Differential Glycosylation and Cell Surface Expression of Lysosomal Membrane Glycoproteins in Sublines of a Human Colon Cancer Exhibiting Distinct Metastatic Potentials*

(Received for publication, August 12, 1991)

Osamu Saitoh†, Wei-Chun Wang‡, Reuben Lotan§, and Minoru Fukuda††

From the †La Jolla Cancer Research Foundation, Cancer Research Center, La Jolla, California 92037, the §Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington 98121, and the ‡University of Texas, M. D. Anderson Cancer Center, Houston, Texas 77030

Changes in the glycosylation of asparagine-linked oligosaccharides have been shown in various tumor cells, including human colon cancer. Attempts were made to elucidate the difference in Asn-linked oligosaccharides attached to lysosomal membrane glycoproteins isolated from sublines of human colon carcinoma exhibiting high and low metastatic potentials in nude mice. Lysosomal membrane glycoproteins (lamp) 1 and 2 were immunoprecipitated from the cells after labeling with radioactive sugars, and the glycopeptides prepared were fractionated by serial lectin affinity chromatography employing immobilized concanavalin A, Datura stramonium agglutinin, and tomato lectin. Comparison of Asn-linked oligosaccharides from the different colonic carcinoma cells revealed the following features. First, the highly metastatic carcinoma cells express more poly-N-acetyllactosaminyl side chains with branched galactose residues than cells with low metastatic potential. Second, sialylation is more significant in the highly metastatic carcinoma cells than in the poorly metastatic ones. Conversely, N-acetyllactosamine units are less fucosylated in the highly metastatic than in poorly metastatic cells. These structural changes were apparently caused by the increase in sialyltransferase and the decrease in fucosyltransferase in the highly metastatic cells. The results also suggest that highly metastatic carcinoma cells express more sialyl LeX structures at the termini of poly-N-acetyllactosaminyl side chains than poorly metastatic carcinoma cells. Further, highly metastatic cells were found to express more lamp-1 and lamp-2 on the cell surface. These results were found to be correlated to the increased expression of sialyl LeX structures with high affinity binding of anti-sialyl LeX antibody on highly metastatic cells. Increased expression of sialyl LeX in the poly-N-acetyllactosamines of the cell surface may contribute to the metastatic behavior of the cells, assuming that this structure can serve as a better ligand for selectins present on endothelial cells and platelets.

Polyacetylactosaminoglycans are high molecular weight carbohydrates and are distinct from usual complex-type Asn-linked saccharides by having side chains composed of Galβ1→4GlcNAcβ1→3 repeats, which are susceptible to endo-β-galactosidase. Polyacetylactosaminoglycans can carry various antigenic determinants such as I/i and ABO blood groups (for review, see Refs. 1–3). Furthermore, the structures of polyacetylactosaminoglycans are often characteristic to different cell types and stage of differentiation. For example, granulocytes and monocytes are enriched in the structures of Galβ1→4(Fucα1→3)GlcNAcβ1→R, LeX, or NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→R, sialyl LeX, which reside at the termini of polyacetylactosaminoglycans in these cells (4, 5). Very recently, it was discovered in several laboratories that these unique structures of poly-N-acetyllactosaminyl glycoproteins and granulocytes and monocytes serve as ligands for adhesive molecules, selectins, in endothelial cells and platelets (6–9). Reports from several other laboratories demonstrated that the level of sialyl LeX or sialyl LeX NeuNAcα2→3Galβ1→4(Fucα1→4)GlcNAcβ1→R are increased in tumor cells, including carcinomas (10–12).

It has been repeatedly observed that malignant transformation of rodent and human cells is associated with an increase in the amount of tetraantennary and triantennary N-glycans containing side chains, which are linked to α-mannose through a GlcNAcβ1→6 linkage (13–15). In particular, the transformation of rat2 fibroblasts with oncogenes such as T24-H-ras, V-K-ras and V-fps results in the increased amount of N-acetylgalactosaminyltransferase V, which forms the above antenna and acquisition of invasive and metastatic potential (16, 17). This increased amount of the tetraantennary and triantennary N-glycans is often associated with the increased amount of poly-N-acetyllactosamine. This is because β1→3-N-acetylgalactosaminyltransferase, the key enzyme for the formation of N-acetyllactosaminyl repeats, preferentially acts on the side chains branched from mannose via GlcNAcβ1→6, which is formed by N-acetylgalactosaminyltransferase V (18).

We have shown previously that lysosomal membrane glycoproteins, lamp-1 and lamp-2, are the major carriers of polyacetylactosaminoglycans in various cells (19, 20). It was also demonstrated that lamp-1 is the major glycoprotein containing GlcNAcβ1→6Manα1→6Man branching in metastatic tumor cells as detected by leukophytohemagglutinin binding (21, 22). It is becoming evident that some of lamp-1 and lamp-2 are expressed on the cell surface, although the majority of these molecules reside in lysosomes (20, 23–25). These results

* This work was supported by Grant R01 CA48737 awarded by the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: La Jolla Cancer Research Foundation, 10601 N. Torrey Pines Rd., La Jolla, CA 92037.

1 The abbreviations used are: lamp-1 and lamp-2, lysosomal membrane glycoproteins 1 and 2; ConA, concanavalin A; DSA, Datura stramonium agglutinin; PBS, phosphate-buffer saline; BSA, bovine serum albumin; Fuc, fucose.
prompted us to examine the carbohydrate structures of lamp-1 and lamp-2 isolated from highly metastatic and poorly metastatic sublines derived from a human colon tumor, in order to determine whether lamp-1 and lamp-2 are the major carriers for polylactosaminoglycans on the cell surface of these tumor cells, and whether the glycosylation of these glycoproteins is different in cells exhibiting distinct metastatic potentials.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Four cell lines derived from a single human colon carcinoma specimen, designated KM12-C, KM12-SP, KM12-SM, and KM12-L4, were kindly provided by Dr. Isabel J. Fidler (M. D. Anderson Cancer Center, Houston, TX). After intrasplenic injection, KM12-C and KM12-SP are poorly metastatic to liver, whereas KM12-SM and KM12-L4 are highly metastatic to liver (26, 27). The sublines were established from a primary colon carcinoma (Duke's stage B2) as described previously (26) and shown schematically in Fig. 1. Briefly, tumor tissue was dissociated into single cells or small cell clumps by treatment with collagenase type I and deoxyribonuclease. The cell suspension was divided into several aliquots, one of which was used directly to establish the cell line designated KM12-C. Other aliquots of the same cell suspension were injected into the spleen of several male athymic BALB/c nude mice, and cell line KM12-SM and KM12-L4 were derived after four cycles of intrasplenic injection and production of experimental hepatic metastases in nude mice (see Fig. 1). Cell line KM12-SP was derived from a rare liver metastasis produced by parental KM12-C cells growing in the cecum wall of a nude mouse (Fig. 1) (27).

**Cell Culture and Metabolic Labeling**—The carcinoma cell lines were cultured in RPMI 1640 medium containing 10% fetal calf serum, 1% potassium phosphate buffer, pH 7.4, 150 mM NaCl, 0.002% NaN3 (28). For metabolic labeling, the cells were cultured in 98% of RPMI 1640 glucose-deficient medium supplemented with 10% dialyzed fetal calf serum and 2% of regular RPMI 1640 that contained 10% fetal calf serum (final glucose concentration, 0.28 mM) (28). [3H]Glucosamine and [3H]galactose, or [3H]fucose or [3H]mannose were added at a concentration of 10 μCi/ml and 20 μCi/ml, respectively, and cells were metabolically labeled for 24 h at 37°C.

