A tumor-associated glycosylation change in the glucose transporter GLUT1 controlled by tumor suppressor function in human cell hybrids

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SUMMARY

Studies of human cell hybrids have provided evidence that the tumorigenicity of a cervical carcinoma (HeLa) is under the control of a putative tumor suppressor on chromosome 11. Using these human cell hybrids, we found a tumor-associated glycosylation change in the glucose transporter GLUT1, which is an N-linked glycoprotein at the plasma membrane. The non-tumorigenic HeLa × fibroblast cell hybrid CGL1 and the normal diploid fibroblast WI38 expressed the 50-55 kDa GLUT1, whereas in a tumorigenic segregant hybrid, CGL4, as well as in parental HeLa cells, GLUT1 glycosylation was altered and its molecular mass was about 70 kDa. However, the altered GLUT1 glycosylation was not observed in SV40-transformed WI38 cells, suggesting a correlation between this glycosylation change and a putative tumor suppressor function. Further investigations using glycosidases, glycosylation inhibitors and lectin-affinity chromatography demonstrated that the tumor-associated glycosylation change in GLUT1 was mainly due to the increase in N-acetyl-lactosamine repeats in the N-linked oligosaccharides. In accordance with the altered glycosylation, affinity for 2-deoxyglucose in the tumorigenic CGL4 cells increased 2-fold, but there was little change in the Vmax. These results suggest there may be a functional role for the modulation by glycosylation of GLUT1 in the tumorigenic behavior of CGL4 and HeLa cells.

Key words: glucose transporter, N-glycosylation, human hybrid cell, tumor suppressor, cervical carcinoma

INTRODUCTION

Tumor formation arises as a consequence of alterations in the control of cell proliferation and disorders in the interactions between cells and their surroundings that result in invasion and metastasis (reviewed by Marshall, 1991). Alterations in genes that positively and negatively regulate cell growth contribute to the malignancy (reviewed by Fearon and Vogelstein, 1990).

Somatic cell hybrid experiments have provided some evidence supporting the notion that genetic alterations underlying neoplastic transformation result from a loss of function of normal alleles (reviewed by Harris, 1988). Stanbridge (1976) constructed karyotypically stable intraspecific human-hamster hybrids from cervical carcinoma HeLa cells and normal diploid human fibroblasts. These hybrids retained the transformed characteristics of the HeLa parent in vitro, such as growth in soft agar, serum requirement and cytoskeletal organization, but the tumorigenic phenotype was completely suppressed in vivo, thereby establishing that separate genetic mechanisms exist for the control of expression of transformed phenotypes and of the tumorigenic phenotype (Stanbridge and Wilkinson, 1978; Stanbridge et al., 1981, 1982). From these stably suppressed hybrid populations, rare tumorigenic segregants arise after prolonged passage in culture. These tumorigenic segregants have lost less than 5% of the original chromosome complement of the nontumorigenic hybrid from which they were derived (Stanbridge et al., 1981). Thus, these paired combinations of nontumorigenic and tumorigenic cell hybrids provide a useful approach by which to identify alterations and genes that may contribute to tumorigenicity.

Further analysis of these human cell hybrids showed that the tumorigenic phenotype is suppressed by the product of a putative tumor suppressor gene present on chromosome 11 (Stanbridge et al., 1981; Saxon et al., 1986). Although an altered expression of a phosphoglycoprotein, an alkaline phosphatase, is noted in tumorigenic human cell hybrids (Der and Stanbridge, 1981; Latham and Stanbridge, 1990), its role in human malignancy remains unknown. Thus, it is important to look further for functional cellular changes associated with tumorigenicity using these human cell hybrids.

An elevated level of facilitated glucose transport is a cellular change associated with cell growth and malignant transformation in vitro (reviewed by Hatanaka, 1974). Recently, several glucose transporter isoforms that mediate facilitated glucose uptake have been isolated from mammalian cells and characterized. They are integral membrane proteins with a molecular
mass of about 50-55 kDa and they possess a single heterogeneous N-linked oligosaccharide (reviewed by Bell et al., 1993). GLUT1 is the most abundant glucose transporter and is prevalent in brain and erythrocytes as well as several cultured mammalian cells including fibroblasts.

