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# Mammalian Sialyltransferase ST3Gal-II: Its Exchange Sialylation Catalytic Properties Allow Labeling of Sialyl Residues in Mucin-Type Sialylated Glycoproteins and Specific Gangliosides

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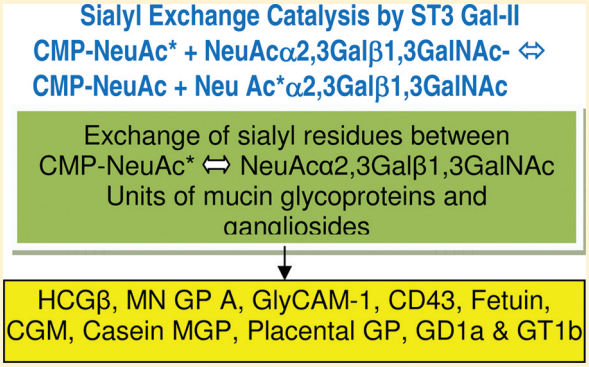
**ABSTRACT:** While glycosyltransferases are known to display unidirectional enzymatic activity, recent studies suggest that some can also catalyze readily reversible reactions. Recently, we found that mammalian sialyltransferase ST3Gal-II can catalyze the formation of CMP-NeuAc from 5′-CMP in the presence of a donor containing the NeuAc$\alpha$2,3Gal$\beta$1,3GalNAc unit [Chandrasekaran, E. V., et al. (2008) *Biochemistry 47*, 320−330]. This study shows by using [9-$^3$H]- or [$^{14}$C]sialyl mucin core 2 compounds that ST3Gal-II exchanges sialyl residues between CMP-NeuAc and the NeuAc$\alpha$2,3-Gal$\beta$1,3GalNAc unit and also radiolabels sialyl residues in gangliosides GD1a and GT1b, but not GM1. Exchange sialylation proceeds with relative ease, which is evident from the following. (a) Radiolabeleling of fetuin was ∼2-fold stronger than that of asialo fetuin when CMP- [9-$^3$H]NeuAc was generated in situ from 5′-CMP and [9-$^3$H]NeuAc$\alpha$2,3Gal$\beta$1,3GalNAc$\beta$1,3Gal$\alpha$-O-Me by ST3Gal-II. (b) ST3Gal-II exchanged radiolabels between [$^{14}$C]sialyl fetuin and [9-$^3$H]NeuAc$\alpha$2,3Gal$\beta$1,3GalNAc$\beta$1,3Gal$\alpha$-O-Me by generating CMP-[$^{14}$C]- and -[9-$^3$H]NeuAc through 5′-CMP; only 20.3% $^{14}$C and 28.0% $^3$H remained with the parent compounds after the sialyl exchange. The [9-$^3$H]sialyl-tagged MN glycophorin A, human chorionic gonadotropin $\beta$ subunit, GlyCAM-1, CD43, fetuin, porcine Cowper's gland mucin, bovine casein macroglycopeptide, human placental glycoproteins, and haptoglobin were analyzed by using Pronase digestion, mild alkaline borohydride treatment, Biogel P6, lectin agarose, and silica gel thin layer chromatography. Sulfated and sialylated *O*-glycans were found in GlyCAM-1 and human placental glycoproteins. This technique has the potential to serve as an important tool as it provides a natural tag for the chemical and functional characterization of *O*-glycan-bearing glycoproteins.




Glycoproteins modulate biological functions such as signaling, immune response, and tissue development by interaction through terminal sugars.[1] These sugars or glycans are commonly either N-linked to asparagine or O-linked to Ser/Thr residues on protein scaffolds. Among these, mucins are *O*-glycan rich glycoproteins that often contain tandem repeats of peptide sequences characterized by a high content of Ser, Thr, and Pro residues.[2−4] Each tissue exhibits a unique pattern of mucinous proteins that can be modified under pathological conditions. Such regulation of mucin expression in cancer epithelial cells influences cell adhesion and tumor invasiveness. Moreover, cancer-associated mucins that contain incomplete *O*-glycan chains are highly immunogenic, and these are potential targets for immunotherapy. Whereas the study of *N*-glycans has progressed well, in part, because of the availability of highly specific endoglycosidases that can release intact *N*-glycans, similar universal glycosidases for mucin-type *O*-glycans are not available. New tools for the study of O-glycosylation are thus desirable. This study addresses this need by presenting a novel strategy for enzymatically radiolabeling *O*-glycans, and also glycolipids.

O-Glycosylation is initiated by the attachment of GalNAc via $\alpha$-linkages to hydroxyl groups of Ser/Thr that are exposed on the protein surface at coils, turns, or linker regions. This step is catalyzed by a family of specific UDP GalNAc:polypeptide *N*-acetylgalactosaminyltransferases.[5,6] Further extension of these glycans is regulated, in large measure, by the distribution of glycosyltransferases and sulfotransferases that are primarily localized in the cellular Golgi.[7,8] Sialic acid residues are typically found at the terminal nonreducing ends of *O*-glycans expressed on both cell surface and secreted glycoproteins. The attachment of these residues is mediated by enzymes belonging to the sialyltransferase family. While these enzymes are typically thought to unidirectionally catalyze the transfer of sialic acid

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from a sugar nucleotide donor (CMP-NeuAc) to an acceptor substrate, we recently showed that this reaction can be reversible at least for the case of one mammalian/rat sialyltransferase ST3Gal-II.[9] Here, using this process, called "reverse sialylation", we demonstrated that ST3Gal-II can synthesize CMP-NeuAc from 5′-CMP and NeuAc$\alpha$2,3Gal$\beta$1,3GalNAc units of *O*-glycans, and glycolipids. In addition to this, we now report that this enzyme can also catalyze the direct exchange of NeuAc between CMP-NeuAc and the NeuAc$\alpha$2,3Gal$\beta$1,3-GalNAc units of *O*-glycans and glycolipids, in the absence of exogenous 5′-CMP. While the precise mechanism for this exchange process is yet to be established, this may be partially attributed to the formation of 5′-CMP in the reaction mixture because of the breakdown of CMP-NeuAc. These unique catalytic properties of ST3Gal-II could be utilized for the facile radiolabeling in vitro of sialyl residues in mucin-type structures.

## MATERIALS AND METHODS

**Materials.** Rat recombinant ST3Gal-II [$\alpha$2,3(O)ST] was purchased from EMD-Chemicals (San Diego, CA).[9,10] CMP-NeuAc was obtained from Sigma (St. Louis. MO). The synthesis of acceptor molecules used in this study was reported previously.[11,12] Human chorionic gonadotropin $\beta$-subunit (HCG$\beta$) was kindly provided by A. F. Parlow (National Hormone & Peptide Program, Harbor-UCLA Medical Center). GlyCAM-1 (200 $\mu$g) and CD43 (28 $\mu$g) were gifts from S. Rosen (University of California, San Francisco, CA) and M. Fukuda (Burham Institute, San Diego, CA). Fetuin, bovine casin macroglycopeptide, and human placenta acetone powder were from Sigma. Cowper's gland mucin (CGM) was available from a previous study.[9] The human placental glycoprotein fraction was isolated from the acetone powder by delipidation with a $CHCl_3/CH_3OH$ mixture (2:1, v/v) twice. The air-dried material was suspended in water and mixed in the cold room using Speci-Mix for 16 h and then centrifuged at 10000*g* and 4 °C for 0.5 h. An aliquot of the supernatant (2 mL) was subjected to Biogel P60 column (100−200 mesh, 1.0 cm × 116.0 cm) chromatography using 0.1 M pyridine acetate (pH 5.4) as the eluent. The anthrone positive fractions emerging first as a broad peak were pooled and lyophilized to dryness (25 mg).

**Enzymology Studies.** All enzymatic sialylation reactions were conducted in sodium cacodylate buffer (pH 6.0) in the presence of CMP-[9-$^3$H]NeuAc or CMP-[$^{14}$C]NeuAc (NEN-DuPont). The total CMP-NeuAc concentration, in some cases, was adjusted in individual reaction mixtures by supplementation with cold CMP-NeuAc. Products formed were subjected to chromatography using either (a) Biogel P2 or P6 chromatography (fine mesh, 1.0 cm × 116.0 cm) columns with 0.1 M pyridine acetate (pH5.4) as the eluent or (b) lectin-agarose affinity chromatography using columns of with a 7 mL bed volume of WGA-, VVL-, AAL-, or ConA-agarose (Vector Lab, Burlingame, CA) under conditions recommended by the supplier.[9,10] WGA binds terminal GlcNAc residues of glycans and also glycoproteins via sialyl residues. Using synthetic mucin core 2 compounds,[10] we have noted that WGA binds to mucin core 2 [9-$^3$H]NeuAc$\alpha$2,3Gal$\beta$1,3(GlcNAc$\beta$1,6)GalNAc$\alpha$ (**1***) and its derivatives containing substituents on GlcNAc such as 6-*O*-sulfo, $\beta$1,4-linked Gal, or both and $\beta$1,4-linked (3-*O*-sulfo)Gal. It does not bind to [9-$^3$H]NeuAc$\alpha$2,3 or $\alpha$2,6Gal$\beta$1,4GlcNAc$\beta$1,6(Gal$\beta$1,3)GalNAc$\alpha$. VVL binds the Tn epitope (GalNAc$\alpha$-O-Ser/Thr) of mucin glycoproteins. ConA binds to complex biantennary N-linked carbohydrate chains. AAL binds $\alpha$1,6- or $\alpha$1,3-linked Fuc residues in carbohydrates.