**Immunoprecipitation and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—Radiolabeled cells were lysed in PBS (5.7 mM potassium phosphate buffer, pH 7.4, 150 mM NaCl, 0.002% Na2EDTA) containing 1% Nonidet P-40 and protease inhibitors as described (28). Lamp-1 and lamp-2 were sequentially immunoprecipitated from the cell lysates by addition of rabbit anti-human lamp-1 antibodies or rabbit anti-human lamp-2 antibodies as described (29). Anti-lamp-1 and lamp-2 antibodies were kindly provided by Dr. Sven Carlsson, University of Umeå, Umeå, Sweden. Aliquots of radiolabeled samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% polyacrylamide gel according to Laemmlli (29), followed by fluorography using Enlighting (Du Pont-New England Nuclear).

**Lectin Affinity Chromatography of Glycopeptides**—Glycopeptides were released by Pronase digestion of immunoprecipitates and fractionated by serial lectin affinity chromatography employing concanavalin A (Con A)-Sepharose, tomato lectin-Sepharose, and dextran transferrin stramonium (DSA)-agarose. The conditions for these chromatographies were described previously (28, 30). Briefly, the glycopeptides were first applied to a column of ConA-Sepharose, and complex-type triantennary and tetraantennary asparagine-linked glycopeptides (Fraction I) were separated from branched asparagine-linked glycopeptides (Fraction II) and high-mannose glycopeptides (Fraction III). Fraction I that contains tri- and tetraantennary glycopeptides was then applied to a column of tomato lectin-Sepharose. The glycopeptides that bound to this column, IB, contain poly-N-acetyllactosaminyl side chains with three or more N-acetyllactosamine repeats (28, 30). Glycopeptides unbound to tomato lectin-Sepharose, IA, were then applied to a column of DSA-agarose. DSA-agarose binds glycopeptides containing N-acetyllactosamine repeats and/or containing R-4GlcNAcβ1→6(R-4GlcNAcβ1→2)Man→, branching (Fraction IA2) (31).

**Endo-β-galactosidase Treatment**—Radiolabeled glycopeptides were digested with endo-β-galactosidase under the conditions described previously (28). Endo-β-galactosidase was purified from Escherichia freundii (32) and kindly donated by Dr. Michiko N. Fukuda of our institute. In the second set of experiments, radiolabeled glycopeptides were hydrolyzed in 0.1 M sodium pyruvate at 100°C for 1 h (4) before they were subjected to the transglucosidation assay. The hydrolysate was neutralized with 1 N NaOH and desalted by Sephadex G-15 gel filtration using water as eluent. This acid hydrolysis removes both fucose and sialic acid.

**Methylation Analysis**—[3H]Mannose-, [3H]galactose-, or [3H]glucosamine-labeled glycopeptides were methylated, together with unlabeled fetuin glycopeptides as carriers by the procedure of Hakomori (33) as described previously (28). The methylated galactose, mannose, and fucose residues were separated directly by thin layer chromatography as described (28). The methylated glucosamine derivatives were methylated (4) and then treated with endo-β-1,2-mannosidase from Pseudomonas aeruginosa (US9934, Sigma) on a Silica Gel G, using the solvent system of acetone/water/ammonium hydroxide (250:3:1.5, v/v/v). The sample lane was separated into 0.5-cm sections, and the radioactivity was determined by scintillation counting. Methylated standards of [3H]glucosamine were obtained from lamp-1 IB glycopeptides of HL-60 cells (28).

**Estimation of the Number of Sialic Acid Residues**—In order to estimate the number of sialic acid residues/mole of glycopeptide, glycopeptides were applied to a column of QAE-Sephadex A-25 equilibrated with 10 mM pyridine/acetic acid buffer, pH 5.5. After washing with the same buffer, monosialo, disialo, trisialo, tetrasialo, and pentasialo (plus more highly sialylated) glycopeptides were eluted step-wise in 20, 70, 150, 200 mM NaCl, and 1 M NaCl in the same buffer (28).

**Assay of Glycosyltransferases**—All enzymes except α→3 fucosyltransferase were assayed under the same conditions as described previously (28, 34). Briefly, Gα1→3Galβ1→4GlcNAcβ1→4GalNAcβ1→4Glcβ1→3(Fucα1→3Glcβ1→4Galβ1→4Glcβ1→3(CH2)5), and GlcNAcβ1→2Manα1→6Glcβ1→0(CH2)5, were used as acceptors for UDP-GlcNAc:β-Gal β1→3-N-acetylgalcosaminyltransferase, "extension enzyme," and UDP-GlcNAc:Man β1→6-N-acetylgalcosaminyltransferase (V), respectively. These two substrates were kindly provided by Dr. Ole Hindsgaul, University of Alberta, Canada. Asialo α→2-acid glycoprotein was used as an acceptor for CMP-NeuNAc β-Galα2→3 sialyltransferase and α→2→6 sialyltransferase and the product was treated with Newcastle disease virus neuraminidase as described (34). The radioactivity remaining after neuraminidase digestion was regarded as the product due to α→2→6 sialyltransferase, whereas the difference between nontreated and neuraminidase-treated products was regarded as the product due to α→2→3 sialyltransferase (34). In order to measure the activity of α→1→3 fucosyltransferase, Fucα1→2Galβ1→4Glc was used as an acceptor under the conditions described (36).

**Estimation of Number of Lamp-1 and Lamp-2 Molecules and Sialyl Leα Structures on the Cell Surface**—To estimate the number of lamp-1 and lamp-2 molecules and sialyl Leα termini on the surface of the colon tumor cell lines, the number of binding sites of monoclonal antibody specific to sialyl Leα (CSLEX1) provided by UCLA tissue typing laboratory, Ref. 10] is an IgM, the sandwich method was used for the determination of binding.
sites. Thus, adherent carcinoma cells were washed with PBS containing 4 mg/ml BSA, and then various amounts of the monoclonal antibodies were added. After incubation at 4 °C for 1 h, the cells were washed with cold PBS containing BSA and 125I-labeled goat anti-mouse IgM diluted in PBS containing BSA was added. In preliminary experiments, the amount of the second antibody was determined to saturate the binding sites. The number of binding sites was calculated from the determination of the specific activity by measuring the total radioactivity of the aliquots used for each experiment. Similarly, the binding sites of lamp-1 and lamp-2 were estimated by using mouse monoclonal antibodies BB6 (specific to lamp-1) and CD5 (specific to lamp-2, Ref. 20) followed by the addition of 125I-labeled goat anti-mouse IgG. 125I-Labeled goat anti-mouse IgM and anti-mouse IgG were purchased from Du Pont-New England Nuclear. In order to estimate the binding sites at the cell surface, the whole procedure was carried out on ice. In order to estimate the binding sites including the cytoplasm, the cells were fixed with 0.5% formaldehyde, and permeabilized with 0.5% saponin, as described (38). The monoclonal antibodies were then added on the fixed, permeabilized cells on ice.

Indirect Immunofluorescence—Indirect immunofluorescence was carried out exactly as described previously (38). Briefly, the cells were fixed first with 0.5% formaldehyde in PBS for 20 min at room temperature and then permeabilized with 0.5% saponin and 4 mg/ml BSA in PBS. The fixed and permeabilized cells were then incubated with anti-lamp-1 or lamp-2 monoclonal antibody followed by fluorescein isothiocyanate-conjugated F(ab')2 fragment of goat anti-mouse IgG. Alternatively, the cells were incubated on ice with appropriate antibody, then fixed with paraformaldehyde. The cells were then incubated in the second antibody conjugated with fluorescein isothiocyanate. The latter procedure is to detect lamp molecules on the cell surface while the former procedure can detect the intracellular lamp molecules as well. Similarly, the cells were incubated with mouse anti- sialyl Le" monoclonal antibody (purchased from Signet Laboratories, Dedham, MA) followed by fluorescein isocyanate-conjugated F(ab')2 fragment of goat anti-mouse IgG.

