Liver Membrane Proteome Glycosylation Changes in Mice Bearing an Extra-hepatic Tumor

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In this study, proteomic and glycomic analyses were used in combination to determine whether liver membrane protein glycosylation was affected in mice bearing the Engelbreth-Holm Swarm sarcoma. Peptide IPG-IEF and label-free quantitation determined that many enzymes involved in the protein glycosylation pathway specifically; mannosidases (Man1a-I, Man1b-I and Man2a-I), mannoside N-acetylgalcosaminyltransferases (Mgat-I and Mgat-II), galactosyltransferases (B3GalT-VII, B4GalT-I, B4GalT-III, C1GalT-I, C1GalT-II, and GaINT-I), and sialyltransferases (ST3Gal-I, ST6Gal-I, and ST6GalNac-VI) were up-regulated in all livers of tumor-bearing mice (n = 3) compared with nontumor bearing controls (n = 3). In addition, many cell surface lectins: Sialoadhesin-1 (Siglec-1), C-type lectin family 4f (Kupffer cell receptor), and Galactose-binding lectin 9 (Galectin-9) were determined to be up-regulated in the liver of tumor-bearing compared with control mice. Global glycan analysis identified seven N-glycans and two O-glycans that had changed on the liver membrane proteins derived from tumor-bearing mice. Interestingly, α (2,3) sialic acid was found to be up-regulated on the liver membrane of tumor-bearing mice, which reflected the increased expression of its associated sialyltransferase and lectin receptor (sialic-1). The overall increased sialylation on the liver membrane of Engelbreth-Holm Swarm bearing mice correlates with the increased expression of their associated glycosyltransferases and suggests that glycosylation of proteins in the liver plays a role in tumor-induced liver inflammation Molecular & Cellular Proteomics 10: 10.1074/mcp.M900538-MCP200, 1–18, 2011.

Despite membrane proteins constituting ~70% of all human protein based drug targets, studying membrane proteins is often hindered by their low abundance, large size, and relatively high hydrophobicity. Isolation and separation of membrane proteins by two-dimensional gel electrophoresis has been particularly problematic because of their poor solubility in various isoelectric focusing buffers. Alternative proteomic approaches such as shotgun proteomics have recently become the preferred method for identifying membrane proteins using high resolution separation of proteolytic peptides by liquid chromatography (4), capillary isoelectric focusing (5) or peptide immobilized pH gradient isoelectric focusing (peptide IPG-IEF) (6) in combination with reverse phase chromatography and tandem mass spectrometry.

We have recently demonstrated the use of broad and narrow range peptide IPG-IEF as an alternative method for shot-

1 The abbreviations used are: Peptide IPG-IEF, peptide immobilized pH gradient-isoelectric focusing; EHS, Engelbreth Holm Swarm; NSAF, normalised spectral abundance factor; Mgat-I, Mannoside N-acetylgalcosaminyltransferase 1; Mgat-II, Mannoside N-acetylgalcosaminyltransferase 2; Mgat-V, Mannoside N-acetylgalcosaminyltransferase 5; B3GalT-VII, Beta-1,3-galactosyltransferase 7; C1GalT-I, Beta1,3-galactosyltransferase 1; NeuAc, N-acetyl neuraminic acid; NeuGc, N-glycolyl neuraminic acid.
gun proteomics of rat liver membrane proteins (6). Peptide IPG-IEF has several advantages as a high-resolution shotgun proteomics technique most notably because of its high sample loading capacity. It has previously been demonstrated to provide more confident and reproducible protein identifications compared with 1D SDS-PAGE (7) and/or strong cation exchange (8) by using the theoretical pI of peptides as an additional filtering criteria to reduce false discovery rates (6). In addition, peptide IPG-IEF followed by reverse phase liquid chromatography tandem MS (RP-MS/MS) can be used for comparative label-free quantitative proteomics by using a protein’s calculated normalized spectral abundance factor (NSAF) (9–12).

Shotgun proteomics has demonstrated its use in identifying membrane proteins; however information on post-translational modifications is not obtained. Membrane proteins can be classified as peripheral, integral, and/or lipid-anchored and are often glycosylated. The extracellular surface of the plasma membrane is often decorated with glycans, glycolipids, and glycoproteins, which mediate many cellular events such as cell signaling, ion transport, and cell recognition and adhesion. The carbohydrates attached to the proteins are covalently linked to the amide nitrogen of asparagine residues (i.e. N-linked) via the consensus sequence Asn-X-Ser/Thr, where X can be any amino acid except proline or to the hydroxyl groups of serine or threonine (i.e. O-linked).

Analogous to genomics and proteomics, which are the studies of a whole set of genes and proteins respectively, glycomics is the study of the whole set of glycans produced in a cell, tissue, or organism at a particular time in health and disease. Glycans can act as markers to characterize cell types and states and they are also involved in numerous biological functions and processes such as modulating structure and function of proteins, fertilization, cell division, anticoagulation, and inflammatory responses (13–15). Glycans are composed of monosaccharides that are covalently linked by glycosidic bonds, either in α or β conformations. However, unlike proteins, which are linked in a linear structure, oligosaccharides often display diverse microheterogeneity as they are often branched-like structures formed by the different linkages of one or more monosaccharides.

The hydrophilicity of glycans can affect the physicochemical properties of glycoproteins thus influencing the folding, solubility, subcellular distribution, and stability of the mature protein. The heterogeneity of glycans and lack of a template make it difficult to predict the type of glycans that are produced and their specific site(s) of attachment to proteins. How a protein is glycosylated can determine the localization, activity, and function of the glycoprotein, therefore glycosidases and glycosyltransferases play crucial roles in catalyzing and maintaining the correct glycosylation of proteins. Many glycan-specific diseases such as congenital disorders of glycosylation, are caused by defects in the protein glycosylation machinery (16). For example, diseases such as cancer and autoimmunity have been reported to possess altered glycosylation as a consequence of defective or altered glycosyltransferases (2, 17, 18).

Glycosylation is known to play a role in cancer carcinogenesis and metastasis in many common cancers, and it has been shown to affect the function of many membrane proteins such as CD44 (19), matrix metalloproteinases (MMPs) (20–22), selectins (23) and cadherins (24) on cancer cells. Glycosyltransferases are a group of enzymes that catalyze the addition of monosaccharides to core proteins. Most glycoproteins are either secreted into the extracellular environment or attached to the plasma membrane of the cancer cell; therefore changes to glycosyltransferases can ultimately influence the overall adhesive properties (2). Reduced levels of sialyltransferases and increased expression of fucosyltransferases, which are known to occur during cancer carcinogenesis and metastasis, extensively modify carbohydrate moieties on the cell surface (25). The degree of sialylation on the cell surface of a cancer cell acts as a confident marker of the aggressiveness of some tumors (26, 27). In addition, Sialyl Lewis X (SLex) antigens, which are sialylated lacto-N-fucopentaose carbohydrate moieties, are commonly found on cell surface glycoproteins of metastatic tumor cells (28). The degree of sialylation of these antigens can affect the polar orientation and adhesion of tumor cells during cancer metastasis (28).