In some cases, peak fractions from chromatography steps containing radioactivity were pooled, lyophilized to dryness, dissolved in a small volume of water, and stored frozen at −20 °C for further experimentation. Thin layer chromatography using silica gel GHLF (250 $\mu$m scored, 20 cm × 20 cm; Analtech, Newark, DE) was also used for further product separation.[9,10]

**Preparation of Radioactive Glycans, Glycoproteins, and Glycolipids.** [9-$^3$H]NeuAc$\alpha$2,3Gal$\beta$1,3(GlcNAc $\beta$1,6)-GalNAc$\alpha$-O-Al (9-$^3$H[**1**]) and [9-$^3$H]NeuAc$\alpha$2,3-Gal$\beta$1,3GalNAc$\beta$1,3Gal$\alpha$-O-Me (9-$^3$H[**3**]) were prepared by incubating synthetic acceptors Gal$\beta$1,3(GlcNAc$\beta$1,6)GalNAc$\alpha$-O-Al and Gal$\beta$1,3GalNAc$\beta$1,3Gal$\alpha$-O-Me, respectively, with ST3Gal-II and CMP-[9-$^3$H]NeuAc as previously described.[10] The radiolabeled compound isolated using Biogel P2 chromatography emerged prior to the unreacted trisaccharide in both cases. NeuAc$\alpha$2,3Gal$\beta$1,3(GlcNAc$\beta$1,6)GalNAc$\alpha$-O-Al and NeuAc$\alpha$2,3Gal$\beta$1,3GalNAc$\beta$1,3Gal$\alpha$-O-Me were synthesized chemically.

$^3$H- and $^{14}$C-labeled bovine brain gangliosides were prepared by exchange sialylation. To prepare the tritiated compound, 13 mg of bovine brain gangliosides (EMD-Chemicals) was incubated with 20 $\mu$Ci of CMP-[9-$^3$H]NeuAc and 0.2 milliunits of ST3Gal II in 1.2 mL of 0.15 M sodium cacodylate (pH 6.0) at 37 °C for 20 h. $^{14}$C-labeled ganglioside was similarly prepared by mixing 25 mg of the bovine brain ganglioside mixture with 4 $\mu$Ci of CMP-[$^{14}$C]NeuAc under identical reaction conditions. Following reaction, the radioactive material emerging at the void volume of a Biogel P2 chromatogram was lyophilized to dryness. This $^3$H-labeled ganglioside mixture was further purified on the Biogel P6 column where it also emerged at the void volume.

A variety of radioactive glycoproteins were prepared using the sialylation properties of ST3Gal-II. $^{14}$C-labeled sialyl fetuin was prepared by incubating 30 mg of fetuin (Sigma) with 1.5 $\mu$Ci of CMP-[$^{14}$C]NeuAc and 0.1unit of ST3Gal-II in a 0.8 mL volume of 0.2 M sodium cacodylate (pH 6.0) for 20 h at 37 °C. All other [9-$^3$H]sialic acid-labeled sialoglycoproteins were made by incubation of CMP-[9-$^3$H]NeuAc as such, without any addition of cold CMP-NeuAc, with 0.2 unit of ST3Gal-II in a total reaction volume of 1.2 mL in 0.1 M sodium cacodylate (pH 6.0) for 16 h at 37 °C. The labeled glycoproteins were isolated using Biogel P2 chromatography. The reaction mixtures were separated using Biogel P2 (1.0 cm × 116.0 cm) chromatography. The first radioactive peak emerging at the void volume contained radiolabeled glycoprotein. This was lyophilized to dryness and used in the characterization studies.

**Proteolytic Treatment and Separation of Glycopeptides.** Pronase digestion of [9-$^3$H]sialyl glyoproteins was conducted in 1.0 mL of 0.1 M Tris-HCl (pH 7.0), 1 mM $CaCl_2$, 1% ethanol, and 0.1% $NaN_3$ containing 10 mg of Pronase CB (EMD-Chemicals) at 37 °C for 24 h. After the treatment, the samples were kept frozen at −20 °C before being fractionated on Biogel P6.

**Release of *O*-Glycans from the Protein Backbone and Their Separation.** Mild alkaline borohydride treatment of [9-$^3$H]sialyl glyoproteins was performed in Teflon-lined screw-capped test tubes using 1.0 M sodium borohydride in 0.1 N NaOH in a total volume of 1.0 mL. Samples were incubated at

45 °C for 24 h; excess borohydride was destroyed via careful addition of drops of acetic acid, and frozen samples were stored at −20 °C before being fractionated on Biogel P6. The outcome of mild alkaline borohydride treatment is illustrated by citing two glycoproteins as examples. As anticipated, TLC of radio sialyl fetuin after this treatment showed one major component representing NeuAcα2,3Galβ1,3GalNAcα-ol and one minor component NeuAcα2,3Galβ1,3(NeuAcα2,6)-GalNAcα-ol, whereas HCGβ showed four components, two being represented by NeuAcα2,3Galβ1,3GalNAcα-ol and mucin core 2 NeuAcα2,3Galβ1,3(Galβ1,4GlcNAcβ1,6)-GalNAcα-ol.

**Liquid Chromatography Coupled with Tandem Mass Spectrometry.** LC separation was performed on a C18 reverse-phase column using a linear gradient of acetonitrile in 0.1% formic acid.[9,10] The sample injection volume was 20 μL. Negative ion electrospray ionization (ESI) was used for the detection of sialylated compounds because of its greater sensitivity versus that of positive ion ESI.[9,10]

**Electrophoresis.** To release *N*-glycans, [$^{14}$C]sialyl-labeled fetuin, haptoglobin, and apotransferrin were treated with 50 units/mL PNGaseF (Sigma) in 50 mM ammonium bicarbonate, 0.2% SDS, and 100 mM 2-mercaptoethanol for 20 h at 37 °C. The enzyme was denatured by being boiled for 5 min in sample buffer. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was then conducted using polyacrylamide gradient gels. Coomassie blue staining of proteins was performed in some cases. In other cases, following transfer to a nitrocellulose membrane, radioactive glycoprotein bands were visualized using phosphorimaging.

## RESULTS AND DISCUSSION

**Exchange of Sialic Acid between CMP-NeuAc and Synthetic Mucin Core 2 Sialyl Compounds.** The cloned rat sialyltransferase α2,3(O)ST (ST3Gal-II) mediates α2,3 sialylation of Gal in the Galβ1,3GalNAcα unit of *O*-glycans. Additionally, we observed that in the presence of ST3Gal-II and CMP-[9-$^{3}$H]NeuAc, NeuAcα2,3Galβ1,3(GlcNAcβ1,6)-GalNAcα-O-Al (**1**) gave rise to a radiolabeled product that behaved like **1**. Like **1**, this product bound to WGA-agarose (a matrix that binds terminal *N*-acetylglucosamine), with the concentration of the product increasing with the concentration of CMP-NeuAc and **1** (Figure 1a). When the same product was eluted using a Biogel P2 column, a distinct radioactive peak appeared at 40−45 mL (Figure 1b), prior to free CMP-[9-$^{3}$H]NeuAc which appeared after 60 mL. This product, obtained from the Biogel column, migrated like **1** in TLC plates in three different solvent systems (Figure 1c).

To determine if the reverse reaction in the scheme at the top left of Figure 1 is feasible, we tested the possibility that CMP-[9-$^{3}$H]NeuAc can be formed upon addition of ST3Gal-II to a mixture containing cold CMP-NeuAc and tritium-labeled compound **1**, [9-$^{3}$H]NeuAcα2,3Galβ1,3(GlcNAcβ1,6)-GalNAcα-O-Al ([9-$^{3}$H]**1**). For this, [9-$^{3}$H]**1** was prepared using ST3Gal-II, CMP-[9-$^{3}$H]NeuAc, and Galβ1,3-(GlcNAcβ1,6)GalNAcα-O-Al,[10] and the radiolabeled product was separated from the unreacted trisaccharide by Biogel P2 column chromatography. Three different reaction mixtures were then prepared with (i) [9-$^{3}$H]**1** and ST3Gal-II but without CMP-NeuAc, (ii) [9-$^{3}$H]**1** and CMP-NeuAc without ST3Gal-II, and (iii) everything. When the reaction mixtures were subjected to WGA-agarose affinity chromatography, the radioactive component from samples i and ii but not sample iii was bound to the column (Figure 1d). The data indicate an efficient (>90%) transfer of radioactive [9-$^{3}$H]NeuAc from 150 μM [9-$^{3}$H]**1** to 1.0 mM CMP-NeuAc in the presence of ST3Gal-II. The extent of enzymatic transfer of [9-$^{3}$H]NeuAc increased with CMP-NeuAc concentration (Figure 1e). These observations were also supported by TLC studies (Figure 1f) in which the radioactive component of sample iii migrated like CMP-NeuAc. The radioactivity from sample ii migrated like [9-$^{3}$H]**1**. When the disialylated synthetic glycan NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1,6(NeuAcα2,3Galβ1,3)GalNAcα-O-Me (**2**) was used instead of **1**, it gave rise to a radioactive peak at 37−41 mL from the Biogel P2 column (Figure 1g), and on TLC, this radioactive product moved like **2** (Figure 1h). The data suggest that ST3Gal-II can catalyze the exchange of NeuAc between CMP-[9-$^{3}$H]NeuAc and NeuAcα2,3Galβ1,3GalNAc as shown in the forward reaction of the scheme at the top left of Figure 1.Taken together, the data support the feasibility of the sialyl exchange reaction between *O*-glycans and CMP-NeuAc in the presence of ST3Gal-II shown in the scheme at the top left of Figure 1.