RESULTS

Lamp-1 and Lamp-2 from High and Low Metastatic Colonic Carcinoma Cells—The colonic carcinoma cell lines with varying metastatic potentials were metabolically labeled in the presence of one of the radioactive monosaccharides, and lamp-1 and lamp-2 were then immunoprecipitated. As shown in Fig. 2A, the major glycoproteins in the total cell lysates are glycoproteins with Mr ~170 to ~180 and Mr ~130. The latter glycoproteins correspond to lamp-1 and lamp-2 as shown in Fig. 2B, indicating that lamp-1 and lamp-2 are the major glycoproteins even in the total cell lysate. Fig. 2 also reveals that [3H]fucose was incorporated more in the poorly metastatic cells than in the highly metastatic cells (compare SP versus L4 in Fig. 2). Examination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of total cell lysates (A) and lamp-1 and lamp-2 (B) after labeling with various radioactive sugars. After labeling, the total cell lysates (A) or lamp-1 and lamp-2, sequentially immunoprecipitated from the total lysates (B), were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by fluorography. In experiment A, 100 μg of protein was applied to each lane. The arrowhead indicates the migration positions of lamp-1 and lamp-2. In experiment B, [3H]mannose-labeled samples are shown. Similar results were obtained on [3H]glucosamine, [3H]galactose, or [3H]fucose-labeled samples. The migrations of standard proteins are indicated at the margins.

Fractionation of Glycopeptides by Serial Lectin Affinity Chromatography—The difference in carbohydrate moieties attached to lamps was demonstrated when the glycopeptides were fractionated by serial lectin chromatography. Glycopeptides were prepared from immunoprecipitated lamp-1 and lamp-2, and the radioactive glycopeptides were fractionated as shown in Fig. 3. The results indicate clearly that lamp-1 from the highly metastatic colon carcinoma cells, L4, yield a greater proportion of glycopeptides that are bound by tomato lectin-Sepharose from glycopeptides from the poorly metastatic SP cells. Similar results were obtained when lamp-2 glycopeptides were analyzed as shown in Fig. 4. Furthermore, the highly metastatic cells (L4) contained a higher amount of

³ Portions of this paper (including part of "Results," Figs. 4-12, and Tables I-V) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
poorly metastatic cell lines, SM and C, was also metabolically
bound to tomato lectin-Sepharose and DSA-agarose than the
tides were prepared from immunoprecipitated lamp-1 and
lamp-2. As shown in Table I, the highly metastatic cell line
(SM) again yielded a greater proportion of glycopeptides
bound to tomato lectin-Sepharose and DSA-agarose than the
poorly metastatic cell line (C). These results indicate that
highly metastatic colon carcinoma cells contain more glycopeptides
bound to tomato lectin-Sepharose and to DSA-Sepharose than poorly metastatic colon carcinoma cells.

Structures of Asparagine-linked Oligosaccharides—To un-
derstand the difference in Asn-linked oligosaccharides be-
tween high and low metastatic colon carcinoma cells, the
structural analysis of glycopeptides was carried out as shown
in the Miniprint. To simplify the description of the structural
analysis, the glycopeptides obtained from lamps of the highly
metastatic carcinoma cell line L4 are described first in detail.

Structures of IB Glycopeptides—Based on the results de-
scribed in the Miniprint, the structures of the glycopeptides
bound to the tomato lectin-Sepharose column can be pro-
scribed, as shown in Fig. 13A. IB glycopeptides are tetraanten-
nary saccharides which contain 5 to 6 mol of N-acetyllacto-
saminyl units/mol of glycopeptides, thus 1-2 mol of N-
acetyllactosamine are present in poly-N-acetyllactosaminyl
extension. The longest poly-N-acetyllactosaminyl extension
has the backbone of linear, Galβ1→4GlcNAcβ1→3Galβ1→
4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→
6Man or branched structure, Galβ1→4GlcNAcβ1→3GlcNAcβ1→6Galβ1→
4GlcNAcβ1→3Man. 65% of the N-acetyllactosaminyl units are
substituted with α1-3 fucose residues, which are apparently
attached uniformly to N-acetyllactosaminyl residues at var-
ious positions. In addition, 0.4 mol of the terminal galactose
is substituted with α1→2 linked fucose. The majority of IB
glycopeptides contain 2 or 3 sialic acid residues. Together,
the termini of IB glycopeptides contain sialyl LeX structure,
NeuNACα2→3Galβ1→4(Fucα1→3)GlcNAc and LeY struc-
ture, Fucα1→2Galβ1→4(Fucα1→3)GlcNAc. Portions of these
structures were released by endo-β-galactosidase (Fig. 7).

In Fig. 13, the structures were proposed for the glycopep-
tides that contain Galβ1→4GlcNAc, namely type 2 chain in
the side chains. However, it has been reported that some of
the oligosaccharide side chains in colonic epithelial cells could
contain the Galβ1→3GlcNAc structure, namely the type 1
chain (39). In order to estimate the ratio of Galβ1→4GlcNAc
to Galβ1→3GlcNAc linkages, the desialylated, defucosylated
IB glycopeptides were digested by β-galactosidase and β-N-
acetylgalosaminidase, both purified from Diplococcus pneu-
monia. Diplococcal β-galactosidase was shown to hydrolyze
only Galβ1→4GlcNAc linkage but not Galβ1→3GlcNAc link-
age (40). The results indicate that more than 70% of the side
chains are composed of Galβ1→3GlcNAc linkages (data not
shown). This number needs to be taken as a minimal estimate
since diplococcal β-N-acetylgalosaminidase may not cleave
all of the GlcNAcβ1→4Galβ1→4GlcNAcβ1→4/6Man→R
structures (39). In order to determine whether these cells
express type 1 structures, we examined the cells by indirect
immunofluorescence using anti-sialyl Leα antibodies. The re-
sults showed that both SP and L4 cells express a small but
detectable amount of sialyl Leα structure (data not shown).
These results therefore indicate that the great majority of the
side chains consists of N-acetyllactosamine units (type 2
chain), whereas a small portion of the side chains (less than
30%) have Galβ1→3GlcNAc structures (type 1 chain).

Structures of IA2 Glycopeptides—Based on the results de-
scribed in the Miniprint, the structures of IA2 glycopeptides
that were bound to DSA-agarose, are proposed as shown
in Fig. 13B. IA2 glycopeptides have on the average 4.6-4.9
mol of N-acetyllactosaminyl units in tetraantenary struc-
tures, thus 0.6-0.9 mol of N-acetyllactosaminyl units are
present as poly-N-acetyllactosaminyl extensions. The major-
ity of IA2 glycopeptides contains 4, 3, or 2 sialic acid residues.
IA2 glycopeptides contain the least amount of fucose residues
attached to N-acetyllactosaminyl side chains among IB, IA1,
and IA2 glycopeptides. A high proportion of N-acetylecto-
saminyl side chains are composed of type 1 chain, Galβ1→
3GlcNAc. IA1 glycopeptides are extensively fucosylated at N-acetyl-
lactosaminyl units. The highly metastatic cells (L4) are more sialy-
lated and less fucosylated than the poorly metastatic cells (SP) (see
text for details).