The clinical relevance of inflammation both in contributing to the development of cancer and in determining prognosis is well established (29, 30). Our interest in tumor-associated inflammation has arisen as a result of our clinical findings that cancer patients with evidence of an acute phase inflammatory response in plasma (elevated C-reactive protein and alpha-1 acid glycoprotein) exhibit reduced clearance of heptatically metabolized cytotoxic drugs resulting in increased toxicity. The murine studies described in this paper have been designed to explain these clinical findings by providing insights into the pathogenesis of the tumor-induced inflammatory effects.

Inflammation is a symptom of a disease or infection that regulates the expression of specific proteins that mediate innate and adaptive immune responses to promote healing of the affected site. Previous studies using lectin and immunoblot analyses have shown that turpentine oil-induced inflammation in mice leads to changes in the sialylation patterns of serum glycoproteins (31). Reverse transcription–polymerase chain reaction (RT-PCR) analysis of the expression profiles of mRNA for the known sialyltransferases involved in the synthesis of these sugars identified increased levels of β-galactoside α (2,3)-sialyltransferases (ST3Gal-I and ST3Gal-III), β-N-acetylgalactosaminide α (2,6)-sialyltransferase (ST6GalNAc-V), and β-galactoside α (2,6)-sialyltransfer (ST6Gal-I) (31). These sialyltransferases are mostly involved in the addition of α (2,3) and α (2,6) sialic acids onto N- and O-glycans. Most plasma proteins are derived from the liver and significant
increases in serum acute phase glycoproteins and their sialylation have generally been observed with most rodent experimental inflammation models (31–33). Previous studies of mice bearing the Engelbreth-Holm Swarm (EHS) sarcoma have demonstrated that increased levels of pro-inflammatory cytokines can initiate a hepatic acute phase response and cause reduced hepatic gene expression of cytochrome enzymes (34, 35), particularly hepatic cytochrome P450 3A4 (CYP3A4). In cancer patients, reduced hepatic CYP3A4 function in response to tumor-associated liver inflammation has been associated with reduced drug metabolism and increased drug toxicity (36). The impact of the EHS tumor and its associated inflammatory response on the liver membrane proteins has recently been reported (30, 36), however little is known about whether tumor-associated liver inflammation can influence membrane protein glycosylation.

In the present study, peptide IPG-IEF and label-free quantitation were used to identify and determine the relative abundance of glycosyltransferases and cell surface lectins on the liver membrane of mice bearing a distal benign tumor. Triton X-114 phase partitioning (37) was employed to further enrich membrane proteins for global glycans profiling to determine whether the changes in glycosyltransferases identified by peptide IPG-IEF reflected the structures of the glycans presented on the membrane. The combination of proteomics and glycomics approaches could help determine potentially important biological changes resulting from the EHS tumor on liver cellular membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tris, sodium chloride, sodium hydroxide, sodium carbonate, EDTA, Bradford reagent, HEPES, phospho-buферased saline tablets, ammonium bicarbonate (NH4HCO3), ammonium acetate (CH3COONH4), formic acid, Triton X-114, dithiothreitol, iodoacetamide, LC grade acetonitrile (ACN) and sodium borohydride (NaBH4) conducted on three biological replicates of individual control and logical reproducibility, all proteomic and glycomic experiments were total body weight over 18 to 21 days. Tumor and control mice were injected with 0.3 ml suspension of EHS sarcoma cells (containing 106 cells) in 0.9% (w/v) phosphate-buffered saline into the quadriceps of the right hind leg. The EHS tumor and its associated inflammatory response on the liver membrane proteins has recently been reported (30, 36), however little is known about whether tumor-associated liver inflammation can influence membrane protein glycosylation.

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topped up with 10 μl water to reduce loss by evaporation and incubated overnight at 37 °C. The sample wells were surrounded by wells containing water to further prevent evaporation, and the plate was sealed tightly with parafilm.

To recover N-linked oligosaccharides, the plate was sonicated in a water bath for 20 min and supernatants from each well containing released N-linked oligosaccharides were collected. Spots were washed twice with 50 μl water, and combined with the supernatant and were reduced to aldithols before LC separation. Before reduction the released N-linked oligosaccharides were incubated with 100 mM CH3COONH4 pH 5 (final concentration 15 mM) for 1 h at room temperature to ensure complete regeneration of the reducing terminus and were reduced with 1 mM NaBH4/50 mM KOH (20 μl) at 50 °C for 2 h. The reduction was quenched with 5 μl glacial acetic acid.

For release of N-linked oligosaccharides from SDS-PAGE separated proteins, gel pieces were equilibrated and dehydrated. Each gel piece was treated with 5 μl PNGase F 0.5 U/μl by passive diffusion, topped up with 20 μl water and incubated at 37 °C overnight. N-linked oligosaccharides were recovered by washing gel pieces with 3×20 μl water and sonicated. Released N-linked oligosaccharides were reduced as above and purified as described below.

Reductive β-elimination of O-linked oligosaccharides was carried out on the same samples after PNGase F release of the N-linked oligosaccharides by re-wetting the protein spots with 2 μl methanol, and incubating overnight with 0.5 M NaBH4/50 mM KOH (20 μl) at 50 °C. The reaction was quenched with 2 μl acetic acid and both reduced N- and O-linked oligosaccharides were purified as described below.

Purification of Reduced N- and O-linked Oligosaccharides—Homemade cation exchange columns comprising 20 μl of AG50W-X8 cation-exchange resin (H+ form) (BioRad, Hercules, CA) were packed on top of μC18 ZipTips (40). Columns were prepared by washing 3×50 μl 1 M HCl, followed by 3×50 μl methanol and 3×50 μl water. The reduced N- and O-linked oligosaccharide aldithols were applied to the column and eluted with 2×60 μl of water and dried. Borate was removed by the addition of 100 μl methanol and drying under vacuum five times. The oligosaccharides were resuspended in 10 μl of water for porous graphitized carbon LC-electrospray ionization (ESI)-MS/MS separation and analysis.