The mechanism of exchange sialylation is unknown, and CMP-NeuAc is resistant to breakdown by NeuAc aldolase, phosphomonoesterase, phosphodiesterase, and neuraminidase.[13] We examined if the breakdown of CMP-NeuAc into 5′-CMP and NeuAc may occur spontaneously under our reaction conditions. To this end, two 80 μL mixtures each containing 0.36 μM CMP-NeuAc and CMP-[9$^{3}$H]NeuAc in 0.1 M sodium cacodylate buffer (pH 6.0) containing 2% Triton CF 54 and 10 mg/mL BSA were prepared; one was incubated at 37 °C for 4 h and the other stored at −20 °C. Both were subjected to Dowex-1-Formate fractionation using stepwise elution at increasing concentrations of NaCl. It is evident from Figure 1i that CMP-NeuAc kept at −20 °C before being subjected to fractionation was quite stable, whereas the one incubated at 37 °C for 4 h had undergone appreciable (25.3%) breakdown into NeuAc and 5′-CMP. On the basis of this observation, it may be possible that 5′-CMP formed under our reaction conditions facilitates NeuAc exchange via the "reversible sialylation" mechanism. Additional studies in the future will be needed to confirm this potential mechanism of action.

**Exchange of 9-$^{3}$H- and $^{14}$C-Labeled Sialic Acid between Fetuin and NeuAcα2,3Galβ1,3GalNAcβ1,3Galα-O-Me.** We determined if it is possible to use the exchange and reverse sialylation properties of ST3Gal-II to transfer sialic acid between NeuAcα2,3Galβ1,3GalNAcβ1,3Galα-O-Me (**3**) and fetuin, a major glycoprotein in fetal calf serum. For this reaction, [9-$^{3}$H]**3** was prepared by reacting Galβ1,3GalNAcβ1,3Galα-O-Me with ST3Gal-II in the presence of CMP-[9-$^{3}$H]NeuAc. $^{14}$C-labeled fetuin was also prepared by incubating fetuin with CMP-[$^{14}$C]NeuAc and ST3Gal-II. [9-$^{3}$H]**3** and [$^{14}$C]fetuin were then mixed with ST3Gal-II, in the absence (Figure 2a) or presence (Figure 2b) of 5′-CMP. After 20 h, the reaction mixture was fractionated using Biogel P2 column chromatography. Exchange of sialic acid between [9-$^{3}$H]**3** and [$^{14}$C]fetuin is noted in Figure 2b in the presence of 5′-CMP. In this panel, 32.3% of the radioactivity from fetuin was transferred to **3**. Also, 18.5% of the [9-$^{3}$H]sialyl residue from **3** was transferred to [$^{14}$C]sialyl fetuin; 20.3% $^{14}$C and 28.0% $^{3}$H remained with the parent compounds, while the rest was in the form of either CMP-[$^{14}$C]NeuAc or CMP-[9-$^{3}$H]NeuAc. These data are consistent with the reactions

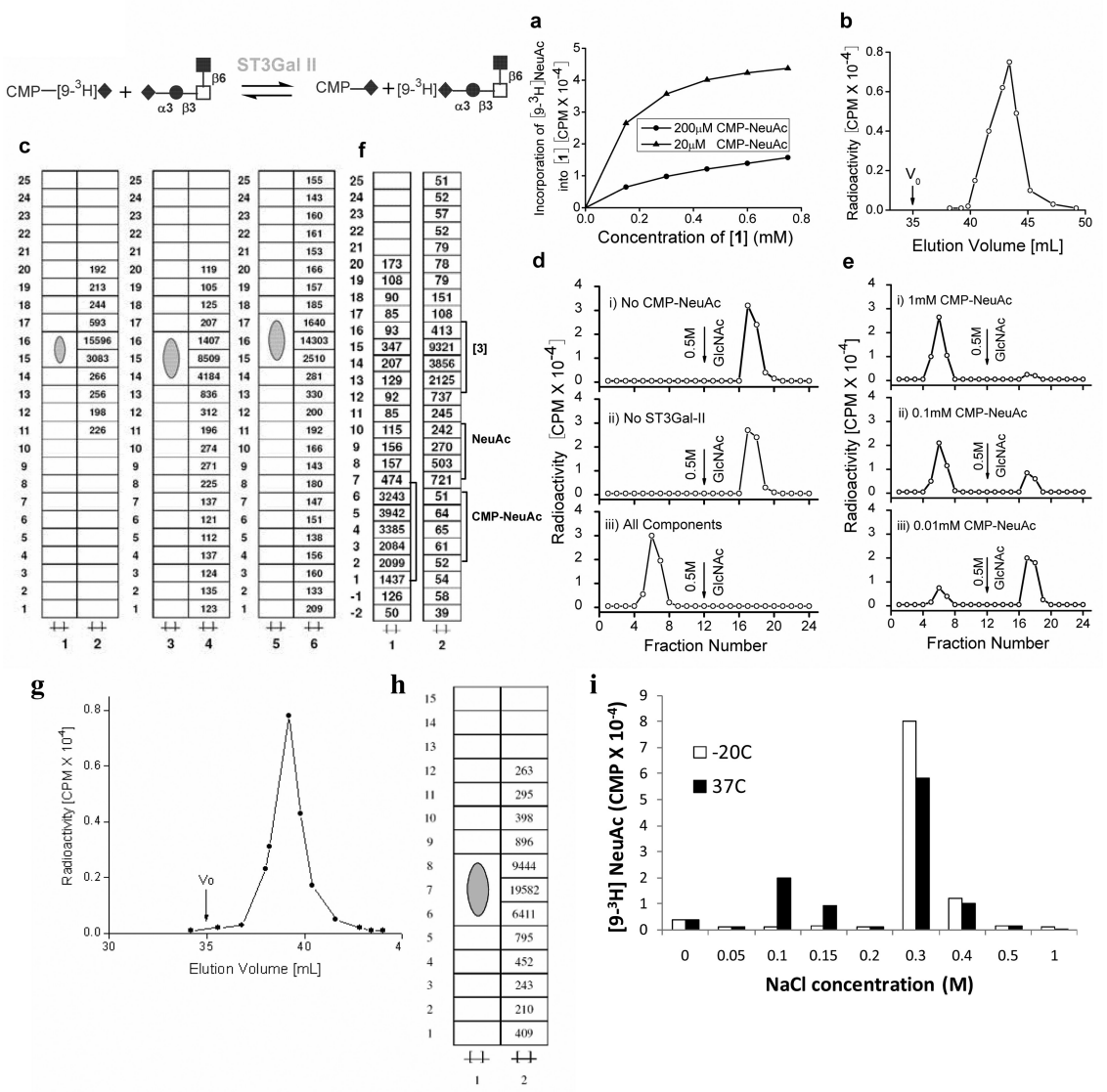


**Figure 1.** Exchange of sialyl residues between CMP-[9-$^3$H]NeuAc and NeuAc$\alpha$2,3Gal$\beta$1,3(GlcNAc$\beta$1,6)GalNAc$\alpha$-O-Al (**1**) catalyzed by ST3Gal-II. In the scheme at the top left, the symbols are as follows: (◆) sialic acid, (●) Gal, (□) GalNAc, and (■) GlcNAc. (a) [9-$^3$H]CMP-NeuAc (0.2 $\mu$Ci) was mixed with cold CMP-NeuAc to concentrations of 20 $\mu$M (low CMP-NeuAc concentration, high specific activity) or 200 $\mu$M (high concentration, low specific activity). This was added to 0−0.8 mM **1** in the presence of 0.16 milliunit of enzyme at 37 °C for 4 h in 100 mM sodium cacodylate buffer (pH 6.0). The reaction volume was 20 $\mu$L. The product was diluted to 1.0 mL using 10 mM Hepes (pH 7.5) containing $CaCl_2$ and $MnCl_2$ and fractioned on a WGA agarose column. The radiolabeled product formed bound to the WGA-agarose affinity column, a matrix that binds **1**. (b) [9-$^3$H]CMP-NeuAc (0.5 mM) and **1** (0.3 mM) were incubated with ST3Gal-II under the reaction conditions described for panel a, and the radiolabeled product was isolated using a Biogel P2 column. Radiolabeled **1** appeared as a peak between 40 and 45 mL with unreacted [9-$^3$H]CMP-NeuAc appearing at 60 mL (not shown). $V_0$ denotes the void volume. (c) The [9-$^3$H]NeuAc-labeled product from the Biogel P2 column in panel b was subjected to TLC plates in three different solvent systems: 1-propanol/$NH_4OH$/$H_2O$ (12:2:5, v/v) developed once (lanes 1 and 2), $CHCl_3$/$CH_3OH$/$H_2O$ (5:4:1, v/v) developed twice (lanes 3 and 4), and ethyl acetate/pyridine/$H_2O$/acetic acid (5:5:3:1, v/v) developed once (lanes 5 and 6). In each case, the first lane contains the unreacted acceptor and the second lane quantifies the radioactivity associated with the product scraped from sections of the TLC plate. (d) Reaction mixtures (RM) with the following compositions were incubated at 37 °C for 2 h in 100 mM sodium cacodylate buffer (pH 6.0): (i) RM containing 150 $\mu$M (0.4 $\mu$Ci) [9-$^3$H]NeuAc$\alpha$2,3Gal$\beta$1,3(GlcNAc$\beta$1,6)GalNAc$\alpha$-O-Al ([9-$^3$H]**1**) (donor) along with 0.8 milliunit of ST3Gal-II but no CMP-NeuAc, (ii) RM containing [9-$^3$H]**1** and 1.0 mM CMP-NeuAc, but lacking ST3Gal-II, and (iii) RM containing all components, including the donor, CMP-NeuAc, and enzyme. Products formed were fractionated on a WGA-agarose column. Only sample iii contained components that did not bind WGA agarose. (e) WGA-agarose affinity chromatography was performed as described for panel d with all components in RM except that the amount of CMP-NeuAc was varied. (f) TLC of RMs ii (lane 1) and ii (lane 2) from panel d using as a solvent a $CHCl_3$/$CH_3OH$/$H_2O$ mixture (5:4:1, v/v) developed twice. The position at which the radioactivity peak appears is marked with a bracket, along with the migration distances for NeuAc, CMP-NeuAc, and [9-$^3$H]**1** that were determined in independent runs. (g) Separation of the radiolabeled product from the acceptor NeuAc$\alpha$2,3Gal$\beta$1,4(Fuc$\alpha$1,3)GlcNAc$\beta$1,6(NeuAc$\alpha$2,3Gal$\beta$1,3)GalNAc$\alpha$-O-Me using a Biogel P2 column. (h) TLC runs of the radiolabeled product and the acceptor using the 2-propanol/$NH_4OH$/$H_2O$ solvent system (12:2:5, v/v). (i) CMP-NeuAc was incubated in cacodylade buffer for 4 h at either −20 or 37 °C. Dowex-1-formate chromatography was then performed by elution with 3.0 mL each of water and various NaCl concentrations as indicated. CMP-NeuAc eluted from the column at 0.3−0.4 M NaCl and free NeuAc at 0.10−0.15 M NaCl.


