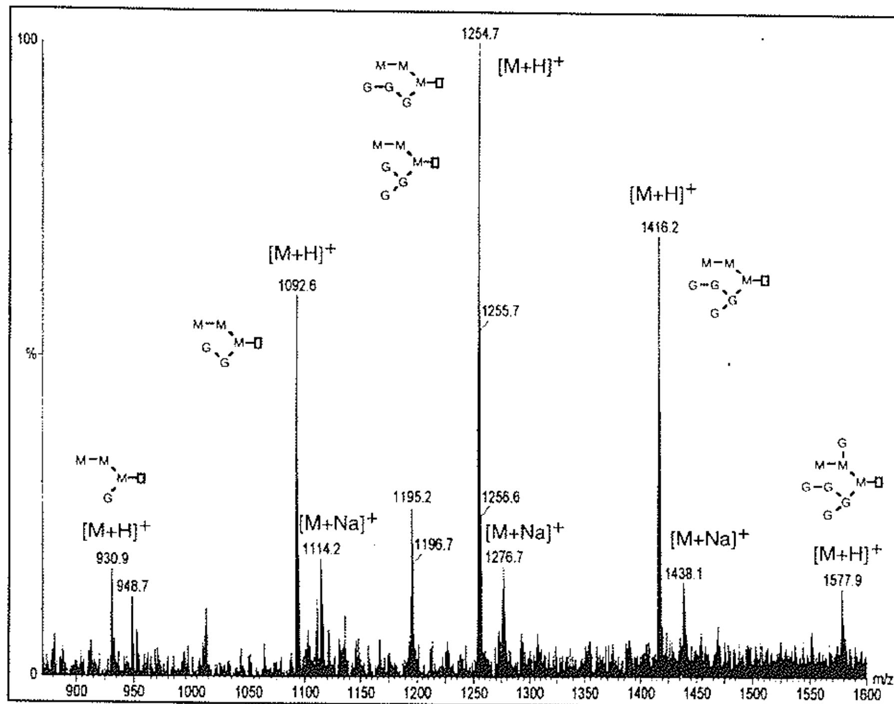

Figure 8D. ES MS of 2AB labelled VSG 117 neutral glycans (NGs). Positive ion mode electrospray mass spectrum of 2AB labelled VSG 117 NGs. 30 pmol were injected in methanol/acetic acid/water (1:1:1). The NG structures giving rise to major peaks are indicated. The empty square represents 2AB labelled anhydromannose.

Conclusion

There are three main post-translational modifications of proteins which involve glycosylation. The technology described here has been developed to enable all of these three types, N- and O-linked sugars and GPI anchor glycans, to be analysed rapidly and routinely. The minimum need is for an HPLC system equipped with an NP column and a fluorescence detector, a series of glycan standards and a set of endo- and exo-glycosidase enzymes are required. The ability to sequence N- and O-linked sugars directly from gels and GPI anchor glycans from blots has eliminated the need for exhaustive protein purification, making feasible the glycosylation analysis of biological samples available only in low yields.

The aim of developing such a strategy is to enable glycoproteins to be viewed in their entirety so that more insight can be gained into the complementary roles which sugars, proteins and anchors play in the structure and function of the glycoprotein.

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