Neuraminidase Digestion—To determine sialic acid linkages, purified N-linked oligosaccharides were digested in either 3 μl α (2,3) neuraminidase (Takara Bio, Japan) or 1 μl α (2,3,6) neuraminidase (Northstar Bioproducts, MA) in 100 mM NH4HCO3, pH 8 at 37 °C for 10 min and overnight respectively. Neuraminidase activity was terminated by heating at 99 °C for 3 min before carbon LC-ESI-MS/MS re-analyses of the desialylated oligosaccharides.

SDS-PAGE—Approximately 10 μg of protein was applied per lane onto NuPAGE 10% Bis-Tris precast gradient gels with 3-(N-morpholino)propane sulfonic acid running buffer. Electrophoresis conditions were set to 200 V, 125 mA for 60 min. The gels were fixed in 7% (v/v) acetic acid and 10% (v/v) methanol for 30 min and stained overnight with SYPRO Ruby (Invitrogen, San Diego, CA). Gel images were obtained using the Typhoon Trio Variable Mode Imager (GE Healthcare, Uppsala, Sweden). Lanes were cut into six equal fractions and equilibrated with 100 mM NH4HCO3 pH 7.8 for 10 min. Gel pieces were dehydrated in 50 mM NH4HCO3/50% (v/v) ACN for 20 min with intermittent vortexing followed by 100% (v/v) ACN for 20 min. Dehydrated gel pieces were reduced with 25 mM dithiothreitol at 37 °C for 30 min and alkylated with 55 mM iodoacetamide in the dark at room temperature for 45 min.

Peptide IPG-IEF Separation—Trypsin-digested proteins (1 mg) in 8 M urea were used to passively rehydrate linear pH 3–10 (in which the actual pH range of the IPG strip is 3.85–9.36, information available at www.gehealthcare.com) 18 cm IPG strips (broad range) for 6 h at room temperature in triplicate. Isoelectric focusing was conducted on an IPGPhoril (GE Healthcare) with a current limit of 50 μA per strip for the BR IPG strip at 20 °C with the following focusing program: 300 V for 1 h, a gradient to 1000 V for 1 h, a gradient to 4000 V for 3 h, a gradient to 8000 V for 3 h, and 8000 V until 100 kVh was reached (6, 9). The strips were then cut (with plastic backing still in place) with a scalpel blade into 24 equal length pieces. Peptides were extracted from each fraction by incubation in 100 μl of 0.1% (v/v) formic acid for 1 h at room temperature. The extraction was repeated twice and subsequently combined with the initial fractions. Combined peptide extracts were desalted using C18 tips (Omix, Varian, Inc., CA) and the eluate was dried using a vacuum centrifuge followed by resuspension in 0.1% (v/v) formic acid.

RP LC-ESI-MS/MS—Each of the 24 IPG-IEF separated peptide fractions (from a 1 mg trypsin digest) from triplicate experiments were analyzed by nanoLC-MS/MS using a LTQ-XL ion-trap mass spectrometer (Thermo). Reversed phase columns were packed in-house to ~7 cm (100 μm i.d.) using 100 Å, 5 μm Zorbax C18 resin (Agilent Technologies, Santa Clara, CA) in a fused silica capillary with an integrated electrospray tip. A 1.8 kV electrospray voltage was applied via a liquid junction upstream of the C18 column. Samples were injected onto the C18 column using a Surveyor autosampler (Thermo). Each sample was loaded onto the C18 column followed by initial wash step with Buffer A (5% (v/v) ACN, 0.1% (v/v) formic acid) for 10 min at 1000 nL/min. Peptides were subsequently eluted from the C18 column with 0–50% Buffer B (95% (v/v) ACN, 0.1% (v/v) formic acid) over 58 min at 500 nL/min followed by 50–95% Buffer B over 5 min at 500 nL/min. The column eluate was directed into a nanospray ionization source of the mass spectrometer and spectra were scanned over the range 400–1500 amu. Automated peak recognition, dynamic exclusion, and tandem MS of the top six most intense precursor ions at 35% normalized collision energy were performed using the Xcalibur software (Thermo).

Carbon LC-ESI-MS/MS—N- and O-linked oligosaccharide alditols released from ~10 μg of Triton X-114 phase partitioned membrane proteins were analyzed by capillary LC-MS/MS using a High Capacity Trap Ultra ETD II ion-trap mass spectrometer (Bruker Biosciences Corporation, Billerica, MA) and a capillary LC-MS/MS using a Agilent 6330 ion-trap mass spectrometer (Agilent Technologies). The samples were applied to a Hypercarb porous graphitized carbon high performance liquid chromatography (HPLC) column (5 μm Hypercarb, 0.32 × 150 mm, Thermo Hyprersil, Runcorn, UK). N-linked oligosaccharides were separated using a linear gradient with 0–45% (v/v) ACN/10 mM NH4HCO3 for 83 min, followed by a 10 min wash with 90% (v/v) ACN/10 mM NH4HCO3 at a flow rate of 5 μL/min. O-linked oligosaccharides were separated using a linear gradient with 0–90% (v/v) ACN/10 mM NH4HCO3 for 45 min, followed by a 10 min wash with 90% (v/v) ACN/10 mM NH4HCO3 at a flow rate of 5 μL/min. ESI-MS was performed in negative ion mode with three scan events: full scan with mass range m/z 100–2500 amu, dependent zoom scan, and dependent MS/MS scan after collision induced fragmentation. Automated peak recognition, dynamic exclusion, and tandem MS of the top three most intense precursor ions at 40% normalized collision energy was performed with an activation time of 30 ms. The composition of glycanics was determined using the monoisotopic masses of detected ions and was verified manually by their associated MS/MS. Monoisotopic masses were searched using the GlycoMod software (available at http://www.expasy.ch/tools/glycomod) with parameters of ±0.7 Da to predict the possible glycan compositions corresponding to these masses. Ratios of glycanics were determined by calculating the peak area of the extracted ion chromatogram (EIC) corresponding to the glycanics of interest, and glycan differences were considered significant if observed in all three biological replicates.
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**RESULTS**

We have applied peptide IPG-IEF separation and RPLC-ESI-MS/MS to identify mouse liver membrane proteins from control (n=3) and tumor-bearing mice (n=3) and quantitated the relative abundance by calculating the NSAF (9–12) of all proteins whose gene ontology mapping suggests involvement with the glycosylation machinery. Mouse liver membrane proteins were further enriched by Triton X-114 phase partitioning and the detergent phase subjected to PNgase F digestion and reductive β-elimination to release both N- and O-linked oligosaccharides respectively for analysis by carbon LC-ESI-MS/MS. In this study we have defined membrane proteins as comprising of integral membrane proteins, lipid-anchored proteins and membrane-associated proteins as determined by Transmembrane Hidden Markov Model together with information derived from UniProt, Gene Ontology (GO) annotation, and the Swiss-Prot databases.