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[$^{14}$C] Sialyl Fetuin + 5'-CMP ⇔ asialo fetuin + CMP-[$^{14}$C]NeuAc

[9-$^{3}$H]NeuAcα2,3Galβ1,3GalNAcβ1,3Galα-O-Me + 5'CMP ⇔ Galβ1,3GalNAcβ1,3Galα-O-Me + CMP-[9-$^{3}$H]NeuAc

Galβ1,3GalNAcβ1,3Galα-O-Me + CMP-[$^{14}$C]NeuAc ⇔ [$^{14}$C]NeuAcα2,3Galβ1,3GalNAcβ1,3Galα-O-Me + 5'-CMP

Asialo Fetuin + CMP-[9-$^{3}$H ]NeuAc ⇔ [9-$^{3}$H] Sialyl Fetuin + 5'-CMP

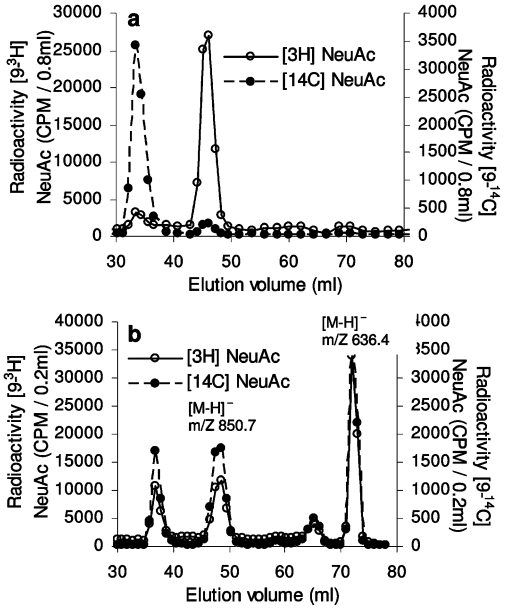


**Figure 2.** Enzymatic exchange of sialyl residues between [$^{14}$C]sialyl fetuin and [9-$^{3}$H]NeuAcα2,3Galβ1,3GalNAcβ1,3Galα-O-Me. In the scheme at the top, 5′-CMP acts as the sialic acid acceptor to form radiolabeled CMP-NeuAc via the reverse sialylation reaction. The newly formed CMP-NeuAc participates in the transfer of sialic acid to fetuin and NeuAcα2,3Galβ1,3GalNAcβ1,3Galα-O-Me via the exchange mechanism. [$^{14}$C]Sialyl fetuin (2 mg in 100 μL of water) and 100 μL of [9-$^{3}$H]NeuAcα2,3Galβ1,3GalNAcβ1,3Galα-O-Me (0.75 mM) was diluted into a 400 μL volume using 0.15 M sodium cacodylate (pH 6.0). One hundred milliunits of cloned ST3Gal-II was added in the absence (a) or presence (b) of 10 mM 5′-CMP. The reaction proceeded at 37 °C for 20 h. Reaction mixtures were diluted to 1.0 mL with water and fractionated using the Biogel P2 column. The product identity was verified by mass spectrometry as indicated by the molecular masses in the figure.

outlined in the scheme at the top of Figure 2, where reverse sialylation results in the formation of radiolabeled CMP-NeuAc. Following this, the exchange of [$^{14}$C]- and [9-$^{3}$H]sialyl moieties occurs because of the normal sialylation reactions, which results in the formation of $^{3}$H-labeled fetuin and $^{14}$C-labeled **3**.

To determine the efficacy of the exchange reaction in the experiment described above compared to the efficacy of the forward reaction, we compared the extent to which radioactive sialic acid from the newly formed radioactive CMP-NeuAc in the scheme in Figure 2 is transferred to fetuin versus asialo fetuin. Here, 1 mg of fetuin (~0.016 μmol) or 1 mg of asialo fetuin (~0.018 μmol) in 80 μL of 64 mM sodium cacodylate (pH 6.0) was incubated with [9-$^{3}$H]**3** (0.037 μmol, 252000 cpm), 5′-CMP (1.0 μmol or 12 mM), and 10 milliunits of ST3Gal-II for 16 h at 37 °C. The product was separated by Biogel P2 column chromatography, and the radioactive fractions corresponding to [9-$^{3}$H]**3**, 9-$^{3}$H-labeled fetuin, and CMP-[9-$^{3}$H]NeuAc were quantified (Table 1). Here, in studies with asialo fetuin, we expect to form CMP-[9-$^{3}$H]NeuAc from [9-$^{3}$H]**3** using reverse sialylation. This would be followed by

**Table 1. Extent of Exchange versus Normal Sialylation**

| | asialo fetuin (cpm) | fetuin (cpm) |
|---|---|---|
| [9-$^{3}$H]sialylated asialo fetuin or fetuin (peak I) | 28265 | 53800 |
| unused [9-$^{3}$H]sialyl donor [9-$^{3}$H] NeuAcα2,3Galβ1,3GalNAcβ1,3Galα-O-Me (peak II) | 171120 | 115119 |
| unused CMP-[9-$^{3}$H]NeuAc (peak III) | 50570 | 82086 |
| CMP-[9-$^{3}$H]NeuAc formed (peaks I and III) | 78835 | 135886 |

transfer of a portion of this radioactive sugar nucleotide to asialo fetuin via the conventional forward sialylation process. Consistent with this, ~11% of the total radioactivity (28265 cpm) was transferred to asialo fetuin (Table 1). In studies with fetuin, however, we expect to form both CMP-[9-$^{3}$H]NeuAc from [9-$^{3}$H**3** and CMP-NeuAc from the sialylated macromolecule. Here, the continuous replenishment of CMP-NeuAc by fetuin is likely to result in greater amounts of CMP-[9-$^{3}$H]NeuAc released from [9-$^{3}$H]**3** and consequently greater transfer of [9-$^{3}$H]NeuAc to fetuin. This proposition is consistent with the data in Table 1, where ~54% (135886 cpm) of the total radioactivity was released from [9-$^{3}$H]**3** and ~21% (53800 cpm) of the [9-$^{3}$H]NeuAc was transferred to fetuin. Overall, these results confirm that the exchange−reverse sialylation reaction mediated by ST3Gal-II proceeds efficiently. This provides a feasible means of radiolabeling sialylated macromolecules.

**Incorporation of [$^{14}$C]- and [9-$^{3}$H]Sialyl Residues into Gangliosides Containing NeuAcα2,3Galβ1,3GalNAc via the Exchange Reaction.** We tested the possibility that the exchange reaction can be used to label gangliosides containing the NeuAcα2,3Galβ1,3GalNAc unit because such molecules may participate in cancer progression,[14,15] and because gangliosides are targets of specific immunotherapy in patients with melanoma, colon carcinoma, and pancreatic adenocarcinoma.[16−19] Further, because serum gangliosides may be more reliable than CA19-9 as an indicator of tumor burden,[19] methods for radiolabeling them can be useful in the context of cancer.

In this study, we used a bovine brain ganglioside mixture containing $GM_1$ (18%), $GD_1a$ (55%), and $GT_1b$ (10%) (Figure 3). ST3Gal-II along with either 9-$^{3}$H- or $^{14}$C-labeled CMP-NeuAc was added to this ganglioside mixture. After reaction, the radiolabeled ganglioside mixture was analyzed using TLC. Upon comparison of lane $A_2$ with lanes $A_1$ and $A_3$ of Figure 3, we noted that both 9-$^{3}$H and $^{14}$C radioactivities could be localized with $GD_1a$ and $GT_1b$ but not with $GM_1$. Autoradiography (lane $A_1$) further showed a band with a mobility closer to that of $GD_1a$. As this band was minor compared to other bands and lane $A_2$ (charring TLC plate) did not show such a band, attempts to identify it were not undertaken. Thus, sialic acid residues in NeuAcα2,3Galβ1,3GalNAc of $GD_1a$ and $GT_1b$, but not NeuAcα2,3Galβ1,4Glc of $GM_1$, may be exchanged with CMP-NeuAc in the presence of ST3Gal-II. While a small portion of $GM_1$ containing Galβ1,3GalNAc may be converted into radioactive $GD_1a$ via forward sialylation, the labeling of $GT_1b$ likely proceeds purely via the exchange mechanism.

