Asparagine-linked Oligosaccharides in Murine Tumor Cells: Comparison of a WGA-resistant (WGA<sup>r</sup>) Nonmetastatic Mutant and a Related WGA-sensitive (WGA<sup>s</sup>) Metastatic Line

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ABSTRACT MDW40, a wheat germ agglutinin-resistant (WGA<sup>r</sup>) mutant of the highly metastatic tumor cell line called MDAY-D2, is restricted to local growth at the subcutaneous site of inoculation. The WGA<sup>r</sup> tumor cells acquire metastatic ability by fusing spontaneously with a normal host cell followed by chromosome segregation, a process accompanied by reversion of the WGA<sup>r</sup> phenotype (i.e., WGA<sup>s</sup>). Since lectin-resistant mutant cell lines often have oligosaccharide alterations that may affect membrane function and consequently metastatic capacity, we compared the major Asn-linked glycopeptides in WGA<sup>r</sup> and WGA<sup>s</sup> cell lines. [2-<sup>3H</sup>]mannose-labeled glycopeptides were separated into four fractions on a DEAE-cellulose column and then further fractionated on a concanavalin A-Sepharose column. Glycopeptide structures were determined by: (a) sequential exoglycosidase digestion followed by chromatography on lectin/agarose and Bio-Gel P-4 columns and (b) proton nuclear magnetic resonance analysis. The metastatic WGA<sup>s</sup> cells had a sialylated poly-N-acetyllactosamine-containing glycopeptide which was absent in the nonmetastatic mutant cell line. Unique to the mutant was a neutral triantennary class of glycopeptide lacking sialic acid and galactose; the WGN lesion therefore appeared to be a premature truncation of the antennae of the poly-N-acetyllactosamine-containing glycopeptide found in the WGA<sup>s</sup> cells. High mannose glycopeptides containing five to nine mannose residues constituted a major class in both WGN and WGA<sup>s</sup> cells. Lysates of both wild-type and mutant cells had similar levels of galactosyltransferase activity capable of adding galactose to the N-acetylglucosamine-terminated glycopeptide isolated from mutant cells; the basis of the WGA<sup>r</sup> lesion remains to be determined.

Specific changes in cell surface structures have long been associated with viral or chemical neoplastic transformation of mammalian cells (1); similar changes may also be required for benign tumor cells to acquire malignant (i.e., invasion and/or metastatic) properties (2–7). In particular, alterations in oligosaccharide structures of glycoproteins (2–5) and glycolipids (6, 7) have been observed following both chemical and viral transformation. The asparagine-linked oligosaccharides of transformed cells appear to be larger due to increased terminal sialylation (2, 8) and in some cases due to increased branching (3). Santer and Glick (5) found that chemically transformed hamster embryo cells initially had glycopeptides of the same size as the normal cell counterpart. However, after being serially transplanted in animals (i.e., in vivo selection for increased malignancy) the glycopeptides assumed a size profile similar to that of other malignant lines. The larger sialylated oligosaccharides may be more closely related to the acquisition of metastatic capacity than to the initial transformation of the cell. Consistent with this interpretation are the results of Yogeeswaran and Salk (9) who showed that the metastatic capacity of a range of murine tumor cell lines correlated with the degree of sialylation of available galactose and N-acetylgalactosamine residues on cell surface glycoconjugates.

The isolation of poorly metastatic tumor cell mutants from metastatic wild-type cells by selection in vitro for resistance...
to the toxic effects of lectins such as wheat germ agglutinin has added to the evidence that certain cell surface oligosaccharides are conducive or even necessary for expression of the metastatic phenotype. WGA-resistant (WGA') mutants of the PG19 melanoma (10), of the B16 melanoma (11), and of the MDAY-D2 tumor line (12, 13) have all been shown to be less malignant than the respective parental tumor cell lines. Mutants selected with other lectins such as ricin, leucoagglutinin (PHA-L), and concanavalin A (Con A) have shown either no reductions in their metastatic potential or even an increase (14, 15). These observations suggest that specific lesions in the WGA-binding glycoconjugates of WGA' mutants may be directly related to the decreased (11), and for some types of mutants loss of, metastatic capacity (12).

WGA binds most avidly to oligosaccharides containing sialic acid (16) and N-acetylgalcosamine (17). As might be expected, many WGA' mutants have under-sialylated Asn-linked glycoconjugates (18–20). An example of this is the poorly metastatic WGA' mutant of the B16 melanoma called Wa-4 which has three to four times less cell surface sialic acid (19) and an increase in fucose linked α1→3 to N-acetylgal cosamine (21). Wa-4 has been shown to have a 60–70-fold increase in α1,3-fucosyltransferase and it was suggested that competition between α1,3-fucosyltransferase and α2,3-sialyltransferase for Galβ1-4GlcNAc-terminated substrates could result in the observed mutant oligosaccharide structures (22).