**Label-free Quantitation Using Peptide IPG-IEF Separation**—Mouse liver membranes were prepared by differential ultracentrifugation using a modified sodium carbonate stripping method (38). The membrane pellet was pulse sonicated and digested with trypsin in the presence of 60% (v/v) methanol as this solvent has been previously shown to improve membrane protein coverage (6). In our previous study in which we characterized the mouse liver membrane proteome by this approach, we identified 1625 proteins of which 236 were determined to be differentially expressed proteins involved in metabolic pathways such as liver metabolism, solute carrier transport, and drug detoxification.²

In this study, we have identified 33 membrane proteins involved in glycosylation of which 15 were found to be significantly and differentially expressed (i.e. fold change greater than 1.3 and p ≤ 0.05) in the livers of all tumor-bearing mice (Fig. 1, supplemental Table S1 and supplemental Fig. S1). These include mannosidases (Man1a-I, Man1b-I, and Man2a-I), which were found to be up-regulated (1.38-, 5.98-, and 1.86-fold respectively); and mannoside N-acetylglucosaminyltransferases (Mgat-I, Mgat-II, and Mgat-V) which were also determined to be up-regulated (4.22, 2.33, and 16.31 fold respectively) in the liver of tumor-bearing mice. Mannosidases are often found in the endoplasmic reticulum and cytoplasm and are mostly involved in the hydrolysis and removal of mannose monosaccharides from glycans that are attached to lipids and proteins. They are also involved in the synthesis of complex glycoconjugates by the degradation of immature glycoproteins that permit further modification by other glycosyltransferases. Mannoside N-acetylglucosaminyltransferase


**Protein Identification and Data Analysis**—Spectra files were converted to mzXML format and processed through the global proteome machine software (GPM) (version 2.1.1), an open source protein identification system that uses the X!Tandem algorithm (41). For each experiment, 24 fractions were processed sequentially with output files for each individual fraction and merged, nonredundant output file generated for protein identifications with Log(e) values less than −1. Peptide identification was determined using a parent ion mass error of ±3 Da and −0.5 Da and fragment ion tolerance of 0.4 Da (41). Mass spectra were searched based on peptide trypticity with up to three missed cleavages. Carbamidomethyl was considered as a complete modification and partial modifications were also considered, including oxidation of methionine and threonine, and deamination of asparagine and glutamine. MS/MS spectra were searched against the Mus musculus database (Database derived from SwissProt, Ensemble, and NCBI, released 01/06/2006) and reverse database searching was used for estimating false discovery rates (42). The actual number of proteins in the database that were actually searched was 64,684.

Mass spectra for single peptide identifications are provided as supplementary information. Peptide identification was validated using peptide pl filtering as described previously (6). Briefly, pl was calculated through an open source pl calculator, Compute pl from ExPASy (http://au.expasy.org/tools/pi_tool.html), which calculates peptide pl from amino acid values (43). Peptide pl filtering was conducted on the data obtained from each of the fractions by removal of statistical outliers that were defined as peptides with pl values that fall outside the pl boundaries, determined using ±0.5 of the standard deviation (calculated over the entire fraction) from the median pl value of the fraction. For analysis of total protein identifications, proteins were further validated using a 1% false discovery rate by searching the MS spectra against a reverse database and applying the following formula: FDR = reverse/(reverse + forward) × 100.

The assignment of an identified protein as being located in the membrane was made using a combination of prediction using Transmembrane Hidden Markov Model (TMHMM) (available at http://www.cbs.dtu.dk/services/TMHMM) (44) together with information on membrane location derived from UniProt, GO annotation and the Swiss-Prot database. In this study we have defined membrane proteins as comprising of integral membrane proteins, lipid-anchored proteins, and membrane-associated proteins.

**Calculation of Peptide Normalized Spectral Abundance Factors (NSAF)—** NSAF were calculated according to Zybailov et al., (12) using the following formula: NSAF = (Spc/L) / √(Spc/L), where Spc refers to spectral count (number of nonredundant peptide identifications for a given protein), L is the length of the protein. Protein identifications were only included in NSAF data analysis if a given protein was identified in each of the triplicates. The reduced protein list was then adjusted with a fraction of a spectral count (in these data sets a factor of 0.18 was used) to allow the incorporation of proteins with zero spectral counts for statistical analysis (9, 12). The protein and peptide identifications used to generate this protein data set are available in supplemental Table S1. An optimal adjustment factor was determined through fitting the natural log of the NSAF values to a Gaussian curve, assessed by computing both R² values and applying a Shapiro-Wilk test. To satisfy the Shapiro-Wilk test, a W value > 0.05 was required. Statistical analysis was conducted with Microsoft Excel using the Student t test with equal sample sizes and p ≤ 0.05 for assignment of statistical confidence. Origin8 software package was used for assessing Gaussian distribution of data for label-free quantitation by computing both R² values and a Shapiro-Wilk test. The rigorous statistical approach used in the experiments (through the use of isoelectric point filtering, a reduced protein list, Shapiro-Wilk analysis, and Student t test) was designed to address concerns of analytical reproducibility.
ses (Mgats) are involved in the catalysis of GlcNAc from uridine diphospho-GlcNAc (UDP-GlcNAc) to terminal branched /H9251-Man residues on glycolipids and glycoproteins in both the endoplasmic reticulum and Golgi apparatus.

The six galactosyltransferases (B3GalT-VII, B4GalT-I, B4GalT-III, C1GalT-I, C1GalT-II and GalNT-I) were all found to be up-regulated in the liver of tumor-bearing mice (Fig. 1) with a range between 6- and 11-fold change. Galactosyltransferases and sialyltransferases are usually localized to the Golgi apparatus membrane and are involved in N- and O-glycan biosynthesis. Galactosyltransferases are responsible for catalyzing the transfer of galactose from UDP-galactose to N- and O-linked glycans via β(1–4), β(1–3), α(1–3), and α(1–4) linkages and are also involved in lactose biosynthesis. Sialyltransferases (ST3Gal-I, ST6Gal-I, and ST6GalNAc-VI) were also found to be up-regulated (5.45-, 5.25-, and 11.67-fold respectively) in the liver of tumor-bearing mice. These enzymes are responsible for catalyzing the addition of α(2,3) and α(2,6) neuraminic acid to the nonreducing termini of glycolipids and of N- and O-linked glycans on glycoproteins. Other glycan-processing enzymes such as alpha(1–3)-mannosyltransferases (Alg2, Alg3), ST3Gal-III, ST3Gal-IV, and GalNT-II were determined to have no significant change in abundance (Fig. 1, supplemental Table S1). Collectively, the up-regulation of these enzymes suggests an overall increase in activity of the glycosylation machinery in the liver of tumor-bearing mice.