**Characterization of Radiolabeled Glycoproteins.** Studies were performed to analyze the nature of sialylated glycoconjugates labeled with ST3Gal-II in the presence of CMP-NeuAc. Two analysis protocols were followed. Pronase-digested glycoproteins were separated using Biogel P6 (Figure 4A),

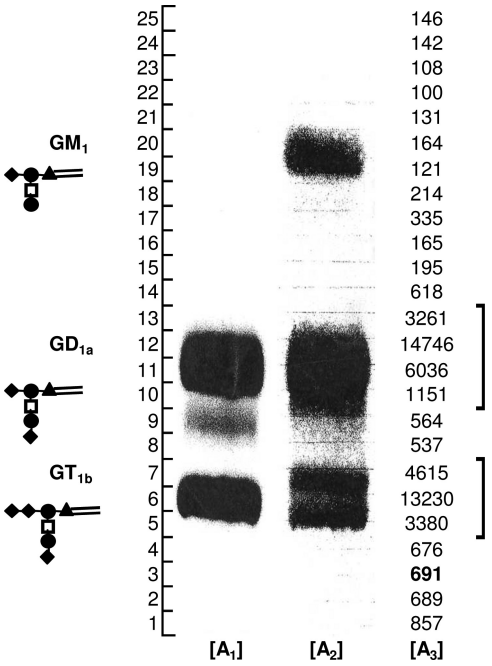


**Figure 3.** Enzymatic exchange of sialyl residues between CMP-NeuAc and gangliosides. [9-$^3$H]- and [$^{14}$C]sialic acid were incorporated into bovine brain gangliosides upon addition of either CMP-[$^{14}$C]NeuAc or CMP-[9-$^3$H]NeuAc to bovine brain gangliosides in the presence of ST3Gal-II. TLC separation used a $CHCl_3/CH_3OH$/0.2% aqueous $CaCl_2$ mixture (60:40:9, v/v) as the mobile phase. A TLC plate containing a [$^{14}$C]sialyl ganglioside mixture was developed by autoradiography (lane $A_1$). A TLC plate containing the same sample was also charred with $H_2SO_4$ in ethanol (lane $A_2$) to locate the gangliosides. We located tritium on the TLC plates by scraping silica gel from 0.5 cm width segments, soaking silica in 2.0 mL of water, and then counting the radioactivity by scintillation counting (lane $A_3$). The structure of gangliosides is shown as a cartoon: (▲) glucose, (◆) sialic acid, (●) Gal, and (□) GalNAc. Radioactive exchange occurs at the NeuAc$\alpha$2,3Gal$\beta$1,3GalNAc arm of gangliosides.

and this was followed by lectin affinity chromatography. Mild alkaline borohydride treatment of [9-$^3$H]sialyl-labeled glyoproteins was used to release *O*-glycans; the released products were separated using Biogel P6 column chromatography (Figure 4B), and this was followed by TLC analysis. Tables 2 and 3 summarize the results from Pronase digestion and mild alkaline borohydride treatment of [9-$^3$H]sialyl-labeled glyoproteins, respectively.

**HCG$\beta$.** Human chorionic gonadotropin (HCG) is a glycoprotein hormone produced by placental trophoblasts and trophoblastic tumors. HCG is a heterodimer composed of $\alpha$- and $\beta$-subunits (HCG$\beta$). The $\alpha$-subunit is shared with the other glycoprotein hormones, whereas the $\beta$-subunit is specific for each hormone. HCG$\beta$ contains two Asn-linked carbohydrate units (Asn13 and Asn30) and four Ser-linked oligosaccharides (Ser121, -127, -132, and -138). The carbohydrate structure of HCG$\beta$ derived from pregnancy was found to differ from malignant tumors by Valmu et al.[20] They identified core 2 glycans at Ser121, Ser127, and Ser132 and core 1 glycans at Ser138. They observed that the peptide chain carrying the two O-glycosylation sites, Ser127 and Ser132, could not be specifically cleaved by known proteases. The major *N*-glycan was a biantennary complex type, but increased levels of triantennary structure linked to Asn20 as well as increased levels of fucosylation of Asn13-bound glycans were identified in cancer. Antibody B152 recognizing mainly the core 2 *O*-glycans at Ser132 was shown to be useful for the prediction of Down syndrome during pregnancy and in the diagnosis and monitoring of cancer.[21]

With regard to the previous paragraph, this study shows that [9-$^3$H]sialyl-labeled HCG$\beta$ contained 31.4% Con A-agarose binding and 10.2% AAL-agarose binding glycoproteins. This is consistent with the data that show that HCG$\beta$ contains biantennary complex-type *N*-glycans with some inner core $\alpha$1,6 fucosyl residues. An extensive Pronase digestion of [9-$^3$H]sialyl-labeled HCG$\beta$ followed by Biogel P6 chromatography showed four distinct glycopeptide fractions (Figure 5a) probably arising from three to four linkage sites containing variable mucin carbohydrate chains as discerned in this study from their differential binding to WGA-agarose. The largest glycopeptide fraction (A) bound completely (Figure 5b), whereas the smallest one (fraction D) did not bind at all to WGA-agarose (Figure 5e). The two middle fractions (B and C) contained additional WGA-binding glycopeptide fractions (Figure 5c,d). Thin layer chromatography of the Biogel P6 fractions (Figure 5f) from mild alkaline borohydride-treated [9-$^3$H]sialyl HCG$\beta$ indicated the presence of four distinct carbohydrate bands (Figure 5g, 1−3). The results suggest that this radiolabeling technique for labeling mucins may be useful for studying changes in HCG$\beta$ due to cancer and other diseases.

**Fetuin.** Of the total carbohydrates of fetuin, 21% are located in the *O*-glycans and the remainder in Asn-linked chains.[22] It contains three triantennary complex-type sialylated oligosaccharides linked to asparagine and three alkali-labile sialylated *O*-glycan chains.[22] The two monosialylated disaccharide units (NeuAc$\alpha$2,3Gal$\beta$1,3GalNAc) are linked to Ser and Thr residues that are situated in the proximity within no more than four amino acid residues.[22] The disialylated NeuAc$\alpha$2,3Gal$\beta$1,3(NeuAc$\alpha$2,6)GalNAc chain is linked to another Ser residue.

In this study, Pronase digestion of [9-$^3$H]sialyl fetuin gave rise to one minor and two major Biogel P6 glycopeptide fractions, all being included by Biogel P6 (Figure 6a). While [9-$^3$H]sialyl fetuin showed complete binding to WGA-agarose (Figure 6b), its glycopeptides did not bind to this column, except for the binding of a small portion of the high-molecular mass fraction (Figure 6c−e). Thin layer chromatography of the Biogel P6 fractions from mild alkaline borohydride-treated [9-$^3$H]sialyl fetuin (Figure 6f) indicated one major and one minor *O*-glycan band (Figure 6g,h). Treatment of [$^{14}$C]sialyl fetuin with PNGase F followed by SDS−PAGE (Figure 6i) showed a decrease in the molecular mass of [$^{14}$C]sialyl fetuin. Further, the radioactive bands had the same intensity before and after PNGase treatment. The data would indicate the specific sialyl radiolabeling of *O*-glycan chains in fetuin by ST3Gal-II.

**MN Glycophorin A.** Glycophorin A (GPA) is a highly glycosylated sialoglycoprotein containing ~12 *O*-glycans and one *N*-glycan.[23] Blood groups M and N glycophorins differ at the N-termini (Ser1/Gly5 in the M antigen and Leu1/Glu5 in the N antigen), and the adjacent amino acid residues (Ser2, Thr3, and Thr4) carry O-linked chains.[24] In this study, [9-$^3$H]sialyl MN GPA gave upon Pronase digestion a major glycopeptide fraction excluded from the Biogel P6 column. This [9-$^3$H]sialyl glycopeptide fraction showed complete binding to WGA-agarose and complete nonbinding to VVL-agarose, indicating the clustering of sialylated *O*-glycans and the absence of Tn epitopes.[4]