Other structural lesions in the oligosaccharides of WGA' cells have been described (23, 24) and appear to be due to different lesions in the biosynthetic pathway for glycoconjugates. Five genetic complementation groups of WGA' Chinese hamster ovary (CHO) cells have been identified (24), each having a characteristic phenotype as determined by cross-resistance to Con A, PHA(L), and ricin, and by WGA binding to the cell surface (18). The degree of perturbation of the metastatic phenotype observed for any tumor cell mutant selected for resistance to WGA may therefore depend on the specific oligosaccharide structures of the mutant in question.

In this report, we examined the lesions in Asn-linked oligosaccharides of MDW40, a WGA' mutant of MDAY-D (i.e., an ouabain-resistant subline of MDW4) which has been shown to be completely nonmetastatic (12). In preliminary experiments, MDW4 cells appeared to be less metastatic than MDAY-D2 as indicated by the survival times of mice injected subcutaneously with cells and the number of tumor nodules in the livers of these mice (Fig. 1). However, upon closer examination, the tumor cells in the organs of MDW4-injected mice were found to be revertant for the WGA' phenotype (12), i.e., they had the following properties: they were WGA-sensitive (WGA'), retained the dominant genetic marker carried by the MDW4 subline W40T3 (i.e., ouabain resistance), had acquired 30–40 more chromosomes, had acquired host-derived histocompatibility markers (i.e., when DBA/2 F1 hybrid hosts were used), and expressed the malignant phenotype of MDAY-D2 when injected into a new group of mice (25, 26). These results clearly demonstrated that the tumor cells in metastases of MDW4-injected mice were the progeny of a host cell–MDW4 cell hybrid. By fusing with a host cell,

the recessive WGA' phenotype of MDW4 was suppressed and the hybrid acquired the metastatic phenotype. WGA' hybrid cells are a detectable minority of the tumor cell population at ~30 d after injection (26), become gradually dominant, and kill the mice by day 70. Unlike the WGA' hybrids and MDAY-D2 cells, MDW4 does not give rise to experimental metastasis when less than 10³ cells are injected intravenously (12), adding further support to the contention that the mutant is nonmetastatic. MDW4 is however tumorigenic since a small dose (i.e., 10³ cells) will grow in a subcutaneous site forming, 30 d after injection, a tumor mass with a diameter of ~0.5 cm containing more than 10⁷ WGA' MDW4 cells.

The MDW4 mutant has previously been shown to have a three- to fourfold reduction in cell surface sialic acid. Furthermore, the mutant cells attached more readily to fibronectin- and type IV collagen-coated surfaces than the WGA' hybrids or MDAY-D2 (i.e., wild-type phenotype for lectin resistance and metastasis) (20). Neuraminidase treatment of the WGA' cell lines increased their attachment to the same substrates indicating a possible role for unsialylated glycoconjugates in cell attachment which may in turn influence metastatic capacity. As a first step towards identifying specific glycoconjugates that may be involved in cell attachment and the malignant phenotype, we have compared the Asn-linked glycopeptides isolated from the WGA' mutant and related WGA' lines.

MATERIALS AND METHODS

Materials: α-[2-3H]mannose was purchased from Amersham Corp. (Oakville, Ontario, Canada) and UDP-N-acetyl-[3H]glucosamine (300 mCi/mmol) from New England Nuclear (Lachine, Quebec, Canada). Con A-Sepharose 4B was obtained from Pharmacia Inc. (Dorval, Quebec), ricin 1/agarose 120 from Miles Laboratories (Kankakee, IL), and Bio-Gel P-2 (200–400 mesh), Bio-Gel P-4 (minus 400 mesh), Bio-Gel P-6 (100–200 mesh), Chelex 100 (100–200 mesh), and AG-1-X8 (100–200 mesh) from Bio-Rad Laboratories (Mississauga, Ontario). DE52, cellulose came from Whatman Laboratory Products, Inc. (St. Louis, MO). Sigma Chemical Co. (St. Louis, MO) supplied Jack bean α-galactosidase, Jack bean β-N-acetylglucosaminidase, UDP-galactose, and UDP-N-acetylglucosamine. Pronase was purchased from Calbiochem-Behring Corp. (Montreal, Quebec) and deuterium oxide (99.96%) from Aldrich Chemical Co. (Milwaukee, WI). Endo-β-N-acetylglucosaminidase Cl from Clostridium perfringens (endo-Cl) was purified as previously described (27).