We identified siglec-1 up-regulated (~43-fold) in liver membrane proteins from tumor-bearing mice in this study compared with the controls (Fig. 2). Other lectins including calcium dependent-type (C-type), lectin 4f (Clec4f) and galactose-binding lectin 9 (Lgals9) were also up-regulated in tumor-bearing mice (Fig. 2). Clec4f has been reported to mediate pathogen recognition and cell-cell interactions using structurally related Ca²⁺-dependent carbohydrate-recognition domains on macrophages and natural killer cells in the immune system (45). Lgals9 is known to promote anti-inflammatory responses in the innate and adaptive immune system when stimulated by pro-inflammatory cytokines (46).
sion of glycosylation-related proteins was converted to observable enzymatic activity reflected in altered substrate glycosylation status. N-linked oligosaccharides were released from the membrane glycoproteins by PNGase F and analyzed using graphitized carbon LC-ESI-MS in negative ion mode.

The combined mass spectra of the global liver membrane N-linked oligosaccharides attached from control and tumor-bearing mice are shown in Fig. 3 and the N-glycan differences observed in the liver membrane proteins of tumor-bearing mice are shown in Table I. Ions corresponding to N-glycans were found as doubly charged [M-2H]2− ions in the combined mass spectra. Several masses assigned to N-glycan compositions were seen in the liver membranes of tumor-bearing mice and not in the control. Both control and tumor-bearing mouse liver membrane proteins expressed high mannose structures (for example [M-2H]2− of m/z 860.3 and 941.3) and sialylated complex type N-glycan structures (for example [M-2H]2− of m/z 1127.4). Specifically, six N-glycan structures were found to be present only on liver membrane proteins from EHS tumor-bearing mice ([M-2H]2− of m/z 965.9, 1022.4, 1038.9, 1111.4, 1184.4, and 1280.9). Of these, four are complex N-glycans terminating with N-acetyl neuraminic acid (NeuAc), with two containing a fucosylated core. Two low abundance ions corresponding to N-glycan masses of m/z (1022.4)2− and (1038.9)2− appeared to be present in higher amounts in the tumor-bearing mice in the average MS taken over the chromatography retention time of 20–65 min (Fig. 3A and 3B). We have substantiated the tumor-specific difference in the abundance of these ions by using a combined EIC of m/z 941.3, 1022.4, and 1038.9 (± 0.5 Da) to detect the specific abundance of each relative to nearby peaks (Fig. 3C and 3D). Average MS of the narrower retention time (21–28 min) of where the m/z ion (1022.4) elutes confirms that this mass ion is present in the tumor-bearing mice but is not detectable in the normal mice. The masses corresponding to the assigned glycan structures have been previously identified in the mouse by the references indicated in Table I. Assigned glycan structures and MS/MS fragmentation have been included as supplementary information (supplemental Table S2 and supplemental Figure S2).

The expression of di-N-glycoly neuraminic acid (NeuGc) biantennary N-glycan structure ([M-2H]2− of m/z 1127.4), which was verified by MS/MS fragmentation (Fig. 4C) was observed in both control and tumor-bearing mice. This mass was seen to exist as two isomers in the EIC at 30 min and at 41 min (Fig. 4A and 4B). Based on the EI width and peak area, the control mice (Fig. 4A) expressed a different ratio of these two isomers (9:1) (isomer 1:2, n = 3) whereas the tumor-bearing mouse had a ratio of 2.3:1 (isomer 1:2, n = 3).

The presence of two isobaric N-linked structures at ([M-2H]2− of m/z 1127.4) suggests that the isomers may have different linkages to the two terminal NeuGc. The N-linked oligosaccharides from membrane proteins of tumor-bearing mice were digested with α(2,3) neuraminidase to determine the specific linkages of the NeuGc attached specifically to the nonreducing termini of the biantennary N-glycan. This enzyme has been shown to remove NeuGc as well as NeuAc (47). The first isomer eluted at 54 min was not affected by digestion and thus must consist of two NeuGcαβ(2,6)Galβ1 moieties attached to the nonreducing termini (Fig. 5B). The isomer observed at 62 min (Fig. 5A) was no longer present after digestion, implying that this isomer consisted of two α(2,3) linked NeuGc moieties attached to the nonreducing termini. Interestingly, after α(2,3) neuraminidase digestion two additional peaks corresponding to the same mass ([M-2H]2− of m/z 1127.4) appeared, eluting at the retention times different to those above relative to the NeuGcαβ(2,6)Galβ1. That is, α(2,3) linked NeuGc had been removed from a precursor oligosaccharide producing two different biantennary di-sialylated glycans different from the double α(2,6) NeuGc terminated structure eluting at 54 min. Therefore, this suggested that in the original sample, a tri-sialylated biantennary N-glycan consisting of three NeuGc may also be present. A peak of [M-2H]2− of m/z 1280.9 was present at 55 and 60 mins, which would correspond to a composition of (NeuGc)3−(Hex)2(HexNAc)2 + (Man)3(HexNAc)2. The structural isomers after treatment with neuraminidase, corresponding to m/z (1127.4)2− observed in Fig. 4 and in the EIC of m/z (1127.4)2− in Figs. 5 and 6 appear to differ in elution times. These samples had been analyzed by carbon chromatography on two different mass spectrometric systems; Agilent HCT and the Bruker Ultra ETD II ion-trap mass spectrometers, and resulted in different retention times. The consistent identity of these isomers was confirmed by their having the same MS/MS fragmentation pattern of the mass ion m/z (1127.4)2− and the same elution position as that of standard glycans chromatographed on the same instruments.