The elevated glucose transport in rat and mouse fibroblasts induced by growth factors, tumor viruses, and oncogenes such as ras and src, is mainly due to the increased GLUT1 expression at the transcriptional level (Flier et al., 1987; Birnbaum et al., 1987; Hiraki et al., 1988; Kitagawa et al., 1989). The increase in expression of the GLUT1 mRNA in quiescent mouse 3T3 cells induced by calf serum or growth factors is enhanced synergistically by TGF-β1 (Kitagawa et al., 1991), which also modulates glycosylation of the GLUT1 protein and enhances its affinity for 2-deoxyglucose (Masumi et al., 1993, 1994). These results indicate that GLUT1-mediated glucose uptake, which is required to provide an energy source for mammalian cell growth, is regulated by GLUT1 expression at the level of transcription and by glycosylation. However, the regulation and role of glucose uptake during human tumor growth as well as metastasis are poorly understood, although the expression of several glucose transporter genes for GLUT1 and GLUT3 (Yamamoto et al., 1990) as well as affinity for glucose (White et al., 1981, 1983) is modulated in some human tumor cells.

Here, we investigated human cell hybrids and found that the glycosylation of GLUT1 is modulated in a human cervical carcinoma. Furthermore, this glycosylation change as well as the tumorigenicity of the cells, may be controlled by a putative tumor suppressor on chromosome 11. The altered oligosaccharide structures of GLUT1 were also characterized.

MATERIALS AND METHODS

Materials

A rabbit antibody against the C-terminal peptide 480-492 of GLUT1 was purchased from Transformation Research, Inc., MA. An enhanced chemiluminescence kit (ECL) and 2-deoxy[1-3H]glucose (2-DG; 17 Ci/mmol) were obtained from Amersham International, enhanced chemiluminescence kit (ECL) and 2-deoxy[1-3H]glucose was purchased from Transformation Research, Inc., MA. An A rabbit antibody against the C-terminal peptide 480-492 of GLUT1 as induced by growth factors, tumor viruses, and oncogenes such as ras and src, is mainly due to the increased GLUT1 expression at the transcriptional level (Flier et al., 1987; Birnbaum et al., 1987; Hiraki et al., 1988; Kitagawa et al., 1989). The increase in expression of the GLUT1 mRNA in quiescent mouse 3T3 cells induced by calf serum or growth factors is enhanced synergistically by TGF-β1 (Kitagawa et al., 1991), which also modulates glycosylation of the GLUT1 protein and enhances its affinity for 2-deoxyglucose (Masumi et al., 1993, 1994). These results indicate that GLUT1-mediated glucose uptake, which is required to provide an energy source for mammalian cell growth, is regulated by GLUT1 expression at the level of transcription and by glycosylation. However, the regulation and role of glucose uptake during human tumor growth as well as metastasis are poorly understood, although the expression of several glucose transporter genes for GLUT1 and GLUT3 (Yamamoto et al., 1990) as well as affinity for glucose (White et al., 1981, 1983) is modulated in some human tumor cells.

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Cell culture

WI38 (human diploid normal fibroblasts), and WI38VA13 (SV40-transformed WI38) cells were obtained from JCRB (Japanese Cancer Research Resources Bank), Tokyo. The human cervix carcinoma, HeLa-S3 was obtained from Dr O. Kuge of our institution. CGL1 (non-tumorigenic) and CGL4 (tumorigenic) cells are human cell hybrids derived from cell fusion between D98/AH2 (HeLa-derived) and human diploid normal fibroblasts GM0077, which were established by Stanbridge et al. (1981). All cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Flow Laboratories, MA) containing 5% fetal calf serum (FCS, Flow Lab.), penicillin (100 U/ml) and streptomycin (100 µg/ml) in humidified 5% CO2/95% air at 37°C in 10 cm plastic dishes (Corning, NY), except WI38 cells, which were supplemented with 10% FCS. These cells were free of mycoplasma contamination.