Tumor Cell Lines:

The origin and properties of the highly metastatic DBA/2 strain tumor called MDAY-D2 has been detailed in (28, 29). The WGA' nonmetastatic mutant MDW4 (laiine, Quebec, Canada) Con A-Sepharose 4B was obtained from Pharmacia Inc. (Dorval, Quebec) and deuterium oxide (99.96%) from Aldrich Chemical Co. (Milwaukee, WI). Endo-β-N-acetylglucosaminidase Cl from Clostridium perfringens was purified as previously described (27).

Glycopeptide Fractionation:

Tumor cell extracts at a density of 10⁶/ml were grown for 24 h in Eagle's alpha minimum essential medium plus 10% fetal calf serum. Duplicate 5-mL cultures received additions of 10 μCi/mL of [2-3H]mannose and the cells were harvested 24 h later, washed, and pooled with 1–5 x 10⁶ unlabelled cells of the same lineage. The cold cells were grown in 6-liter spinner flasks in Eagle's alpha minimum essential medium plus 5% fetal calf serum and were harvested at subconfluent density (i.e., approximately 5 x 10⁵ cells/ml). The cells were extracted twice with 200 ml chloroform/methanol (2:1) (vol/vol) and the residue was taken up in 0.1 M Tris HCl (pH 7.9), 1 mM CaCl₂ and digested with 1% (wt/wt) pronase under tol enone as previously described (30). After 3 d with daily additions of pronase, DNA, RNA, and glycosaminoglycans were precipitated with cetylpyridinium chloride (30). The glycopeptides were desalted on a Bio-Gel P-2 (200–400 mesh) column and again digested with pronase as above. After desalting on a Bio-Gel P-6 column (200–400 mesh), the samples were applied to a DE52 cellulose column (1 x 20

Abbreviations used in this paper: CHO, Chinese hamster ovary; Con A, concanavalin A; endo-Cl, endo-β-N-acetylglucosaminidase Cl; α-mG, methyla-d-glucopyranoside; NMR, nuclear magnetic resonance; PHA(L), leucoagglutinin; WGA', wheat germ agglutinin-resistant; WGA', WGA-sensitive.
Asn (i.e., GnGn). Biantennary glycopeptides are named according to the sugar
branching GlcNAc-transferases (34) were assayed at pH 7.0 using GlcNAc-1-
MGn) isolated from a human myeloma immunoglobulin G as described (31).

Glycosidase Digestion: Glycopeptide samples containing 10 nmol or less were digested with 0.05 U of either Jack bean \( \beta \)-galactosidase or Jack bean \( \beta \)-N-acetylgalcosaminidase or endo-Cl. For preparative columns, a small aliquot of each 1-ml fraction was used for determination of radioactivity. The recovery of radioactivity from the columns was 90-100%.

Nuclear Magnetic Resonance (NMR) Spectroscopy: Trace amounts were removed from samples by passage through 3-ml columns of Chelex 100 which had been extensively washed with water. Samples were exchanged three times with 99.96% deuterium oxide for analysis. The spectra were taken with a Nicolet 360-MHz spectrometer at the Tokyo Biomedical Centre (32).

Fractionation of Glycopeptides from WGA\'/Nonmetastatic and WGA\'/Metastatic Cells

MDW40 cells are confined to growth at the subcutaneous site of inoculation where, at low frequency, bone marrow-derived host cells hybridize with MDW40 cells (Fig. 1) (25). The WGA\' hybrids display a selective growth advantage over the mutant cells at the site of injection and readily metastasize (i.e., W40M1). This transition from a nonmetastatic tumor growth to a metastatic lesion in situ bears some similarities to the phenomena of tumor progression described for other transplantable tumors (i.e., genetic instability, phenotypic heterogeneity, and selective outgrowth of the more malignant cells) (29). Therefore we began by comparing the Asn-linked glycopeptide from MDW40 and WGA\' cells removed from a metastases in an MDW40-injected mouse (i.e., W40M1).

A aliquots of MDW40 (WGA\', nonmetastatic) and W40M1 cells (WGA\', metastatic) grown in the presence of 2-[\( ^3 \mathrm{H} \)]-mannose were pooled with approximately 5 \( \times 10^5 \) unlabeled cells of the same lineage, and glycopeptides were isolated from pronase digests of total cell proteins. The samples were desalted and applied to a DEAE-cellulose column to separate neutral and sialylated glycopeptides. The mutant had previously been shown to have three to four times less cell surface sialic acid (20) and, as expected, MDW40 had relatively more neutral glycopeptides (fraction I, Fig. 2) than did the WGA\' cells. W40M1 glycopeptides contained a fraction IV (17.5% of the total) which eluted from the DEAE-cellulose column near the end of the NaCl gradient and was not found in glycopeptides from MDW40 cells.