Structures of IA1 Glycopeptides—Based on the results de-
scribed in the Miniprint, the structures of IA1 glycopeptides
that were not bound to DSA-agarose are proposed as shown
in Fig. 13C. The majority of IA1 glycopeptides are tetraanten-
nary saccharides containing 0 or 1 mol of poly-N-acetyllac-
tosaminyl extensions. IA1 glycopeptides contain, on the average, 2 sialic acid residues. IA1 glycopeptides are highly fucosylated and more than 75% of N-acetyllactosaminyl units are substituted with fucose. These fucose residues are uniformly distributed. Thus, a majority of IA1 glycopeptides must have at least 1 mol of sialyl Le^a and a maximum of 0.5 mol of Le^a/mol of the glycopeptides.

Comparison of Glycopeptide Structures from Poorly Metastatic and Highly Metastatic Colon Carcinoma Cell Lines—Glycopeptides obtained from poorly metastatic cells were subjected to structural analysis in the same way as was done for those from highly metastatic cells, and the following conclusions can be drawn. (a) Lamps from highly metastatic cells contain more polyglactosamine-containing Asn-linked oligosaccharides, both IB and IA2 glycopeptides, than poorly metastatic carcinoma cells (Table I). Concomitantly, much less IA1 glycopeptides were obtained from highly metastatic cells compared to poorly metastatic cells. (b) The glycopeptides from highly metastatic carcinoma cells are appreciably more sialylated than the counterparts from poorly metastatic cells (Table IV). Conversely, the fucose content is much higher in the glycopeptides isolated from poorly metastatic carcinoma cells (Tables III and V). Methylation analysis and fucose content indicated that IA1 glycopeptides from poorly metastatic carcinoma cells contain almost fully fucosylated N-acetyllactosamine units. (c) Since ConA I and ConA II glycopeptides contain 3 mol of mannose/mol of glycopeptides, the number of Asn-linked oligosaccharides can be estimated as shown in Table VI, assuming that high mannose oligosaccharides contain about 7.5 mol of mannose/molecules as shown in the previous studies for HL-60 cells (28). The results indicate that the major difference between highly and poorly metastatic cells is that more sialylated and less fucosylated in the N-acetyllactosamines in the highly metastatic carcinoma cells. In addition, slightly more N-glycosylation sites are modified to acquire poly-N-acetyllactosamine in the highly metastatic cells. On the other hand, the number of N-acetyllactosamine units in each glycopeptide fraction does not differ irrespective of the metastatic potential of the cells.

Comparison of Glycosyltransferase Activities between Highly and Poorly Metastatic Colon Carcinoma Cell Lines—To understand further the mechanisms underlying the difference in glycosylation, β-Gal-β1→3-4-N-acetylgalactosaminyltransferase, extension enzyme, α-man-β1→6-N-acetylglucosaminyltransferase, “GlcNAc-transferase V,” β-Glc(NAc)-α1→3 fucosyltransferase, α2→3, and α2→6 sialyltransferases were assayed. Although no appreciable difference was observed in the first two enzymes, the latter two enzymes show a dramatic difference between highly and low metastatic cells. Table VII reveals that poorly metastatic SP cells express twice as much α1→3 fucosyltransferase as highly metastatic L4 cells, whereas highly metastatic L4 cells express 50% more α2→3 sialyltransferase than poorly metastatic SP cells. These results are in good agreement with the above results that the highly metastatic cells contain more sialylated but less fucosylated glycopeptides than the low metastatic cells.

Comparison of Cell Surface Lamp Expression between High and Low Metastatic Carcinoma Cells—It has been shown that a small portion of lamp molecules can be expressed on the cell surface, and these molecules can be the carriers of poly-N-acetyllactosamine chains on the cell surface (20, 23–25). To test if highly metastatic carcinoma cells express more lamp on the cell surface, the expression of lamp molecules was examined by indirect immunofluorescence. As shown in Fig. 14A, the intensity of immunofluorescence staining of the lamp-1 molecule on the surface of the poorly metastatic cells was relatively weak. In contrast, the immunofluorescence on the surface of highly metastatic cells was obvious and stronger than that on the poorly metastatic ones (compare C with A).

TABLE VI

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Lamp-1 (18^a)</th>
<th>Lamp-2 (16^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex-type containing poly lactosa-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mine with 3 repeats of N-acetyllacto-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>saminyl (IB)</td>
<td>0.5</td>
<td>1.9</td>
</tr>
<tr>
<td>Complex-type containing poly lactosa-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mine with 1–2 repeat(s) of N-acetyl-</td>
<td>1.3</td>
<td>6.0</td>
</tr>
<tr>
<td>tallowactosaminyl (IA2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heavily fucosylated complex-type tri-</td>
<td>14.1</td>
<td>7.9</td>
</tr>
<tr>
<td>and tetraantennas (IA1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biantennary complex-type (II)</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>High mannose-type (III)</td>
<td>1.8</td>
<td>2.0</td>
</tr>
</tbody>
</table>

^a The number of total carbohydrate chains/lamp-1 or lamp-2 molecule (see the previous report of Ref. 20).

TABLE VII Glycosyltransferase activities in extracts of poorly and highly metastatic colon cell lines

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Poorly metastatic SP cells</th>
<th>Highly metastatic L4 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme metastatic metastatic metastatic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP-GlcNAc:βGal β1→3-4-N-acetylgalactosaminyltransferase (extension enzyme)</td>
<td>0.56</td>
<td>0.51</td>
</tr>
<tr>
<td>UDP-GlcNAc:α-Man β1→6-N-acetylgalactosaminyltransferase (GlcNAc-transferase V)</td>
<td>0.037</td>
<td>0.030</td>
</tr>
<tr>
<td>GDP-Fuc:β-Glc(NAc) α1→3-fucosyltransferase</td>
<td>0.42</td>
<td>0.20</td>
</tr>
<tr>
<td>CMP-NeuNAc:β-Gal α2→3-sialyltransferase</td>
<td>0.12</td>
<td>0.19</td>
</tr>
<tr>
<td>CMP-NeuNAc:β-Gal α2→6-sialyltransferase</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

^a The amount of this enzymatic activity is almost close to the limit of the detection.

FIG. 14. Expression of lamp-1 molecules on the cell surface and in the cytoplasm of poorly metastatic and highly metastatic colon carcinoma cells examined by immunofluorescence. SP (A and B) and L4 (C and D) cells were examined either as intact cells for surface expression of lamp-1 (A and C) or after permeabilizing with saponin for visualization of the intracellular distribution of lamp-1 (B and D). The cells were incubated with monoclonal anti-(h-lamp-1) antibody followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG. The bar indicates 20 nm.
in Fig. 14). When the cells were permeabilized, intracellular lysosomal structures were stained with similar intensity in both cell types (Fig. 14, B and D). These results suggest that more lamp molecules are exposed on the surface of highly metastatic carcinoma cells than on poorly metastatic ones.

In order to quantitate this difference, the number of lamp-1 and lamp-2 molecules was estimated by determination of the number of antibody-binding sites. As shown in Fig. 15A, more of the antibodies were bound to the surface of highly metastatic cells than poorly metastatic cells. The number of binding sites/cell was found to be $2.2 \times 10^6$ and $1.4 \times 10^6$ for highly and poorly metastatic cells, SP and L4, respectively (Table VIII). A similar difference was also found between another pair of cells, and $2.4 \times 10^6$ and $1.2 \times 10^6$ lamp-1/cell, respectively, were found to express on the cell surface of SM and C cells. In these experiments, nonspecific binding was less than 2% of the total binding. In addition, the size of the highly and poorly metastatic cells is very similar. We also estimated the number of lamp-1 and lamp-2 molecules in the whole cell, including the cytoplasm by the analysis of permeabilized cells. Table VIII reveals that highly metastatic cells contain slightly more total lamp-1 and lamp-2 molecules than poorly metastatic cells in both pairs of sublines (see also Fig. 15C).