Identification of a Biantennary N-glycan with Three N-glycoly Neuraminic Acids—To determine the structures of the tri-sialylated N-glycan, the N-linked oligosaccharides were digested with α(2,3,6) neuraminidase to remove both terminal NeuGcαβ(2,3)Galβ1 and NeuGcαβ(2,6)Galβ1 linkages. After 10 min digestion all di-sialylated and tri-sialylated masses (m/z of (1127.4)2− and (1280.9)2−) had disappeared with only nonsialylated and mono-sialylated structures remaining (Fig. 6B). After further overnight digestion with α(2,3,6) neuraminidase there remained evidence of a single resistant NeuGc residue with m/z of (973.8)2− corresponding to a mono-sialylated biantennary N-glycan. MS/MS fragmentation of the remaining mono-sialylated glycan mass (m/z of (973.8)2−) showed a diagnostic ion (c2, m/z ion (509.1)−) which corresponded to the mass of a NeuGc attached to GlcNAc suggesting possible existence of the two structures depicted in Fig. 6D. It is of course possible that the α(2,3,6) sialidase digestion could have been incomplete and/or that some of the three NeuGc residues were resistant to cleavage. In particular, it is possible that the unusual NeuGc-GlcNAc linkage may not be cleaved.
Fig. 3. Combined LC-ESI-MS (retention time 20–65 min) of globally released N-linked oligosaccharides from mouse liver membrane proteins from control (A) and tumor-bearing mice (B). Combined extracted ion chromatogram (EIC) of m/z (941.3)$^{2-}$, (1022.4)$^{2-}$, and (1038.9)$^{2-}$ and average MS (retention time 21–28 min) of control (C) and tumor-bearing mice (D). Ions corresponding to m/z (1022.4)$^{2-}$ and (1038.9)$^{2-}$ were observed in low abundance in the average MS spectra in both control (A) and tumor-bearing (B) mice but were clearly observable by their combined EIC (C and D). Averaging MS spectra of the narrower retention time (21–28 min) reveals the presence of m/z (1022.4)$^{2-}$. Some major ions corresponding to N-glycans have been assigned possible structures (supplemental Table S2). *Representative [M-2H]$^{2-}$ ions correspond to identified structures found only in tumor-bearing mice (Table I).
by this enzyme so that the exact linkages of this tri-sialylated biantennary structure cannot be unequivocally assigned. Interestingly, liver membrane proteins from EHS tumor-bearing mice expressed this tri-sialylated glycan structure ([M-2H]2^- of m/z 1280.9), which the control mice did not express. This particular N-glycan has recently been identified in the mouse liver (48).

Analysis of O-linked Oligosaccharides—EIC of the O-linked oligosaccharides released by β-elimination from liver membranes of control and tumor-bearing mice show distinct differences. The combined EIC of m/z (675.3)^1-, (691.3)^1-, (966.2)^1-, (982.3)^1-, (998.4)^1-, and (681.3)^2- (Fig. 7A and 7B) monitors the masses corresponding to O-glycans previously identified in the mouse (49–51). These ions were mostly singly charged ions [M-H]^-1. Liver membranes from both control and tumor-bearing mice mostly expressed similar compositions containing both NeuAc and NeuGc ([M-H]^-1 of m/z 675.3 ([Hex],(HexNAc),(NeuAc)_1), 691.3 ([Hex],(HexNAc)_1- (NeuGc)_1), 966.2 ([Hex],(HexNAc),(NeuAc)_2) and 982.3 ([Hex], (HexNAc)_1, (NeuAc), (NeuGc)_1) as shown in Fig. 7A and 7B.

Interestingly, the average MS between retention time 17.2 to 17.9 min indicated an ion of m/z (998.4)^1- in tumor-

<table>
<thead>
<tr>
<th>N-glycan changes</th>
<th>m/z</th>
<th>N-glycan structure based on known structures found in the mouse</th>
<th>Reference</th>
</tr>
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<tr>
<td>Only expressed in tumour-bearing</td>
<td>(1038.9)^2-</td>
<td>(75, 76)</td>
<td></td>
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<tr>
<td></td>
<td>(1111.4)^2-</td>
<td>(77, 78)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(965.9)^2-</td>
<td>(77, 78)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1022.4)^2-</td>
<td>(79)</td>
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</tr>
<tr>
<td></td>
<td>(1184.4)^2-</td>
<td>(77, 78)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1280.9)^2-</td>
<td>(80)</td>
<td></td>
</tr>
<tr>
<td>Different ratios of glycan isomers</td>
<td>(1127.4)^2-</td>
<td>(50, 80)</td>
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</table>

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Only expressed in tumour-bearing</td>
<td>[core 1]</td>
<td>(49, 50)</td>
</tr>
<tr>
<td></td>
<td>[core 2]</td>
<td>(51)*</td>
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*O-glycan structure has been previously identified by high pH anion-exchange chromatography in mouse erythrocytes and consists of two NeuAc rather than two NeuGc on the non-reducing terminal.

<table>
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<tr>
<th>N-acetyl-neuraminic acid</th>
<th>Galactose</th>
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<th>Mannose</th>
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<th>N-glycolyl-neuraminic acid</th>
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<td>Fucose</td>
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<td>N-acetyl-neuraminic acid</td>
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</table>

**TABLE I**

Summarized results of glycan differences observed on liver membranes of control and tumour-bearing mice.

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10.1074/mcp.M900538-MCP200–9
boring but not in control mice. MS/MS fragmentation of this ion identified a core 1 structure consisting of (HexNAc)1(Hex)1(NeuGc)2 (Fig. 7C). In addition, a O-glycan of composition (HexNAc)2(Hex)2(NeuGc)2 ([M-H]2/-H11002 of m/z 681.3) was shown to be a core 2 structure by MS/MS fragmentation (Fig. 7D).

Glycan Profile Changes on Fractionated Membrane Proteins—To determine whether the differences in glycans observed in the liver of EHS tumor-bearing mice were occurring on all membrane proteins or on specific protein(s), Triton X-114 phase partitioned membrane proteins were separated
by 1D SDS-PAGE and gel lanes cut into six equal fractions of descending mass with each fraction digested with PNGase F to release N-linked oligosaccharides. The analysis of N-linked oligosaccharides released from each of the six fractions showed that the same N-glycans seen in the global N-glycan profile were overall reflected similarly on all membrane proteins (Fig. 8).

**DISCUSSION**

The liver has been well studied at both the genomic and proteomic level with strong emphasis on liver diseases such as hepatitis (52, 53), hepatocellular carcinoma (53) and alcoholism (54). Mouse models have been widely used as model systems to study parallel aspects of human disease to further improve our understanding of the mechanisms involved with these diseases. The specific model used in the current study involves systemic inflammation produced by the EHS sarcoma, which has been previously shown to result in repression of multiple hepatic drug metabolizing enzymes and transporters (30, 36). The inflammatory reaction is associated with elevated plasma concentrations of pro-inflammatory cytokines produced by the tumor and the surrounding stromal cells (29, 35, 55). These changes can lead to reduced liver metabolism and clearance of drugs resulting in increased toxicity from chemotherapy. In this study we determined that the glycosylation of liver membrane proteins was also affected by the presence of this tumor.