Glycopeptides separated on DEAE-cellulose were desalted and further fractionated on Con A/Sepharose columns. Non-biased biantennary complex and high mannose glycopeptides are known to bind to Con A/Sepharose and may be separated from tri- and tetra-antennary structures which pass unimpeded through the column (31, 34). The binding specificities of the Con A/Sepharose used in these experiments (31, 34); they elute from the origin.
The glycopeptide fraction IA from mutant cells appeared to have a highly branched (more than two antennae) oligosaccharide lacking terminal sialic acid since it bound neither to DEAE-cellulose nor to Con A/Sepharose. Sialic acid is usually attached to galactose in complex structures. The sample was therefore applied to a ricin I/agarose column with unsubstituted Gal residues (Fig. 4A); fraction IA eluted from the ricin I/agarose column in the void volume. This suggested that the nonreducing terminal residues of fraction IA may be N-acetylgalactosamine and thus the fraction was treated with β-N-acetylgalactosaminidase followed by Con A/Sepharose chromatography (Fig. 4C). Most (85%) of the previously Con A-negative fraction now bound tightly to the column and was eluted in a sharp peak with αmG indicating that terminal N-acetylgalactosamine residues had been removed to expose the trimannosyl core. The β-N-acetylgalactosaminidase-treated fraction IA was reduced in size but separated into three major peaks on a Bio-Gel P4 column (compare Fig. 4, B and D). The three peaks were pooled and digested with endoglycosidase C1 followed by chromatography on the Bio-Gel P4 column. The sample now eluted as a sharp peak coincident with authentic (Manα1-6)Manα1-3Manα1-4GlcNAc (Fig. 4D). This indicated that the size heterogeneity in the β-N-acetylgalactosaminidase-treated fraction IA was due to the peptide portion and not to incomplete glycosidase digestion.

The 1H NMR spectra of the glycopeptides of fraction IA can only be tentatively interpreted at this time. Because of the small amount of material, the best spectra were obtained at 70°C where the resonance from residual HDO obscures the fewest resonances; however, the data for reference compounds are limited at this temperature. Even with these problems, a number of conclusions can be drawn which support the interpretation of the biochemical studies. Overall this fraction appeared to be a mixture of complex triantennary glycopeptides with terminal GlcNAc residues. Very little intensity appeared at 4.47–4.48 ppm where the C1-H resonances of terminal Galβ1-4 residues are found. However, a complex of two overlapping doublets (4.585 ppm, J = 8.3 Hz; 4.561 ppm, J = 8.8 Hz; 70°C) was found with N-acetyl resonances at 2.078, 2.051, 2.049, 2.010 (70°C). Although such resonances are not unique to unsubstituted GlcNAc, they are consistent with such residues being present.

Furthermore, the branching pattern was difficult to assign with confidence. Mannose C2-H corresponding to triantennary to tetra-antennary structures were present (4.215, 4.086 ppm; 17°C) as were mannose C2-H resonances characteristic of biantennary and triantennary structures (4.255, 4.188, 4.086 ppm; 17°C) (35). There was no evidence for bisecting GlcNAc. In view of the failure of this fraction to bind to Con A/Sepharose, the most consistent interpretation is that it represents a mixture of ~33% triantennary with the Manα1-3, 2,4 disubstituted...
tuted with GlcNAc and ~66% triantennary with the Manα1-6, 2,6 disubstituted with GlcNAc (35).

Structure of Fraction IV from WGA* Cells

W40M1 glycopeptides separated into four fractions on the DEAE-cellulose column, fraction IV being the most negatively charged species and found only in the WGA* cells (Fig. 2). Most of fraction IV did not bind to the Con A/Sepharose column (Table I) suggesting that the glycopeptides had more than two antennae on the trimannosyl core. Fraction IVA (i.e., excluded from Con A/Sepharose) did not interact with ricin I/agarose; removal of terminal sialic acid by mild HCl treatment resulted in retardation by the ricin column indicating the presence of sialyl-Gal units at the nonreducing termini (Fig. 5). Treatment of sialic acid-free fraction IVA with β-galactosidase only marginally reduced the size of the glycopeptide as observed by its elution position from a Bio-Gel P-4 column (Fig. 6). If fraction IVA had the antennary structure commonly found on many complex glycoproteins (i.e., sialyl-Gal-GlcNAc), the removal of sialic acid and Gal residues should reduce the size of the glycopeptide to approximately that of the mutant GlcNAc-terminated fraction IA. This was not the case since sialic acid-free, β-galactosidase-treated fraction IVA eluted from Bio-Gel P-4 well ahead of fraction IA, indicating that the antennae on fraction IVA were not sialyl-Gal-GlcNAc (Fig. 6).