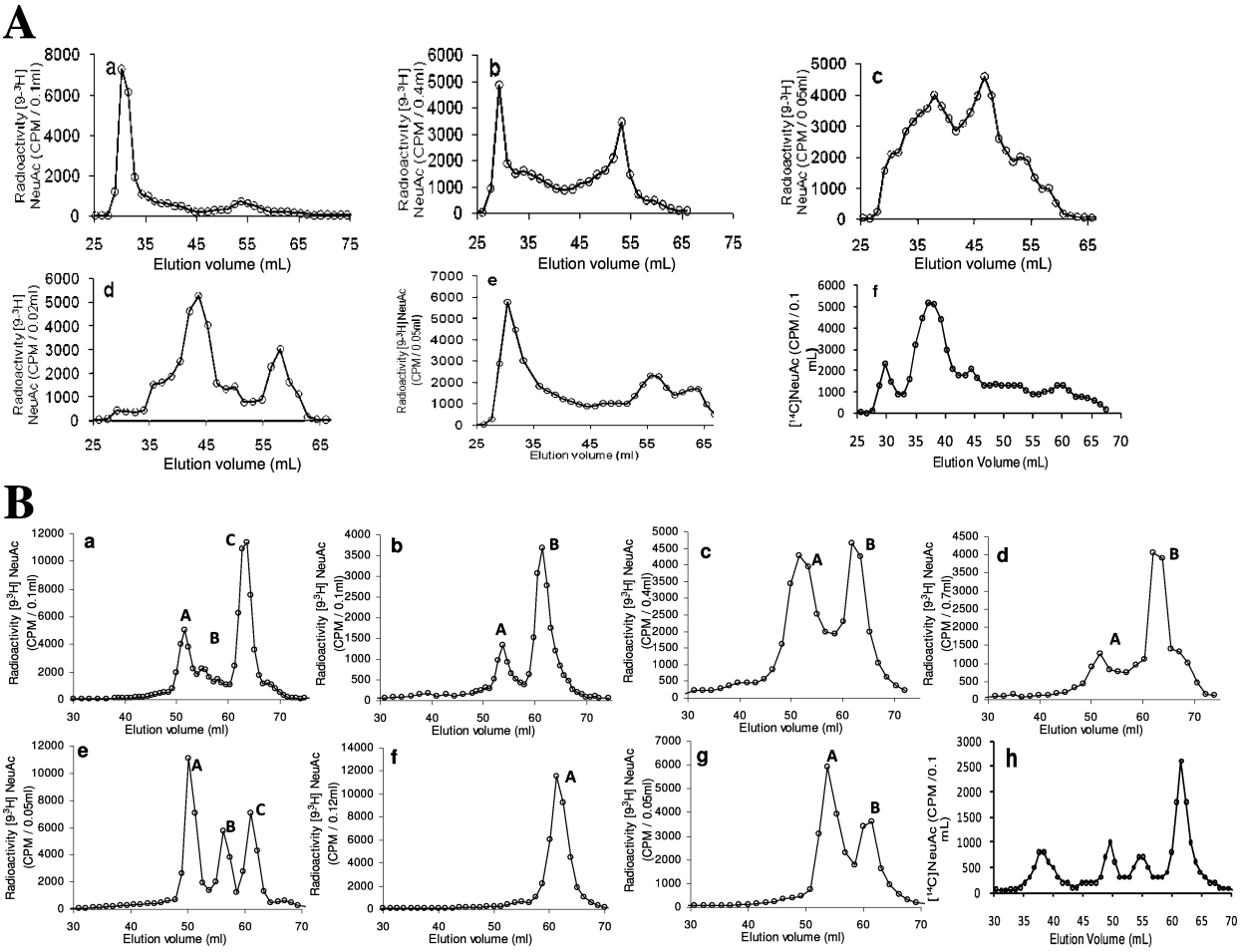


**Figure 4.** Characterization of [9-$^3$H]sialyl-tagged glycoproteins. (A) Comparison of the fragments arising from Pronase digestion of [9-$^3$H]sialyl-tagged glycoproteins by Biogel P6 column chromatography: (a) MN glycophorin A, (b) GlyCAM-1, (c) human placental glycoproteins, (d) bovine casein macroglycopeptide (MGP), (e) Cowper's gland mucin (CGM), and (f) human haptoglobin. (B) Biogel P6 chromatography of [9-$^3$H]sialyl-labeled products arising from mild alkaline borohydride treatment of various glycoconjugates: (a) HCG$\beta$, (b) MN glycophorin A, (c) GlyCAM-1, (d) CD43, (e) human placental glycoproteins (HPG), (f) bovine casein macroglycopeptide (MGP), (g) Cowper's gland mucin (CGM), and (h) human haptoglobin.

**Table 2. Characterization of [9-$^3$H]Sialyl-Labeled Glycopeptides Obtained following Pronase Digestion**

| | [9-$^3$H]sialyl label containing Biogel P6 fractions resulting from Pronase digestion | |
|---|---|---|
| [9-$^3$H]sialyl-labeled glycoprotein | major glycopeptide fractions | minor glycopeptide fractions |
| HCG$\beta$ (31.4 and 10.2% of the glycoprotein binds ConA- and AAL-agarose, respectively, whereas 100% of it bound WGA-agarose) | four (the largest binding to WGA-agarose and the smallest not binding to WGA-agarose; the middle two fractions contained both WGA-binding, nonbinding, and loosely binding glycopeptides) | none |
| MN glycophorin A | one (binding to WGA-agarose and not binding to VVL-agarose) | none |
| GlyCAM-1 | two (the large one to WGA-agarose and the small one not binding to WGA-agarose) | none |
| fetuin (100% of glycoprotein binds WGA-agarose) | two (both did not bind to WGA-agarose) | one (mostly not binding to WGA-agarose) |
| human placental glycoproteins (98% bound to WGA-agarose) | two (apparently heterogeneous) | none |
| bovine casein macroglycopeptide (60% bound to WGA-agarose) | one (large in size) | one (small in size) |
| porcine Cowper's gland mucin | one (large in size) | two (smaller in size) |

Alkaline borohydride treatment resulted in one major and one minor Biogel P6 fraction (Figure 4B, b). Thin layer chromatography showed one major carbohydrate band and three distinct minor bands (Figure 7, lanes 1and 2). Fukuda et al.[25] reported the minor occurrence of novel sialylated large *O*-glycans in human erythrocyte glycoproteins.

**GlyCAM-1.** Hemmerich et al.[26,27] determined that GlyCAM-1, the high endothelial venule-derived ligand for L-selectin,


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**Table 3. Thin Layer Chromatographic Separation of Biogel P6 Fractions Obtained from Alkaline Borohydride-Treated [9-$^3$H]Sialyl-Labeled Glycoproteins**

| | alkaline borohydride released and separated by Biogel P6 fractions containing [9-$^3$H]sialyl label and their TLC components | | |
|---|---|---|---|
| [9-$^3$H]sialyl-labeled glycoprotein | A | B | C |
| HCG$\beta$ | one major band | one minor and one major bands distinct from A and C | one major band |
| MN glycophorin A | three distinct bands | one major band distinct from A | |
| GlyCAM-1 | two distinct bands | one band distinct from A | |
| fetuin | one distinct band | one distinct band | |
| human placental glycoprotein | one distinct band | one distinct band | one distinct band |
| bovine casein macroglycopeptide | | only one major band | |
| porcine Cowper's gland mucin | two distinct bands | two distinct bands different from A | |

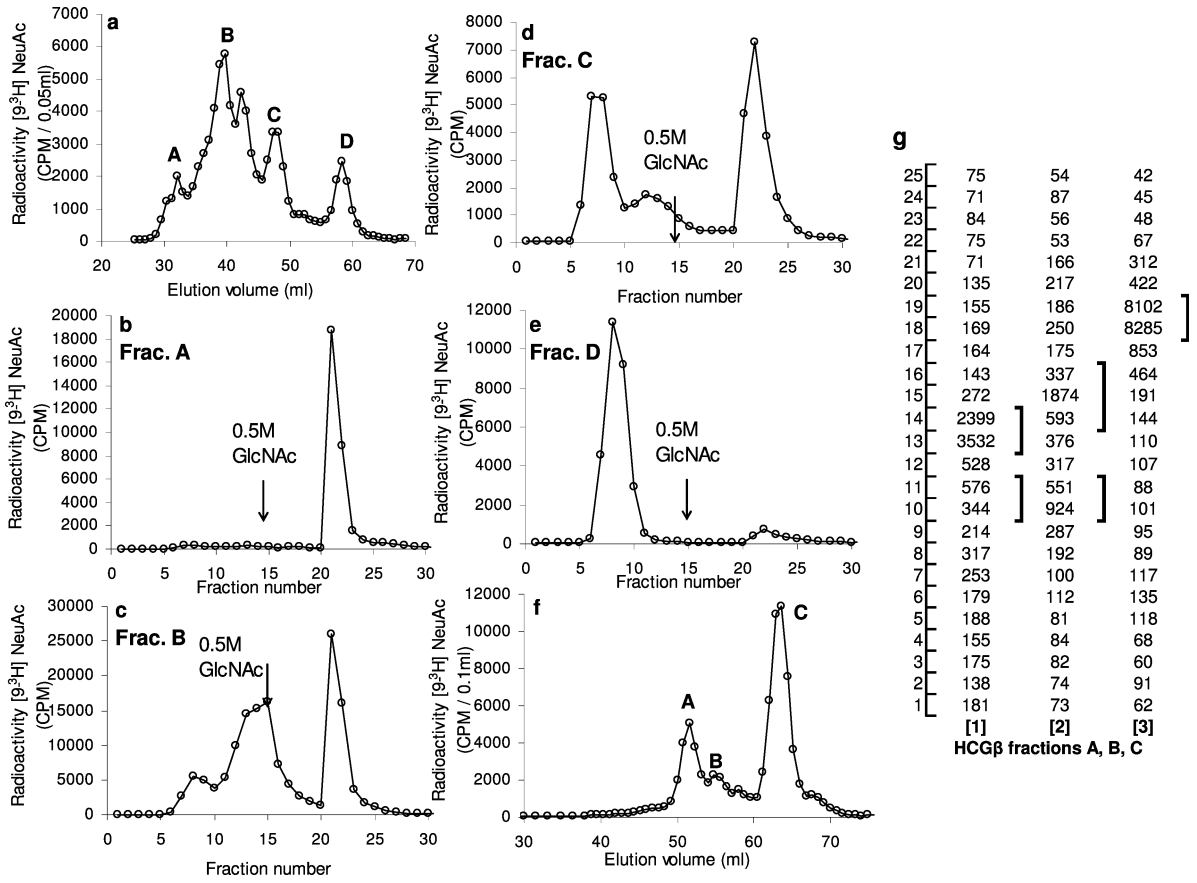


**Figure 5.** Analysis of HCG$\beta$. WGA-agarose affinity chromatography of the Biogel P6 fractions arising from Pronase digestion of [9-$^3$H]sialyl HCG$\beta$. (a) Biogel P6 fractionation of Pronase-digested [9-$^3$H]sialyl HCG$\beta$. WGA-agarose chromatography of (b) Pronase fraction A, (c) Pronase fraction B, (d) Pronase fraction C, and (e) Pronase fraction D. (f) Biogel P6 chromatography of [9-$^3$H]sialyl-labeled products arising from mild alkaline borohydride treatment of HCG$\beta$. (g) TLC analysis of fractions A–C from panel f.

contains 6′-sulfo and 6-sulfo LacNAc as the major disaccharides and NeuAc$\alpha$2,3(6-SO$_4$)Gal$\beta$1,4(Fuc$\alpha$1,3)GlcNAc as the major capping group. The specificities of $\alpha$1,3-fucosyltransferases FT-III, FT-IV, and FT-V[28] and $\alpha$2,3(N)sialyltransferase[10] indicate the possible existence of the structural unit NeuAc$\alpha$2,3Gal$\beta$1,4-(Fuc$\alpha$1,3)(6-SO$_4$)GlcNAc in GlyCAM-1.