Comparison of Cell Surface Sialyl Le^X Expression between Highly and Poorly Metastatic Carcinoma Cells—The number of sialyl Le^X determinants on the cell surface was estimated in order to test whether the expression of cell surface sialyl Le^X correlates with the expression of lamp-1 and lamp-2 molecules. Table VIII shows that the number of binding sites for anti-sialyl Le^X antibodies/cell is close to that estimated for lamp-1 and lamp-2. Simple calculations, based on the number of binding sites, suggest that as much as 30% of sialyl Le^X structures can be carried by cell surface lamp molecules. However, the anti-sialyl Le^X antibody is of the IgM type, so that some antibodies could bind pentavalently, whereas some of the anti-lamp antibody binds divalently due to its IgG nature. Because of this difference in valency, these experiments do not provide the actual number of sialyl Le^X determinants carried by cell surface lamp molecules.

Fig. 15B and Table VIII also reveal that the highly metastatic cell expresses more of sialyl Le^X structures with high affinity to the monoclonal antibody. This difference in the binding curve was reproduced in repeated experiments. This result is consistent with the above findings that highly metastatic cells express more of poly-N-acetyllactosaminyl glycopeptides (see Table VI), assuming that sialyl Le^X structures at the termini of poly-N-acetyllactosaminyl side chains have higher affinity to the monoclonal antibody than those at short chains. The same experiments also showed that the number of sialyl Le^X sites is almost the same at saturating antibody concentrations, regardless of cell types. This is probably due to the fact that low metastatic cells express more of shorter N-glycans enriched with sialyl Le^X structure (IA1 glycopeptides). As a result, both highly and poorly metastatic tumor cells express a similar amount of sialyl Le^X structure which has low affinity to the anti-sialyl Le^X antibody.

**Table VIII**

<table>
<thead>
<tr>
<th>Number of binding sites to monoclonal antibodies specific to lamp-1, lamp-2, and sialyl Le^X</th>
</tr>
</thead>
<tbody>
<tr>
<td>The numbers are expressed as $10^4$/cell.</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Lamp-1 (BB6)</td>
</tr>
<tr>
<td>Surface</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Lamp-2 (CD3)</td>
</tr>
<tr>
<td>Surface</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Sialyl Le^X (CSLEX1)</td>
</tr>
<tr>
<td>Surface</td>
</tr>
<tr>
<td><em>The numbers in parentheses indicate a proportion of lamp molecules on the cell surface expressed as a percentage of total lamp molecules.</em></td>
</tr>
</tbody>
</table>

*The numbers in parentheses indicate the binding sites with high affinity to the monoclonal antibody.*

**DISCUSSION**

The present studies revealed differences in Asn-linked oligosaccharides attached to lysosomal membrane glycoproteins 1 and 2 synthesized in poorly and highly metastatic human colon carcinoma cells. The results can be summarized as follows. First, the highly metastatic colon carcinoma cell lines (L4 and SM) synthesize more of Asn-linked oligosaccharides that contain poly-N-acetyllactosamines than their poorly metastatic counterparts (C and SP). Second, the poly-N-acetyllactosamine chains are sialylated more extensively in...
the highly metastatic colonic cells. Third, the poly-N-acetyllactosamine units are fucosylated to a lower degree in the highly metastatic colonic carcinoma cell lines. Table VI illustrates that the number of Asn-linked oligosaccharides containing poly-N-acetyllactosaminyl units with branched galactose moieties (IB glycopeptides) increases from 0.5 to 1.9 mol for lamp-1 and from 0.6 to 1.7 mol for lamp-2, after the cells are selected for liver metastasis. The number of Asn-linked oligosaccharides containing linear N-acetyllactosaminyl repeats but fewer fucose residues (IA2 glycopeptides) increases dramatically after the same selection. Concomitant with this increase, the amount of oligosaccharides with a minimum amount of poly-N-acetyllactosamines but with higher fucosylation (IA1 glycopeptides) decreases dramatically in the highly metastatic cells (Table VI). These results indicate that the degree of sialylation increases but fucosylation decreases in the highly metastatic cell lines. These results were obtained because the highly metastatic cells express an increased amount of α2→3 sialyltransferase and a decreased amount of α1→3 fucosyltransferase than the poorly metastatic cells (Table VII). In addition, the increased sialylation may result in the decreased fucosylation in the highly metastatic colonic carcinoma cells, since sialylation in general hinders fucosylation at N-acetyllactosamine units and fucosylation follows after sialylation (42, 43). On the other hand, it is not clear at this point how the highly metastatic cells express a slightly increased amount of poly-N-acetyllactosamine. Consistent with our results it has been reported that a B16 melanoma variant, which is resistant to wheat germ agglutinin, is enriched in the Galβ1→4(Fucα1→3)GlcNAc structure but contains only a trace amount of sialic acid (44).

Our studies demonstrated that serial lectin affinity chromatography is useful in fractionating glycopeptides exhibiting differences in poly-N-acetyllactosamine. As reported previously (28, 30), tomato lectin-bound fraction was preferentially enriched with glycopeptides containing longer poly-N-acetyllactosaminyl repeats. All of the glycopeptides with branched galactose were apparently bound to this immobilized lectin. In contrast, DSA-agarose can bind oligosaccharides even with short poly-N-acetyllactosamines. However, it is significant to note that the glycopeptides with short poly-N-acetyllactosamines were not bound to DSA when they were also heavily fucosylated, which is consistent with a previous report (31). It was reported that triantennary oligosaccharides lacking 2,6-di-O-substituted α-mannose also fails to bind to DSA (45). The present study indicates these observables in that they show not only the extent of poly-N-acetyllactosamine and the number of antennae but also other substitutions, such as fucosylation, can be discriminated efficiently by the serial lectin affinity chromatography employing ConA, tomato lectin, and DSA. It is worthwhile to mention that the effect of fucose substitution was not evident in fractionation of glycopeptides from HL-60 lamp molecule (28). This is most likely because glycopeptides from HL-60 lamps contain longer poly-N-acetyllactosaminyl side chains with less fucose substitution than those from colon carcinoma cells.

The present results are consistent with the previous reports on the expression of polylactosaminoglycans in transformed cells. It has been shown that baboon hamster kidney cells transformed by the Rous sarcoma virus or poliovirus express more polylactosaminoglycans than their untransformed parent cell lines (13–15). It is noteworthy that metastatic tumor cells transformed by various oncogenes also increase in the synthesis of the side chain arising from GlcNAcβ1→6Manα1→6, which is formed by N-acetylgalactosaminyltransferase V (16, 17). Since this side chain is the most preferable site for poly-N-acetyllactosamine extension by β1→3-N-acetylgalactosaminyltransferase, the increased amount of the GlcNAcβ1→6Manα1→R branch leads to an increase in poly-N-acetyllactosamine (18, 45, 46). Conversely, it was reported that tumor metastasis in animal models was reduced by the addition of glycosylation inhibitors such as swainsonine and castanospermine (47–49). Since these two inhibitors block the formation of side chains elongating from C-6 of α-mannose (50), they presumably inhibit poly-N-acetyllactosaminyl formation. Thus, the inhibition of poly-N-acetyllactosamine synthesis apparently reduces tumorigenicity. Similarly, we have shown recently that the major carriers for poly-N-acetyllactosamine in human CaCo-2 colonic tumor cells are lamp-1 and lamp-2 and that their poly-N-acetyllactosamine content is decreased after the CaCo-2 cells are differentiated and lose their tumorigenicity (51). These results indicate that the amount of poly-N-acetyllactosamine in lamp-1 and lamp-2 is positively correlated to the tumorigenicity. Our study extended these findings in that we compared two pairs of highly metastatic and poorly metastatic sublines, all derived from a single human colon cancer specimen. The increased amount of poly-N-acetyllactosamine in lamp-1 and lamp-2 of the highly metastatic cells implies that they are linked to tumor progression and the acquisition of the metastatic phenotype.