Analysis of fraction IVA by 1H NMR revealed a signal at 4.747 ppm which is characteristic for the anomic proton of GlcNAc β1-3 linked to Gal and suggests a poly-N-acetyllactosamine structure. The NMR spectra of fraction IVA made at 70°C was compared with a 70°C spectra of the poly-N-acetyllactosamine-containing complex isolated from CHO cells as described by Li et al. (reference 36 and J. Carver, manuscript in preparation). If repeating units of Galβ1-4GlcNAcβ1-3 are present in the antennae of desialylated fraction IVA, treatment with β-galactosidase followed by inactivation of β-galactosidase and treatment with β-N-acetylglucosaminidase would not reduce the glycopeptide to the Con A-binding trimannosyl core. As expected, this sequential glycosidase digestion yielded only 14% of material binding to Con A/Sepharose; repetition of the sequential digestion resulted in 40% Con A/Sepharose-bound material. When both enzymes were present in the same incubation mixture, however, 60% of the radioactivity bound to Con A/Sepharose (Fig. 7). The sample digested with both glycosidasises simultaneously (Fig. 7C) was analyzed on a Bio-Gel P-4 column and 48% of the material had the size expected for the trimannosyl...
core glycopeptide (Fig. 6). The heterogeneity remaining after glycosidase digestion may be due to fucosyl substitutions of antennae which would block complete degradation to the trimannosyl core (37). To summarize, fraction IVA from W40M1 cells contained sialylated poly-N-acetyllactosamine-containing glycopeptides with intramolecular heterogeneity due possibly to variations in the number of Galβ1-4GlcNAcβ1-3 repeating units and in substitution of the antennae (Fig. 8).

We also examined the parental tumor line MDAY-D2 in an effort to determine whether there was a possible relationship between the presence of the sialylated poly-N-acetyllactosamine-containing complex and metastatic behavior. Total pronase digests of the W40M1 (data not shown) and the parental tumor line MDAY-D2 (Fig. 9) separated on a Bio-Gel P-6 column contained a large glycopeptide eluting in the void volume. The MDAY-D2 sample was separated on DEAE cellulose and analysis of fraction IV by NMR revealed a spectra similar to that of W40M1 fraction IVA including the GlcNAc,6l-3Gallinkage (data not shown). Therefore re-expression of the poly-N-acetyllactosamine-containing structure in the hybrid W40M1 correlated with re-expression of the metastatic phenotype in these cells.

High Mannose Glycopeptides of MDW40 and W40M1

The neutral Con A/Sepharose-bound glycopeptides in fraction IC were a major component in both WGA' and WGA' cells (i.e., 31.6 and 44.7% of W40M1 and MDW40, respectively). Fraction IC from both MDW40 and W40M1 eluted from the Bio Gel P-4 column in the same position as the marker GlnM5 and had a GlcNAc/Asn ratio of approximately 2.5. An aliquot of fraction IC was subjected to β-N-acetylglucosaminidase and rechromatographed on the Bio Gel P-4 column. The glycopeptides from W40M1 were totally resistant while a minor subfracthion of the MDW40 sample was reduced in size by the glycosidase treatment (data not shown). The β-N-acetylglucosaminidase-resistant glycopeptides were the expected size for high mannose glycopeptides containing five to nine mannos residues. The β-N-acetylglucosaminidase-digestible subfraction present only in MDW40 fraction IC appeared to contain neutral nonbiected biantenary complex glycopeptides with terminal GlcNAc residues. The NMR spectra of MDW40 fraction IC shows signals at 4.55 and 4.57 ppm (Fig. 10) typical of antennary GlcNAc residues.