Consistent with these findings, this study shows that an extensive Pronase digestion of [9-$^3$H]sialyl GlyCAM-1 gave rise to two major glycopeptide fractions, one being excluded and the other being included by the Biogel P6 column (Table 2). These two fractions contain mostly WGA binding and non-binding glycopeptides (Table 2). Alkaline borohydride treatment of [9-$^3$H]sialyl GlyCAM-1 led to two major Biogel P6 fractions (Table 3 and Figure 4B, c). TLC of these fractions indicated the presence of three distinct carbohydrate bands in GlyCAM-1 (Figure 7, lanes 3 and 4). Thus, GlyCAM-1 differs distinctively from other glycoproteins with respect to *O*-glycan chains.

**CD43.** In B-cell precursor (BCP) acute lymphoblastic leukemia, the major selectin ligand on BCP cells is CD43, which is a sialomucin.[29] CD43 was also identified as a ligand for E-selectin on CLA+ T cells[30] and in activated T cells.[31] In this study, [9-$^3$H]sialyl CD43 on alkaline borohydride treatment gave two Biogel P6 fractions similar to [9-$^3$H]sialyl MN glycophorin A (Figure 4B, d), indicating the presence of disialylated and monosialylated T-hapten structures.

**Bovine Casein Macroglycopeptide (MGP).** Pisano et al.[32] identified Thr121, Thr131, Thr133, Thr136, Thr142,

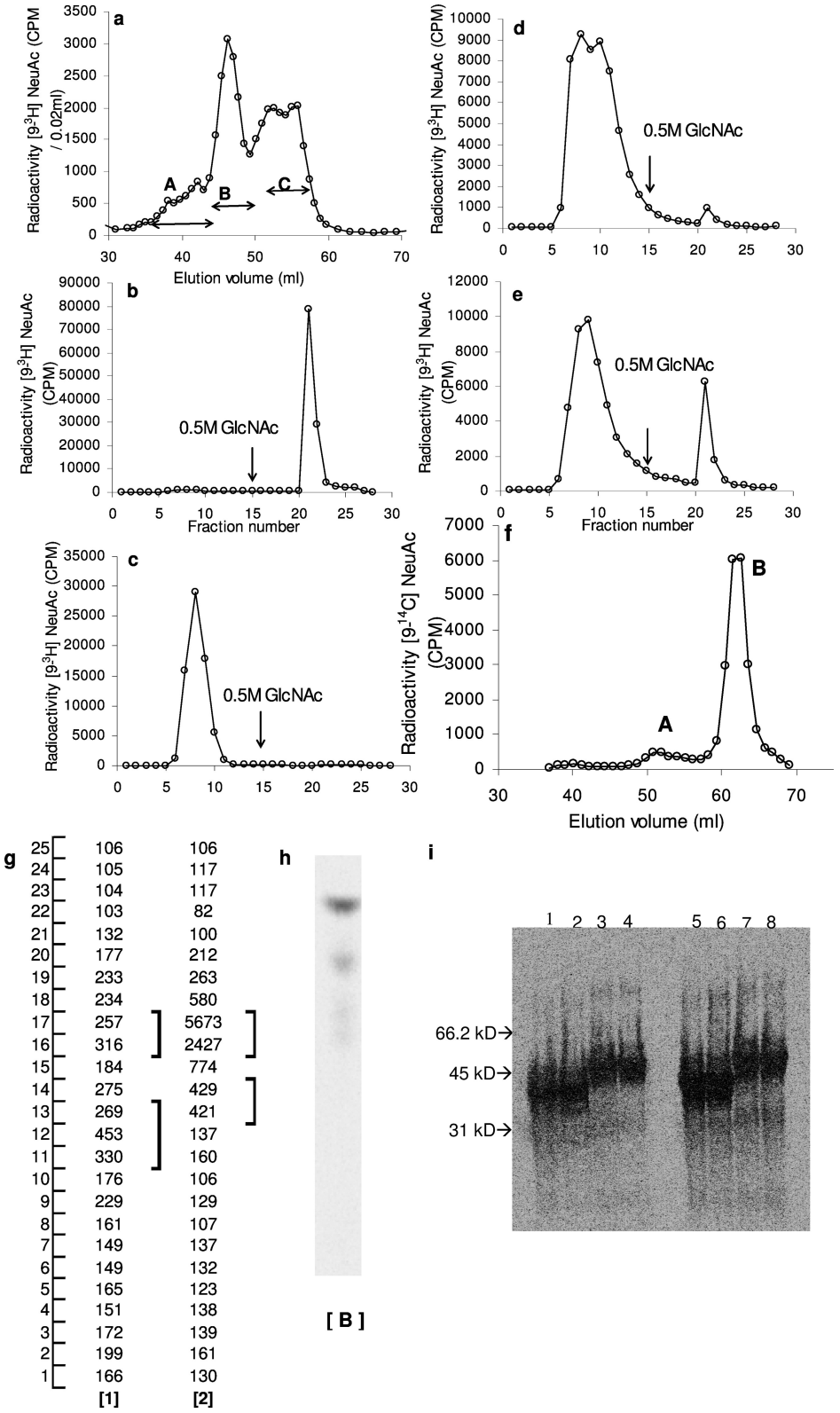


**Figure 6.** WGA-agarose chromatography of [9-$^3$H]sialyl fetuin and Biogel P6 fractions A–C arising from Pronase-digested [9-$^3$H]sialyl fetuin. (a) Biogel P6 fractionation of Pronase-digested [9-$^3$H]sialyl fetuin. WGA-agarose chromatography of (b) [9-$^3$H]sialyl fetuin, (c) Pronase fraction C, (d) Pronase fraction B, and (e) Pronase fraction A. (f) Biogel P6 elution of alkaline borohydride-treated [$^{14}$C]sialyl fetuin. (g) Thin layer chromatography of the Biogel P6 fractions isolated from alkaline borohydride treatment of [9-$^3$H]fetuin showing the presence of one major fraction with radioactivity. (h) TLC of the dominant fraction identified in panel g (fraction B from panel f) developed by autoradiography. (i) SDS–PAGE of [$^{14}$C]sialyl fetuin before and after PNGase F treatment. Ten micrograms ([$^{14}$C]sialyl fetuin) was applied to lanes 1–4 and 20 $\mu$g to lanes 5–8. Lanes 1, 2, 5, and 6 contained PNGase F-treated fetuin and lanes 3, 4, 7, and 8 fetuin that had not been treated with PNGase F.


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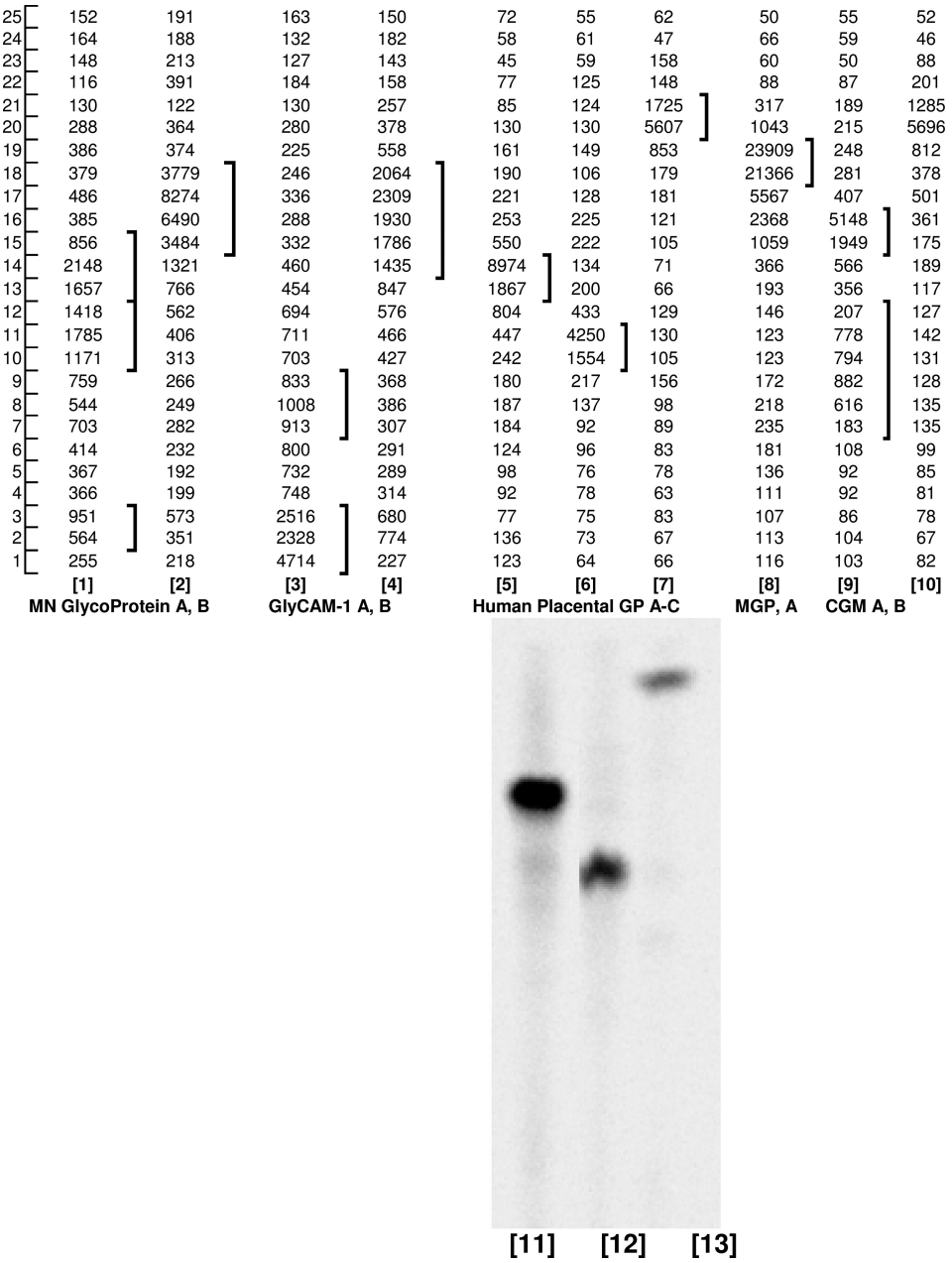