The present study also revealed that the highly metastatic sublines express more lamp molecules on the cell surface than the lower metastatic sublines. The increased expression of lamp-1 and lamp-2 on the surface of the highly metastatic cells may be related to the metastatic properties, especially as mediators of cell adhesion. In order for tumorigenic cells to establish metastasis, blood-borne tumor cells need to lodge in capillary beds at junctions between endothelial cells (52). Studies from several laboratories suggest that this process can be mediated by interaction between adhesive molecules, selectins, on endothelial cells and the surface of tumorigenic cells (53, 54). It has been demonstrated recently that adhesive molecules, named selectins, such as ELAM-1 on endothelium and GMP-140 on platelets bind to sialyl Leα and its related structures on neutrophils and monocytes (6–9, 55). Furthermore, several laboratories showed that human epithelial carcinoma cells in situ, in particular those derived from colon, stomach, and breast, express a significant amount of the sialyl Leα and sialyl Leα structures on the cell surface (10–12, 56). This raises the possibility that blood-borne metastatic tumor cells aggregate with platelets in the circulation and succeed in attaching to endothelial cells through the interaction between tumor cell surface sialyl Leα or sialyl Leα structures and selectins present on endothelial cells. The present study revealed, however, that both highly and poorly metastatic tumor cells express almost the same number of sialyl Leα determinants at saturating antibody concentrations, whereas the highly metastatic cells express more of the sialyl Leα determinants with high affinity to the antibody (Table VIII). The results therefore indicate that the role of sialyl Leα is independent of the total number of sialyl Leα structures, if sialyl Leα plays any role in the metastatic differences shown by these cell lines. It is also possible that sialyl Leα determinants on highly metastatic cell lines are recognized more favorably by mouse ELAM-1 because of a more favorable structure in underlying glycans such as polylactosaminoglycans or protein backbone. However, further studies are necessary to determine if the differences in the affinity of anti-sialyl Leα antibody binding can be extended to differences in ELAM-1 binding affinity.

It has been shown that one of the important factors that
enable tumor cells to become invasive is their ability to secrete lysosomal enzymes that can degrade surrounding extracellular matrix (58-60). This release of lysosomal enzymes could be most efficient if intact lysosomes are transported to the cell surface and the lysosomal enzymes exocytosed en masse. In fact, it has recently been shown that cytotoxic T-lymphocytes release lysosomal enzymes by fusing secretory lysosomes with plasma membrane when they attack target cells (61). In addition, we have shown recently that polylysaccharidolignans attached to lamp molecules apparently contribute to the stability of lamps, by protecting the polypeptide moiety from proteolytic digestion in the lumen of lysosomes (28). It is then most likely that secretory lysosomes can reach the cell surface more efficiently in highly metastatic cells and increase their ability to degrade extracellular matrix components and subsequent invasion. It will be interesting to determine in future studies if poly-N-acetyllactosamines on cell surface lamps are actually major ligands on metastatic tumor cells recognized by selectins and if more stable secretory lysosomes are present in highly metastatic tumor cells.

Acknowledgments—We thank Dr. Issiab J. Fidler for providing the colon carcinoma cell lines, Dr. Kentaro Maemura for his assistance in some of the experiments, Dr. Olé Hindegaul for providing the synthetic substrates, and Henny Bierhuizen for secretarial assistance.

REFERENCES

(*Continued on next page.*)
RESULTS

Structures of Glycopeptides Bound to Tomato Lectin-Saponars

Structure of non-N-acetyllactosaminyl side chains - To analyze the structure of complex A-linked oligosaccharides that were bound to tomato lectin-Saponars, the glycopeptide fractions designated as 1 in Fig. 3 were first subjected to endo-b-galactosidase digestion. Fig. 5 (right upper corner) demonstrates that intact II glycopeptides were strongly hydrolyzed (65% of total radioactivity) by endo-b-galactosidase whereas after deamidation (Figs. 5, right lower panel), the amount of released oligosaccharides is increased to 32.6% of the total radioactivity. Since this increase was not observed after neuraminidase treatment, the results suggest that fucose residues attached to N-acetylglucosamine hinder the hydrolysis. This is because endo-b-galactosidase cleaves galactose linkage much slower when N-acetylglucosamine is substituted with fucose as above (Table 1).

\[ R \rightarrow GaICl - 3Gal, 4GlcNAc - 1+3Gal \]

If N-acetylglucosamine is substituted with fucose, the poly-N-acetyllactosaminyl structure is almost completely resistant to endo-b-galactosidase digestion. Methylation analysis of [3H]-glucosamine-labeled II glycopeptides yielded a significant amount of 6-O-methylglucosamine, supporting the above conclusion (Fig. 6A).

The oligosaccharides recovered in Fractions a and b in Fig. 6 were subjected to paper chromatography. Fig. 7 reveals that fractions a contains oligosaccharides corresponding to NeuAca2-3Gal1-4GlcNAc1-3Gal1-4(Fuc1-2)Gal1-4(Fuc1-3)GlcNAc1-3Gal (peak 3), NeuAca2-3Gal1-4GlcNAc1-3Gal1-4(Fuc1-2)Gal1-4(Fuc1-3)GlcNAc1-3Gal1-4(Fuc1-3)GlcNAc1-3Gal (peaks 2 and 1), NeuAca2-3Gal1-4GlcNAc1-3Gal1-6GlcNAc1-3Gal (peak 5), and NeuAca2-3Gal1-4GlcNAc1-3Gal1-6GlcNAc1-3Gal1-6GlcNAc1-3Gal (peak 6). Among these, NeuAca2-3Gal1-4GlcNAc1-3Gal1-6GlcNAc1-3Gal and NeuAca2-3Gal1-4GlcNAc1-3Gal1-6GlcNAc1-3Gal (peak 6) are the leading compounds of peaks 3 and 2, respectively. The same analysis of glycopeptides from low metastatic clone 10A indicates that NeuAca2-3Gal1-4GlcNAc1-3Gal1-6GlcNAc1-3Gal is the major product. About 5.5% of IA2 glycopeptides yield NeuAca2-3Gal1-4GlcNAc1-3Gal (peak 5) in addition to NeuAca2-3Gal1-4GlcNAc1-3Gal1-6GlcNAc1-3Gal, and NeuAca2-3Gal1-4GlcNAc1-3Gal1-6GlcNAc1-3Gal1-6GlcNAc1-3Gal (peak 6). (Fig. 7A).

Methylation analysis of IA2 glycopeptides - Methylation analysis of IA2 glycopeptides labeled with 3H-glucosamine supports the above conclusion and a small amount of 2,4,6-tri-O-methylgalactose, 3,4,6-tri-O-methylgalactose and 3,4-di-O-methylgalactose was produced in addition to the 2,6-di-O-methylgalactose from IA2 glycopeptides (Fig. 8A, closed circles). After removal of sialic acid, the amount of 2,4,6-tri-O-methylgalactose was decreased with the concomitant increase of 2,3,4,6-tetra-O-methylgalactose (Fig. 8A, open circles, see also Table 1). The results indicate that 21.2% of the galactose is present as internal residues within linear N-acetyllactosaminyl repeats. About 15% of the galactose residues are in the branch point, 24% as (3-O-methylgalactose (3-0GalNAc1-3Gal) based on the amount of N-acetyllactosaminyl (Table II), supporting the above results obtained by anti-galactosidase reagents. Similarly, about 10% of the galactose residues are substituted with sialyl residues, based on the amount of 2,6,4-tri-O-methylgalactose (Table II).