The NMR spectra at 23° and 70°C of fraction IC from both MDW40 and W40M1 cell lines (Fig. 10) contained resonances that can be assigned to a number of different microenvironments (Table II). The resonances for the Manβ1-4 and the 3,6 disubstituted Manα1-6 were present as one equivalent per molecule indicating that the smallest possible structure present was the conventional Manα1GlcNAc2Asn from ovalbumin. If it is assumed that all possible, Manβ, Manγ, and ManΔ isomers occur, then determination of the relative populations is not possible. However, if it is assumed that the only structures present are the postulated intermediates of high mannose processing (38) as was found for CHO cells by Li and Kornfeld (39), then the individual species can be quantified. Since the first mannoe to be removed from Manβ in the processing pathway is the 2-substituent on the Manα1-3 of the 6-arm, the integrated intensity of the resonance corresponding to this microenvironment (~5.40 ppm, 23°C) quantifies the Manβ species (~40% for MDW40 and W40M1). The next residue to be removed is the 2-substituent of the
Manα1-2 of the 3-arm resulting in the loss of the resonance characteristic of a Manα1-2 of the 3-arm resulting in the loss of the resonance characteristic of a Manα1-2-substituted Manα1-2 (~5.30 ppm, 23°C). Therefore this resonance can be used to estimate the amount of (Manβ9 + Manβ1) present and, by difference, Manβ (15% for both cell lines). Similarly the resonance at 5.148 ppm (23°C) is characteristic of the Manα1-2-substituted Manα1-6 of the 6-arm which is only present in the Manα, Manβ, and Manγ species. Conversely, the resonance at 4.917 ppm (70°C) is characteristic of an unsubstituted Manα1-6 which only occurs in Manα and Manβ. Hence the population of Manβ can be deduced and was found to be 10–15% for both cell lines. The relative populations of Manβ versus Manα can be estimated in several ways. One method is to consider the intensity at ~5.34 ppm (at 23°C). All structures except Manβ contribute one hydrogen per molecule to this resonance since it corresponds to Manα1-3 residues α1-2-substituted by either Man or Manα1-2Man. Thus the contribution of Manβ can be calculated, knowing those of Manα1-2, or the population of Manβ can be obtained as the difference in intensity between this resonance and those at ~4.77 and ~4.88 ppm (70°C), both of which arise from all species. On this basis, both cell lines had about 20–30% Manβ and 10–15% Manγ.

Of the structures considered to be present in this analysis, the only GlcNAc-transferase I acceptor would be the Manβ. Therefore, by comparing fraction IC with Manβ (ovalbumin glycopeptide pool E) as substrates for purified hen oviduct GlcNAc transferase I (41), an independent estimate of Manβ was obtained. Fraction IC from both MDW40 and W40M1 accepted approximately three times less GlcNAc compared with authentic Manβ, suggesting that 75% of the glycopeptide in fraction IC had substitutions blocking the addition of...
GlcNAc to the Manα1-3 arm (i.e., Manαβ3), or as much as 25% of fraction IC was Manα.

To summarize, ~40% of the high mannose pools of both the WGA' and the WGA' cells was Manα with 25% of Manβ and 15% each of Manβ, Manαβ, and Manβ. Li and Kornfeld (39) found similar results for CHO cells, ~36% Manα, 27% Manβ, and 36% Manβ.

**Galactosyl- and N-Acetylglucosaminyl Transferases**

The glycopeptides in fraction IA from mutant cells and fraction IV from W40MI cells appeared to have a similar GlcNAc-substituted trimannosyl core (Fig. 8). The W40MI complex structure had elongated poly-N-acetyllactosamine-containing antennae which were absent in the mutant structure due to an apparent lack of galactose. Galactosyltransferase activities in cell lysates of WGA' and WGA' cells were compared using MGn and GnGn as substrates (Table III). Mutant and wild-type cell lysates had similar activities and in addition there was no difference in galactosyltransferase activities when the GlcNAc-terminated fraction IA isolated from MDW40 cells was used as acceptor.

GlcNAc-transferases I and II (33) and GlcNAc-transferases III + IV were measured using as substrates Manα, MGn, and GnGn, respectively. GlcNAc-transferases I (i.e., Manα) was elevated in mutant cell lysates while the other transferases were unchanged. Hen oviduct membrane is known to synthesize GlcNAc-terminated oligosaccharides of a type very similar to those in fraction IA (40). It is interesting that hen oviduct membrane had a high GlcNAc-transferase I and a low Gal-transferase (with MGn) activity when compared with the tumor cells (Table III). The relative proportion of GlcNAc-to galactosyltransferases may regulate the early termination of antennae in hen oviduct but this does not appear to be the case for MDW40 cells.