**Figure 7.** Thin layer chromatography of the Biogel P6 fractions isolated from alkaline borohydride treatment of [9-$^3$H]sialyl-labeled glycoproteins: lanes 1 and 2, MN glycophorin fractions A and B, respectively; lanes 3 and 4, GlyCAM-1 fractions A and B, respectively; lanes 5−7, human placental glycoprotein fractions A−C, respectively; lane 8, bovine casein MGP fraction; lanes 9 and 10, CGM fractions A and B, respectively; lanes 11−13, fractions A−C, respectively, prepared from [$^{14}$C]sialyl like human placental glycoproteins 5−7, except these were labeled using CMP-[$^{14}$C]NeuAc and the TLC images were developed by autoradiography.

and Thr165 as the six sites of O-glycosylation in the k-casein macroglycopeptide from bovine milk. No Ser residues are glycosylated. Moreno et al.[33] identified by the gas chromatographic method GalNAc, Gal, and NeuAc as constituents of the k-casein macroglycopeptide. In this study, Pronase digestion of [9-$^3$H]sialyl casein MGP gave rise to two distinct Biogel P6 fractions (Table 2). A clustering of Thr residues (Thr131, -133, -136, and -142) was reported by Pisano et al.[32] This would have caused noncleavage of this peptide portion by Pronase and resulted in two distinct Biogel P6 glycopeptide fractions, the major fraction being that with the large molecular size. Mild alkaline brohydride treatment resulted in one Biogel P6 fraction (Figure 4B, f), and TLC of this fraction showed a single component (Figure 7, lane 8) corresponding to the structure NeuAc$\alpha$2,3Gal$\beta$1,3GalNAc-ol. These results are consistent with the data reported by others as mentioned above.

**Human Placental Mucin Glycoproteins.** Rettig et al.[34] showed that human placenta expresses the human teratocarcinoma antigen K4, a sulfated and sialylated glycoprotein (160−200 kDa). Zimmer et al.[35] detected a 36 kDa O-glycosylated sialoglycoprotein in human placenta. Higuchi et al.[36] showed the expression of MUC 20 mRNA in human placenta. Human MUC 20 produced in MDCK as well as in HEK 293 cells indicated molecular masses of 76 and 79 kDa, respectively.[35] This labeling technique was able to reveal more details about

the mucin type *O*-glycan chains of human placental glycoproteins. It showed that [9-$^3$H]sialyl-labeled human placental mucin glycoprotein exhibited almost complete binding to WGA-agarose and gave rise to two major heterogeneous Biogel P6 fractions upon Pronase digestion (Table 2). Mild alkaline borohydride treatment resulted in three distinct Biogel P6 fractions (Figure 4B, e). When these fractions were subjected to TLC, one distinct component was present in each fraction (Figure 7, lanes 5−7 and 11−13). The TLC mobility of the larger component A was faster than that of the smaller component B, indicating the presence of a sulfate group in addition to a sialyl group in component A.[10] The third component, C, was identical to NeuAc$\alpha$2,3Gal$\beta$1,3GalNAc-ol.

**CGM.** Cowper's gland mucin (CGM) is a viscus epithelial glycoprotein (molecular mass of ~6 × 10$^6$ Da) and is the principal constituent of the seminal gel secretion by swine Cowper's gland. Its oligosaccharides linked to Ser/Thr [NeuAc$\alpha$2,3Gal$\beta$1,3(NeuAc$\alpha$2,6)GalNAc and NeuAc$\alpha$2,6GalNAc] are distributed all along the polypeptide chains.[37] As anticipated with high-molecular mass mucins containing clustered carbohydrate chains, Pronase-treated [9-$^3$H]sialyl CGM gave rise to a major glycopeptide fraction excluded by the Biogel P6 column. Mild alkaline borohydride treatment followed by Biogel P6 chromatography showed two fractions (Figure 4B, g). Thin layer chromatography showed that CGM contained two distinct major and two minor components (Figure 7, lanes 9 and 10).

**Haptoglobin and Apotransferrin.** Haptoglobin, known for many years to contain asparagine-linked carbohydrates, has been studied in various types of cancer and other diseases for its N-glycosylation status. Fucosylated haptoglobin in serum has been identified as a marker for pancreatic cancer.[38] Fujimura et al.[39] showed recently the presence of mono- and disialyl core 1 type O-linked glycans in haptoglobin of prostate cancer sera. In this study, human plasma haptoglobin (5 mg0 (Calbiochem) was incubated in 1.2 mL of 100 mM sodium cacodylate (pH 6.0) containing CMP-[$^{14}$C]NeuAc (12 $\mu$M) and ST3Gal-II (250 milliunits) for 24 h at 37 °C and then subjected to Biogel P6 column chromatography; it was found that 6.4% of [$^{14}$C]NeuAc was incorporated into haptoglobin. Pronase digestion of [$^{14}$C]sialyl haptoglobin followed by Biogel P6 column chromatography showed a major glycopeptide fraction (46.6% radioactivity) and four minor fractions (Figure 4A, f). Mild alkaline borohydride treatment of the major glycopeptide fractions followed by Biogel P6 column chromatography showed four distinct radioactive peaks containing 21.9, 14.7, 15.1, and 48.3% radioactivity (Figure 4B, h), the pattern apparently being similar to that of human placental mucin glycoproteins (Figure 4B, e).

$^{14}$C-labeled haptoglobin and $^{14}$C-labeled apotransferrin obtained from the sialylation reaction were subjected to PNGase F treatment. When this product was separated using SDS−PAGE followed by Coomassie blue staining and phosphorimaging analysis, it was found that apotransferrin was not sialylated (Figure 8a). This is reasonable because apotransferrin is known to contain only two N-glycosidic carbohydrate chains. The radiolabeled sialylated product of <45 kDa detected in this gel is likely a minor contaminant because this was not detected in the Coomassie-stained gel. Haptoglobin, on the other hand, was sialylated (Figure 8b). The change in molecular mass upon PNGaseF treatment is accompanied by a similar shift in both the Coomassie-stained gel and phosphorimaged membrane. Thus, the *O*-glycans in haptoglobin were specifically labeled by ST3Gal-II in this study.

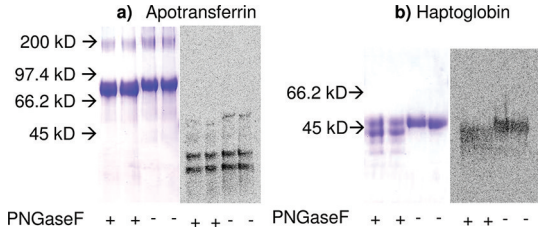


**Figure 8.** Autoradiography of apotransferrin and haptoglobin. Apotransferrin (a) and haptoglobin (b) were sialylated using the exchange reaction. These proteins were treated with PNGase F to remove *N*-glycans as indicated. Ten micrograms of each protein was then analyzed using SDS−PAGE, with Coomassie blue staining being used to visualize protein and phosphorimaging being applied to monitor [$^{14}$C]NeuAc incorporation. All samples were analyzed in duplicate.

## CONCLUSION

The identification and study of proteins bearing mucin-type O-linked glycans remains challenging both because of the complex nature of their biosynthesis of such structures and because of the lack of well-developed experimental tools.[40] While the Bertozzi laboratory has reported a strategy for labeling mucin-type O-linked glyoproteins with a bioorthogonal chemical tag that exploits the presence of a conserved core GalNAc residue in all *O*-glycans,[40] few other tools are available in this field. Such strategies are, however, necessary for the study of carbohydrate chains that are associated with cancer glycoprotein antigens. The structure of such glycans is tightly regulated primarily on the basis of the tissue-specific repertoire and activity of cellular glycosyltransferase genes, and also on the basis of the availability of sugar nucleotides and competition between enzymes for acceptor intermediates during glycan elongation. Among the glycosyltransferases, our previous studies showed that tumor tissues and cancer cell lines display dominant sialyltrasferase activity toward Gal$\beta$1,3GalNAc.[41,42] Further, $\alpha$1,2-fucosyltransferase and Gal:3-0-sulfotransferase acting on Gal$\beta$1,3GalNAc are not widely expressed by cancer cells.[41−43] Thus, we expect that the NeuAc$\alpha$2,3Gal$\beta$1,3GalNAc unit could be a dominant structure in cancer-associated cellular mucins. On the basis of this knowledge and exploitation of the exchange sialylation properties of mammalian sialyltransferase ST3Gal-II, we present here a novel strategy for labeling in vitro glycoproteins containing mucin-type *O*-glycans. This strategy may also be extended to some specific gangliosides that are involved in disease processes, including cancer. As shown in this paper, this method can be used to radiolabel a range of glycoproteins. Such labeling provides a natural tag for the various structural and functional methodologies that aim to characterize sialyl *O*-glycan-bearing glycoproteins.

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## ■ ABBREVIATIONS

Al, allyl; BSA, bovine serum albumin; CMP, cytidine 5′-monophosphate; GlyCAM, glycosylation-dependent cell adhesion molecule; GM, monosialo ganglioside; GD, disialo ganglioside; GT, trisialo ganglioside; NeuAc, *N*-acetylneuraminic acid (sialic acid); RM, reaction mixture; TLC, thin layer chromatography; WGA, wheat germ agglutinin.

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