To estimate the total number of N-acetyllactosaminyl units in the IA2 glycopeptides, the following analysis was carried out. First, methylation analysis of N-acetyllactosaminyl, which was not possible in the above analysis, was performed after acid hydrolysis or after methylation followed by acid hydrolysis. [3H] Glucose was labeled with the metabolic conversion of [3H] glucose, but the specific activity of the [3H] glucose was not analyzed (Table III). Therefore, the total number of N-acetyllactosaminyl units was calculated on the assumption that the specific activity of [3H] glucose is 0.6 in N-acetyllactosaminyl units of IA2 glycopeptides from low metastatic clone 10A. Based on this number, each glycoside derivative can be obtained (for example 3.15 (95/170) = 3.4 (Table I)). Thus, in total IA2 glycopeptides from IA2 lamp-l, on average, 2.8 moles of galactose per mole of glycopeptide. Similarly, the numbers of N-acetyllactosaminyl units were calculated for other glycopeptides. Based on the amount of 2,6,4-tri-O-methylgalactose before and after deamidation, 2.6 glycopeptides of L4 lamp-l contain, on average, 5.8 moles of sialic acid residues. In fact, the results shown in Table IV indicate that the majority of IA2 glycopeptides from L4 lamp-1 are of the triadial and disialyl type.

Estimation of N-acetyllactosaminyl substituted with hexasaccharides - The above results suggest that N-acetyllactosaminyl side chains of IA2 glycopeptides are heavily fucosylated. In order to examine the fucose residues attached to N-acetyllactosaminyl units, the following analyses were carried out. As shown in Table II and Fig. 9, the radioactivity was retained after acid hydrolysis or after methylation followed by acid hydrolysis. [3H] Fucose is formed by the metabolic conversion of [3H] glucose, but the specific activity of the [3H] glucose was not analyzed (Table III). However, the results in Table III and Fig. 4 show that the difference in fucose content among different serum samples varies 13.1:1, whereas the majority of the fucosylated glycopeptides and glycoproteins are derived from low metastasis cells are more fucosylated than those from highly metastatic cells. More definitive data on the number of fucose residues obtained by anti-fucosidase reagents. In this analysis, 10N-acetyllactosaminyl labeled samples. In this analysis, 10N-acetyllactosaminyl labeled samples. By this analysis, NeuAca2-3Gal1-4GlcNAc1-3Gal1-6GlcNAc1-3Gal1-6GlcNAc1-3Gal is produced from N-acetyllactosaminyl residues substituted with fucose at C-6 or C-4. The results shown in Fig. 4 indicate that 85% of NeuAca2-3Gal1-4GlcNAc1-3Gal1-6GlcNAc1-3Gal1-6GlcNAc1-3Gal is fucosylated. 6-O-Methylglucosamine derivatives. The 6-O-methylglucosamine was diminished after the glycopeptides were defucosylated, followed by methylation with the concomitant increase of 3,6-di-O-methylglucosamine (data not shown).

Since IA2 glycopeptides of L4 lamp-l contain 7.7 moles of N-acetyllactosaminyl, the results indicate that 3.8 moles of fucose residues are attached to the same glycopeptides contain, on average, 5.7 moles of N-acetyllactosaminyl units, indicating that 63% of the N-acetyllactosaminyl units are modified by fucose residues.

Structure of IA2 Glycopeptides Bound to DSA-Agarose

Glycopeptides IA2 were not bound to tomato lectin-Saponars but bound to DSA-Agarose column and eluted with a mixture of Chloroform and Methanol (fractions 3 and 4).

Methylation analysis of IA2 glycopeptides - The methylation analysis of [3H]-mannose-labeled glycopeptides indicated that IA2 glycopeptides consist of exclusively tetraantennary saccharides. Thus IA2 glycopeptides should contain 45 moles of terminal galactose residues (unsubstituted and fucose-substituted terminal galactose) (Table II). It is then possible to estimate that 0.5 to 0.9 moles of internal residues within linear N-acetyllactosaminyl repeats. About 15% of the galactose residues are in the branch point. These results suggest the possibility of the presence of any 2,4,6-tri-O-methylgalactose, indicating that branching galactose is absent in these glycopeptides (see also Fig. 11). The results indicate that IA2 glycopeptides contains 4 to 4.5 moles of galactose residues. The methylation analysis obtained before and after deamidation indicates that the number of sialic acid residues in IA2 glycopeptides was not constant, which is consistent with the results obtained by PAGE-Sephadex chromatography (Table IV). Methylation analysis of [3H]-glucosamine-labeled lamp-I agarose showed that compared to glycopeptides, an almost identical amount of 2,4,6-tri-O-methylgalactose was produced IA2 glycopeptides (see Fig. 6). The results indicate that approximately 2.5 moles of fucose is attached to N-acetyllactosaminyl units in IA2 glycopeptides.
Lysosomal Membrane Glycoproteins in Metastatic Carcinoma Cells

Fig. 5  Sephadex G-50 gel filtration of [3H]glucosamine-labeled IB glycopeptides before and after endo-8-galactosidase digestion. The glucosamine-labeled glycopeptides from tann-1 isolated from SP and L4 cells were fractionated as in Fig. 3. IB glycopeptides were subjected to Sephadex G-50 gel filtration before (○) and after (●) endo-8-galactosidase digestion. The second set of experiments (lower panels) were carried out after removal of trypsin and stable acid residues (designated as denatured). The amount of the released glycopeptides was expressed as a percentage of the total radioactivity.

Fig. 6  Thin-layer chromatography of methylated mucosaccharides obtained from [3H]glucosamine-labeled IB glycopeptides. [3H]glucosamine-labeled glycopeptides from tann-1 isolated from L4 cells, fractionated as shown in Fig. 5, were methylated. The resultant methylated mucosaccharides were spotted on thin-layer chromatography as described in Experimental Procedures.

A, B, C, and D are L4-IB, J2-1A1 and HL-60 tann-1 IB (2B) glycopeptides, respectively. The migration points of the methylated sugars are 1, 3-O-Methyl-N-acetylglucosamine; 2, 6-O-Methyl-N-acetylglucosamine; 3, 3,6-Di-O-Methyl-N-acetylglucosamine; 4, 4,6-Di-O-Methyl-N-acetylglucosamine. Under these conditions, 4,6-Di-O-Methyl-N-acetylglucosamine migrates almost at the same position as 3,6-Di-O-Methyl-N-acetylglucosamine. The radioactivity close to the origin is due to incomplete N-acetylation of glucosamine.

Fig. 7  Paper chromatography of glycopeptides released by endo-8-galactosidase treatment of [3H]glucosamine-labeled IB glycopeptides. The glycopeptides denoted as a in Fig. 5 were separately subjected to paper chromatography. A and C, glycopeptides from SP and L4, respectively; B and D, glycopeptides from HL-60 cells and a purified glycopeptide, respectively. The migration points of the glycopeptides are indicated by numbers as follows:


The migrations of the glycopeptides are indicated by numbers as follows:


The migrations of the glycopeptides are indicated by numbers as follows:


The migrations of the glycopeptides are indicated by numbers as follows:


The migrations of the glycopeptides are indicated by numbers as follows:

Lysosomal Membrane Glycoproteins in Metastatic Carcinoma Cells

Fig. 9  Thin-layer chromatography of methylated monosaccharides obtained from [3H]-galactose-labeled L4 glycopeptides.