**TABLE II**

| Microenvironment | W40M1 | MDW40 | \(\xi \) | Shift* | 9 8 7 6 5 |
|-------------------|-------|-------|--------|--------|--------|
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 5.373 | 5.369 | 16 | 5.404 | + - - - - |
| (6-arm) | 5.406 | 5.409 | 3, 4 | 5.34-5.35 | + + + - - |
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 5.332 | 5.331 | 1 | 5.309 | + + - - - |
| (3-arm) | 5.341 | 5.343 | 1 | 5.304 | + + - - - |
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 5.283 | 5.282 | 3 | 5.309 | + + + + - |
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 5.118 | 5.117 | 39 | 5.144 | + + + + - |
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 5.148 | 5.148 | 39 | 5.144 | + + + + - |
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 5.118 | 5.117 | 10 | 5.089 | + + + + + |
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 5.084 | 5.086 | 10 | 5.089 | - - - - - |
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 5.058 | 5.057 | 2 | 5.049 | - - - - - |
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 5.040 | 5.042 | 2 | 5.049 | - - - - - |
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 4.917 | 4.915 | 41 | 4.909 | - - - - - |
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 4.905 | 4.915 | 41 | 4.909 | - - - - - |
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 4.880 | 4.878 | 41 | 4.909 | - - - - - |
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 4.866 | 4.866 | 37 | 4.872 | + + + + + |
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 4.765 | 4.765 | 41 | 4.909 | - - - - - |
| \(\text{Man}_{1-2}\text{Man}_{3-1}\) | 4.765 | 4.763 | 37 | 4.872 | + + + + + |

* For each microenvironment the upper line gives the chemical shift at 70°C, the lower line that obtained at 23°C.
* The chemical shifts are the C1-H resonance of the residue italicized. \(\xi \) and the "shift" refer to the designation and mean chemical shift for that microenvironment as given in Carver and Grey (40).
* These columns indicate whether the microenvironment is present (+) in the structures with the number of mannoses indicated by the corresponding column heading.
* Signal obscured by residual HDO signal.

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**TABLE III**

Glycosyltransferase Activities in Tumor Cell Lysates and in Hen Oviduct Membranes

| Substrate | GlcNAc Transferase | Gal Transferase |
|-----------|--------------------|-----------------|
|           | MDW40 | fraction | IA | |
| Man | 8.1 | 6.4 | 10.3 | 11.1 | 18.6 | 16.4 | 1.9 | 3.2 | 9.6 | 23.1 | 7.6 | 25.4 | |
| MgGn | 8.8 | 3.2 | 10.3 | 7.6 | 1.9 | 9.6 | 2.8 | 3.2 | 9.6 | 23.1 | |
| GnGn | 20.7 | 7.6 | 20.7 | 23.1 | 23.4 | |
| ND | |

GlcNAc and galactosyl-transferase activities in tumor cell lysates and hen oviduct membranes (HOM) were assayed as described in Materials and Methods. The substrates were added at 20 nmol/assay except for MDW40 fraction IA where 5 nmol was used. ND, not determined.

**DISCUSSION**

WGA' mutants selected from the MDAY-D2 tumor line (12, 13), the B16 melanoma (11), and PG 19 melanoma (10) have been shown to be less malignant or nonmetastatic compared with the respective parental line, but selection with other lectins has not resulted in the isolation of nonmetastatic mutants (14, 15). Many lectin-resistant cell lines have lesions in cell surface oligosaccharide structures and it is possible that specific changes found most often in mutants selected in WGA lead to a decrease in metastatic capacity.

Many WGA mutants of CHO cells (23, 24) and of tumor lines (18–20) including MDW40 have less cell surface sialic acid than the parental lines, which may be related to decreased malignancy since the degree of sialylation of available cell surface galactose and N-acetylgalactosamine has been shown to correlate with metastatic capacity (9). Sialylation of tumor cell glycoconjugates has been shown to correlate with platelet aggregation activity (42), inversely with tumor cell susceptibility to natural killer cell lysis (43), and inversely with tumor cell adhesion to the extracellular matrix proteins fibronectin and collagen type IV (20). Each of these membrane-related properties of the tumor cell has been suggested to be an important feature of the metastatic phenotype (1). Simply the presence or absence of sialic acid may affect certain membrane functions or, more likely, both sialic acid and underlying oligosaccharide structures are involved. The galactose receptor on hepatocytes provides an example of the latter. The receptor binds to oligosaccharides terminating in galactose (mono = ≈ bi = ≈ triantennary) but not to the same oligosaccharide with terminal sialic acid (44). In a similar manner, glycoconjugates lacking sialic acid may be involved in membrane binding interactions such as tumor cell recognition by natural killer cells and tumor cell attachment to extracellular matrix proteins.

We have compared the Asn-linked glycopeptides isolated from the total cellular protein of the nonmetastatic mutant MDW40 and the in vivo derived metastatic revertant, W40M1. Approximately 40% of the [3H]mannose-labeled glycopeptides from both mutant and wild-type cells were high mannose structures with five to nine mannose residues. Proton NMR analysis revealed that this class of glycopeptide was very similar in MDW40 and W40M1 cells. However, glycopeptides bearing complex oligosaccharides were dissimilar in mutant and wild-type cells. A large fraction of the glycopeptides found in MDW40 cells was shown to be triantennary with a single GlcNAc as the antenna. Further substitutions on the antennae appeared to have produced the sialylated poly-N-acetyllactosamine-containing glycopeptides found only in wild-type cells (i.e., W40M1 and MDAY-D2). Loss of the metastatic phenotype in the mutant was accompanied by the loss of the sialylated poly-N-acetyllactosamine structure. This relationship is strengthened by the finding that reexpression of the structure in the hybrid line W40M1 was accompanied by reacquisition of the metastatic phenotype.