**Glycosylation Pathway Enzymes Change in the Liver of Tumor-bearing Mice**—Peptide IPG-IEF and label-free quantitation analyses determined that mannosidases and many glycosyltransferases (mgat, galactosyltransferases, and sialyltransferases) were up-regulated in the liver membrane of tumor-bearing mice. Mannosidases are often found in the endoplasmic reticulum and cytoplasm and they are responsible for trimming mannose residues from N-linked glycans that are attached to proteins for correct folding and orientation. They are also involved in the degradation of mature glycoproteins forming glycoconjugate intermediates that permit further modification by other glycosyltransferases such as Mgats.

Mgat I (GlcNAcT-I) and Mgat-II (GlcNAcT-II) are responsible for the transfer of GlcNAc in a β(1,2) glycosidic linkage to the Man(1,3) and Man(1,6) arm of a N-linked glycan respectively in a [Manα(1,3)/Manα(1,6)]β(1,4)GlcNAcβ(1,4)GlcNAc-Asn sequence, and are both essential in initiating the biosynthesis of complex N-glycans within the lumen of the Golgi apparatus. It has been reported that the loss of either enzyme can reduce the number of complex-type N-glycans whereas increasing the number of hybrid-type N-glycans on the cell surface causing alterations to cell adhesion and other cellular interactions (56). Mgat-II deficiencies in mice display phenotypic signs similar to those reported in human congenital disorders of glycosylation. Mgat-V (GlcNAcT-V) transfers GlcNAc to the hydroxyl on carbon 6 of α-linked mannose in the preliminary sequence GlcNAcβ(1,2)Manα(1,6)β(1,4)GlcNAcβ(1,4)GlcNAc-Asn, which is present as the N-glycan intermediate at the medial Golgi apparatus stage of glycosylation. Knockout studies of mouse Mgat-V have found that Mgat-V-deficient mice were susceptible to autoimmune diseases, reduced cancer progression and T-cell hypersensitivity (57, 58).

Galactosyltransferases (B3GalT-VII, B4GalT-I, B4GalT-III, C1GalT-I, C1GalT-II and GalNT-I) were identified be up-regulated in the liver of tumor-bearing mice. These enzymes are responsible for catalyzing the transfer of galactose to N- and O-linked glycans via β(1-4) and β(1-3) linkages, and they are also involved in lactose biosynthesis. B3GalT-VII and C1GalT1 are responsible for the formation of the core 1 di-
FIG. 6. Identification of N-glycan with three glycolyl neuraminic acids. Structures are depicted as biantennary N-glycans because this is the most likely configuration of the compositions in the mouse. EIC of (1127.4)$^2$ and (1280.9)$^2$ and combined MS spectrum (A) over 40–65 min. Digestion of purified N-linked oligosaccharides with α(2,3,6) neuraminidase for 10 min and combined MS spectrum (B) over 40–65 min. Overnight digestion of purified N-linked oligosaccharides with α(2,3,6) neuraminidase and combined MS spectrum (C) over 40–65 min (D). MS/MS fragmentation of the resultant monosialylated N-glycan (m/z of 973.8)$^2$ confirms a NeuGc attached to a GlcNAc/m/z ion 509.1) thus predicting the structures shown.

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10.1074/mcp.M900538-MCP200–12

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Fig. 7. Combined EIC of (675.3)\(^+\), (691.3)\(^+\), (966.2)\(^+\), (982.3)\(^+\), (998.4)\(^+\), and (681.3)\(^-\) and average MS of O-linked oligosaccharides (retention time 17.2–17.9 min) from liver membranes from control (A) and tumor-bearing mice (B) MS/MS fragmentation of \(m/z\) (998.4)\(^+\) (C) and MS/MS fragmentation of \(m/z\) (681.3)\(^-\) (D) confirming the structures shown.
Saccharide O-glycan (Galβ(1–3)GalNAc), known as the T antigen. The core 1 O-glycan is the precursor for the synthesis of many of the extended mucin-type O-glycans, which are commonly found on mammalian glycoproteins. The loss of B3GalT-VII and C1GalT-I enzymatic activity has been reported to cause hemolytic anemia because of the lack of T antigens on blood cells (59). It has been proposed that C1GalT-II functions as a molecular chaperone together with C1GalT-1 in the synthesis of core 1 O-glycans (60). B4GalT-I is ubiquitously expressed and has two enzymatic activities. As a monomer it transfers Gal to GlcNAc-acceptors and when associated with α-lactalbumin, B4GalT-I catalyzes the transfer of Gal to Glc producing lactose (60). B4GalT-I is active toward GlcNAc in a range of glycoconjugates (N- and O-glycans and glycolipids) and deficiencies in this enzyme in mice present with skin lesions, decreased fertility and absence of lactose in milk (60). The function of B4GalT-III appears to be in the synthesis of the first N-acetyllactosamine unit of poly-N-acetyllactosamine chains on glycoconjugates (61). Furthermore, GalNT-I is one of the enzymes responsible for the synthesis of T antigens in blood cells.

Fig. 8. 1D SDS-PAGE followed by in-gel PNGase F digestion of proteins of decreasing mass to release N-linked oligosaccharides. Combined MS of 20–65 min of the N-glycans released from the proteins with each band show a similar global profile to the total protein glycoprofile.
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for the transfer of GalNAc to serine and threonine residues on many O-linked glycoproteins (60).

Sialyltransferases (ST3Gal-I, ST6Gal-I, and ST6GalNAc-VI) were also observed to be up-regulated on the liver membrane proteins of tumor-bearing mice. ST3Gal-I is highly abundant in the spleen, liver, bone marrow, thymus, and salivary glands of humans and mice. It is responsible for the transfer of sialic acid from CMP-NeuAc to the terminal galactose residues through $\alpha(2,3)$ linkages and has been reported to preferentially transfer sialic acid to core 1 O-glycan structures (62). NeuAc is modified to NeuGc in mice by the enzyme CMP-hydroylase, which catalyzes the hydroxylation of CMP-NeuAc to CMP-NeuGc in the presence of hydrogen donor (NADH), cytochrome b5 and NADH-cytochrome b5 reductase (63, 64).