(PR)-galactose-labeled glycopeptides from L4 cells, fractionated in a similar way as shown in Fig. 3, were methylated. The resultant methylated monosaccharides were separated on thin-layer chromatography as described in "Experimental Procedures." A, B glycopeptides; B, IA2 glycopeptides; C, IAI glycopeptides. Most glycopeptides (B-3; defucosylated glycopeptides) were as follows: 1. 2,4-dimethylgalactose; 2, 3,4,6-tetra-O-methylgalactose; 3, 2,4,6-tri-O-methylgalactose.

CM FROM ORIGIN

![Diagram]

Fig. 10  Sephadex G-50 gel filtration of [3H]-galactose-labeled IA2 glycopeptides before and after removal of fucose and sialic acid.

(PR)-galactose-labeled IA2 glycopeptides from SP and L4 lamp-1 molecules were subjected to Sephadex G-50 gel filtration before (-O.) and after (-P.) endo-β-galactosidase digestion. The chromatographic conditions are the same as shown for Fig. 5. The second set of experiments was made after removal of fucose and sialic acid (denoted as defucosylation).

CM FROM ORIGIN

![Diagram]

Fig. 11  Paper chromatography of glycopeptides released by endo-β-galactosidase treatment of IA2 and IA1 glycopeptides.

Oligosaccharides a shown in Fig. 10 and 12 were subjected to paper chromatography. A, IA2 glycopeptides from SP; B, IA2 glycopeptides from L4; C, IA1 glycopeptides from SP; D, IA1 glycopeptides from L4. The oligosaccharides standards are the same as shown in the legend of Fig. 7.

Table 1  Fractionation of glycopeptides labeled with various radioactive monosaccharides by heparin affinity-chromatography. The recovered radiactivity is expressed as % of the total radioactivity.

<table>
<thead>
<tr>
<th>Lamp-1</th>
<th>Gal</th>
<th>Man</th>
<th>Glcn</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>L4</td>
<td>C</td>
<td>SM</td>
</tr>
<tr>
<td>IA2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Torque bound</td>
<td>95.3</td>
<td>94.9</td>
<td>50.2</td>
</tr>
<tr>
<td>IA2 SA bound</td>
<td>11.1</td>
<td>13.5</td>
<td>15.3</td>
</tr>
<tr>
<td>IA2 SA unbound</td>
<td>11.1</td>
<td>13.5</td>
<td>15.3</td>
</tr>
<tr>
<td>IA1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Torque bound</td>
<td>9.9</td>
<td>27.1</td>
<td>30.7</td>
</tr>
<tr>
<td>IA1 SA bound</td>
<td>11.1</td>
<td>13.5</td>
<td>15.3</td>
</tr>
<tr>
<td>IA1 SA unbound</td>
<td>11.1</td>
<td>13.5</td>
<td>15.3</td>
</tr>
</tbody>
</table>

Table 2  Methylation analysis of [3H]-galactose-labeled glycopeptides from lamp-1 and lamp-6 before and after defucosylation.

<table>
<thead>
<tr>
<th>Lamp-1</th>
<th>L4</th>
<th>SP</th>
<th>C</th>
<th>SM</th>
<th>SP</th>
<th>L4</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Torque bound</td>
<td>2.42</td>
<td>2.61</td>
<td>2.42</td>
<td>2.61</td>
<td>2.42</td>
<td>2.61</td>
</tr>
<tr>
<td>IA2 SA bound</td>
<td>11.1</td>
<td>13.5</td>
<td>15.3</td>
<td>21.2</td>
<td>50.2</td>
<td>31.2</td>
</tr>
<tr>
<td>IA2 SA unbound</td>
<td>11.1</td>
<td>13.5</td>
<td>15.3</td>
<td>21.2</td>
<td>50.2</td>
<td>31.2</td>
</tr>
<tr>
<td>IA1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Torque bound</td>
<td>2.42</td>
<td>2.61</td>
<td>2.42</td>
<td>2.61</td>
<td>2.42</td>
<td>2.61</td>
</tr>
<tr>
<td>IA1 SA bound</td>
<td>11.1</td>
<td>13.5</td>
<td>15.3</td>
<td>21.2</td>
<td>50.2</td>
<td>31.2</td>
</tr>
<tr>
<td>IA1 SA unbound</td>
<td>11.1</td>
<td>13.5</td>
<td>15.3</td>
<td>21.2</td>
<td>50.2</td>
<td>31.2</td>
</tr>
</tbody>
</table>
TABLE III. Mutarotation analysis of (α)-mannose-labeled glycoproteins from Lamp-1 and Lamp-2. The analysis was based on the thin layer chromatography of methylated monosaccharide.

<table>
<thead>
<tr>
<th>Lamp</th>
<th>Temp. (°C)</th>
<th>Bound</th>
<th>Unbound</th>
<th>Bound</th>
<th>Unbound</th>
<th>Bound</th>
<th>Unbound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamp-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SP</td>
<td>DSA</td>
<td>IA2</td>
<td>IA1</td>
<td>IA2</td>
<td>IA1</td>
<td>IA1</td>
</tr>
<tr>
<td>Mesquite</td>
<td>3.4:6-O-methyla</td>
<td>1.02</td>
<td>2.00</td>
<td>1.00</td>
<td>2.00</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.4:6-O-methyla</td>
<td>1.00</td>
<td>2.00</td>
<td>1.00</td>
<td>2.00</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.4:6-O-methylb</td>
<td>0.01</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.4:6-O-methylb</td>
<td>0.01</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamp-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SP</td>
<td>DSA</td>
<td>IA2</td>
<td>IA1</td>
<td>IA2</td>
<td>IA1</td>
<td>IA1</td>
</tr>
<tr>
<td>Mesquite</td>
<td>3.4:6-O-methyla</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>1.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.4:6-O-methyla</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.4:6-O-methylb</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4 3,4:6-Trimethylmannose and 3,4:6-D-methylmannose could be separated by HPLC.
5 No correction for differences in the specific radioactivity of (α)-mannose and (α)-fucose was made.

TABLE IV. Determination of the number of static acid residues. The (α)-mannose-labeled glycoproteins were applied to a column of DEAE-dextran and fractionated by stepwise increase of the NaCl concentration.

<table>
<thead>
<tr>
<th>Lamp</th>
<th>SP</th>
<th>L4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of negative charges</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>IB</td>
<td>IA2</td>
</tr>
<tr>
<td>Lamp-1</td>
<td>0</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>5 and higher</td>
<td>3.6</td>
</tr>
<tr>
<td>Lamp-2</td>
<td>0</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>5 and higher</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* Expressed as percentage of the total radioactivity recovered.

TABLE V. Ratio of fucose/mannose of (α)-mannose-labeled glycoproteins obtained from non-metastatic (SP) and metastatic (L4) dextran tumor cell lines. The amount of fucose is expressed by taking the number of mannose being 3.0. No correction for difference in the specific activities of (α)-mannose and (α)-fucose was made.

<table>
<thead>
<tr>
<th>Lamp</th>
<th>SP</th>
<th>L4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con A</td>
<td>IB</td>
</tr>
<tr>
<td>Lamp-1</td>
<td>1.21</td>
<td>1.32</td>
</tr>
<tr>
<td>Lamp-2</td>
<td>1.30</td>
<td>1.47</td>
</tr>
</tbody>
</table>

* No correction for differences in the specific radioactivity of (α)-mannose and (α)-fucose was made.