Since the complex oligosaccharides from MDW40 cells terminated in GlcNAc, synthesis in the mutant appeared to be inhibited at the point of galactose addition. However, the mutant and wild-type cells had similar levels of galactosyltransferase capable of adding Gal to GnGn, MgGn, and, most importantly, to the mutant structures in fraction IA. MDW40 cells thus appear to have both galactosyltransferase and suitable oligosaccharide acceptor for the enzyme, but due to either a deficiency of UDP-galactose or to an error in compartmentalization of the enzyme and substrates, the transfer of galactose to fraction IA oligosaccharides does not occur in vivo. The twofold increase in GlcNAc-transferase I activity in MDW40 cells does not in any obvious way account for the observed oligosaccharide structures in the cells. The action of GlcNAc-transferase I commits the oligosaccharides to hybrid and complex structures (45).

Clone 13, a WGA' CHO mutant, appears to have a phenotype similar to that of MDW40 (46). The mutant did not incorporate galactose into either glycolipids or Asn-linked oligosaccharides and this defect could not be attributed to a change in galactosyltransferase levels, UDP-galactose pools, or endogenous galactose acceptors. Clone 13 produces the galactose-containing tetrasaccharides sequence found in glycosaminoglycans, which is synthesized in the endoplasmic reticulum rather than in the Golgi complex where galactose is added to the antennae of Asn-linked oligosaccharides (47). Consequently, Briles (47) has suggested that a Golgi-specific defect involving compartmentalization of UDP-galactose or galactosyltransferase may be responsible for the defect in clone 13.

MDW40 cells were found to be 10-fold more sensitive to the cytotoxic effect of the terminal GlcNAc-binding lectin from *Bandeteara simplificifolia* seeds (BSII) than were wild-type cells (data not shown). Therefore, MDW40 appears to express the mutant GlcNAc terminating complex on the surface of the cell.

Amino acid heterogeneity remaining after exhaustive pronase digestion made glycopeptide sizing on the Bio-Gel P-4 column difficult as was evident from the analysis of fraction IA (Fig. 4). After endo-C1 digestion of β-N-acetylgalactosaminidase-treated fraction IA, the sample eluted from the Bio-Gel P-4 column as a sharp peak coincident with Manα1-6(Manα1-3)Manβ1-4GlcNAc. Amino acid heterogeneity also complicated the analysis of glycopeptide fraction II and III from the DES2 cellulose column. After mild acid treatment the majority of the glycopeptide in fractions II and III were again retained on DES2 cellulose suggesting that the negative charge resided in the peptide portion rather than terminal sialic acid (data not shown). Therefore fraction II and III were not analyzed further. Separation problems created by heterogeneity in the peptide portion of the glycopeptide will be eliminated in our subsequent studies by releasing the Asn-linked oligosaccharides with peptide N-glycosidase isolated from almond emulsin.
The sialylated glycopeptide found in the metastatic WGA-I cells but not in the mutant containing repeating units of Gal-GlcNAc as has been reported for Asn-linked oligosaccharides isolated from erythrocyte glycoproteins (48), K562 leukemia (49), and rat ethroleukemic cell lines (50). The inability of β-galactosidase plus β-1,3-N-acetylglucosaminidase to completely digest fraction IVA to MM suggests that there may be branching of the antennae similar to that reported for other poly-N-acetylactosamine-containing glycopeptides (37, 49). Some of the Gal residues in the antennae have been reported to have either Fucol-2(37) or Galβ1-4GlcNAcβ1-6 substitutions (49). Proton NMR analysis of fraction IV indicated an α2-3 linkage for terminal sialic acid (16), but more detailed analysis was not possible because of the considerable heterogeneity of this fraction.

Melanoma cells and fibroblasts treated with tunicamycin, an inhibitor of Asn-linked carbohydrate synthesis have been shown to be less adhesive to fibronectin-coated surfaces (51, 52). In addition, some ricin-resistant baby hamster kidney cell lines with lesions in oligosaccharide structure have been shown to be less adhesive on fibronectin (53). The decreased sialylation of the Asn-linked oligosaccharide of MDW40 has previously been implicated in the more adhesive behavior of the cells on fibronectin and type IV collagen (20), a characteristic of the cell line that may inhibit their escape from the primary subcutaneous site.

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