Mice with ST3Gal-I deficiency exhibit decreased cytotoxic T-cell immune responses and increased apoptosis of CD8+ T-cells (65). The sialylation of core 1 and core 2 glycans with SLex structures of acute phase serum proteins is considered a secondary outcome of biantennary $N$-glycans with terminating $\alpha(2,6)$ linkage. ST6Gal-I is ubiquitously expressed and plays important roles in the immune system, as it has been demonstrated that mice lacking ST6Gal-I display reduced serum IgM levels and impaired B-cell proliferation (66). The increased expression of many enzymes involved in glycosylation suggest that this could result in dramatic changes in the structure of metabolically significant $N$- and O-glycans found collectively on membrane proteins as a result of bearing a benign tumor.

Presence of Tumor Increases Sialylation on Liver Membrane Glycoproteins—It has been reported that the release of inflammatory cytokines stimulates the expression of glycosyltransferases in tumor, as well as hepatocyte cells, which results in increased glycan branching and Sialyl Lewis X (SLex*) (NeuAcα(2,3)[Galβ(1,4)Fucα(1,3)Galβ(1,4)GlcNAC] structures on secreted glycoproteins in humans (67). In addition to the expressional changes of acute phase proteins the prevalence of tri- and tetra-antennary glycans with SLex* structures have also been shown to be altered during systemic autoimmune liver inflammation. During short-term acute liver inflammation, $\alpha$1-acid glycoprotein displayed increased expression of biantennary $N$-glycans. However, during long-term acute liver inflammation $\alpha$1-acid glycoprotein expressed primarily tri- and tetra-antennary $N$-glycan forms (67). The increased levels of tri- and tetra-antennary and SLex* structures of acute phase serum proteins is considered a secondary outcome after the secretion of pro-inflammatory cytokines from human diseases such as stomach (67) and ovarian cancer (68), as well as rheumatoid arthritis (69). Although much attention has been paid to identifying alterations in phase serum glycoproteins during acute liver inflammation, our goal was to determine if changes in glycan composition were observed on the liver membrane proteome correlating with observed glycosylation enzyme expression changes.

Liver membrane glycoproteins from tumor-bearing mice showed increased sialylation on complex-type biantennary $N$-linked glycans with terminating $N$-acetyl- and $N$-glycolylneuraminic acid, and increased $N$-glycolyl sialylation on core 1 and core 2 O-glycans. In addition, the ratios of different isomers of biantennary complex-type $N$-glycans with two NeuGc attached to Galβ(1→4)GlcNAcβ(1→2)Manα(1→3)[Galβ(1→4)GlcNAcβ(1→2)Manα(1→6)]Manβ(1→4)GlcNAcβ(1→4)GlcNAc differed between control and tumor-bearing mice. Interestingly, liver membrane proteins from tumor-bearing mice, and not control mice, expressed a tri-sialylated biantennary $N$-glycan with three NeuGc. Neuraminidases with different specificities were used to determine the linkages of the three attached NeuGc. Together with MS/MS fragmentation data, the presence of a NeuGcα(2→6)GlcNAcβ1 linkage was confirmed. This biantennary $N$-glycan structure with three NeuGc attached was shown to be present only in the liver of tumor-bearing mice and not in the control. This $N$-glycan has been reported previously to be present in the liver of control and insulin-resistant mice (48).

Similar glycosylation changes were observed on all SDS-PAGE fractionated membrane proteins from tumor-bearing mice, this was not surprising because hepatocytes make up ~80% (w/w) of the cytoplasmic mass of the liver and both $N$- and O-glycosylation are processed through the same protein glycosylation machinery in the endoplasmic reticulum and Golgi apparatus. The $N$-glycan profiles of the different mass range proteins separated by gel electrophoresis were generally similar among all fractions and reflect the gross overall profile shown in the global glycan analysis (Fig. 8). This observation emphasizes the fact that alterations in the abundance of glycosylation enzymes in a cell affect all expressed glycoproteins. There are some differences observed in the relative intensities of some peaks corresponding to $N$-glycans in the average MS of the different gel fractions and these small variations are most likely attributed to subtle differences in glycosylation of individual membrane glycoproteins. The effect of these observed glycosylation changes on the function of individual membrane glycoproteins remains to be determined experimentally.

Both NeuAc and NeuGc belong to a family of sialic acids that are typically found attached to the terminal positions of several classes of cell-surface and secreted $N$- and O-glycans. They are also presented on glycans to increase the hydrophilicity of glycoproteins for export to the extracellular environment and/or the cell surface for cell interaction events. Sialic acids often act as ligands for selectins and sialic acid-binding lectin (Siglecs) for glycan-mediated cellular interactions, such as B- and T-cell activation in adaptive immunity (65, 70), and the increased expression of NeuAc and NeuGc in the liver of tumor-bearing mice may play roles in response to inflammatory stimuli.
Siglec family are a family of sialic acid-binding immunoglobulin (Ig)-like lectins that are found either secreted into the extracellular environment or attached to the surface of cells where they interact specifically with sialic acid on glycan structures. For example, siglec-1 binds to the sialic acids on the surface of B-cells in activating the adaptive immune system (71). Siglec-1 is also expressed by resident and inflammatory macrophages in the liver, lung, and spleen in response to inflammation and is responsible for binding sialylated ligands. The lectin specifically recognizes (2,3) sialic acid on N- and O-glycans in a NeuAc/NeuGc(2–3)Galβ(1–4)GlcNAc sequence (72) on other hemopoietic cells, neutrophils, monocytes, natural killer cells, B cells, and a subset of CD8+ T lymphocytes (73). The increased expression of sialylation observed in this study on the surface of the liver membrane in response to the distal tumor could therefore act as a ligand for macrophage receptors like siglec-1 whereby it could modify cell adhesion and interaction.

The data presented in this study indicates that many enzymes involved in the protein glycosylation pathway in inflamed livers of mice bearing a distal benign tumor were found to be significantly up-regulated. Cell surface lectins and receptors were also determined to be up-regulated in these tumor-bearing mice. These enzymes and receptors correlated with the changes in N- and O-linked glycans structures, which were expressed only on the liver membrane of tumor-bearing mice and were reflected on all membrane glycoproteins. Specifically, increased N-acetylated and N-glycolyl sialylation were only identified on the membrane proteins from livers of tumor-bearing mice. The presence of cancer is well-known to be associated with alterations in membrane protein glycosylation but differential expression of these glycan structural differences, together with their associated synthetic enzymes and receptors demonstrate that downstream effects of cancer are seen in the liver which is distant from the tumor itself.

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This article contains supplemental Figs. S1 and S2 and Tables S1 and S2.

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