Cell lysates, membranes and solubilized membranes

As described (Masumi et al., 1993, 1994), the cells cultured in 10 cm dishes were washed twice with PBS, scraped into sonication buffer (250 mM sucrose, 100 mM sodium phosphate, 5 mM EDTA, 1 mM PMSF, 25 mM benzamidine, 50 mM NaF, pH 7.4) and lysed by sonication (3x 30 seconds) on ice. The lysate was centrifuged at 550 g for 10 minutes to remove nuclei and unbroken cells, and the supernatant was then centrifuged at 100,000 g for 1 hour at 4°C. The pellet (whole membranes) was resuspended in membrane buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, pH 7.4). The membranes were solubilized in membrane buffer containing 2% Triton X-100 at 4°C for overnight with gentle shaking, and the supernatant (solubilized membranes) was collected after centrifugation at 100,000 g. Protein contents in cell lysates, membranes and Triton X-100-soluble membranes were determined using the BCA reagent (Pierce, IL) and bovine serum albumin as a standard, and stored at -20°C until use.

Immunoblot and lectin blot analyses

Sample proteins (15-30 µg) were diluted 1:1 with 2× sample buffer (4% SDS, 20% glycerol, 0.01% BPP, 100 mM dithiothreitol, 125 mM Tris-HCl, pH 6.8), then incubated for 30 minutes at 37°C and resolved by separation on SDS-10% PAGE gels. The proteins in the gels were electrophoretically transferred to durapore filters (Millipore Co. Ltd) as described (Masumi et al., 1993). The filters were then incubated for 1-2 hours at room temperature in TBS-Tween (500 mM NaCl, 20 mM Tris, pH 7.5 plus 0.1% Tween-20) containing either anti-GLUT1 antibody (1:1,000) and 5% skim milk or the indicated biotinyl lectin plus 3% BSA. For GLUT1 blotting, the filters were then washed twice with TBS-Tween for 10 minutes, incubated for 1 hour with HRP-conjugated anti-rabbit second antibody (Amersham), followed by washes with TBS-Tween, and visualized with the ECL-detection kit. For lectin blotting, the filters were washed twice with TBS-Tween for 10 minutes, incubated with streptavidin-peroxidase conjugate for 15 minutes at 37°C, extensively washed with TBS-Tween and visualized with tetramethylbenzidine.

Chromatography of the solubilized membrane proteins on immobilized lectin columns

The solubilized membrane proteins in Tris buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM PMSF, 2% Triton X-100) were separated on a lectin-agarose affinity column. Immobilized lectins (0.5-1 ml) were packed into 0.5× 7 cm columns. Columns containing either WGA, DSA or Con A were equilibrated in buffer composed of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.02% NaN3, and 0.5% Triton X-100. The solubilized membrane proteins (1-1.5 mg) in Tris buffer were applied to the immobilized lectin columns. The columns were washed with 5 ml of equilibrating buffer, then bound materials were eluted with the sugars or reagents as follows: 0.75 M α-methyl D-mannoside for Con A, 0.5 M N-acetylglucosamine for WGA, 1% N,N′-diacytethylchitobiose for DSA. Proteins in the column void and the bound fractions were collected and concentrated, solubilized in sample buffer for separation by SDS-PAGE and for immunoblotting as well as lectin blotting, as described above.

Digestion of glycoproteins with glycosidases

For N-glycosidase F digestion, 15-30 µg of proteins (cell lysates) were suspended in 25 µl buffer containing 10 mM EDTA, 0.75% NP-40, 10 mM β-mercaptoethanol, 10 mM sodium phosphate, pH 7.4, and 0.6 units of the enzyme. The reaction mixture was then incubated for 18 hours at 37°C. The sample proteins (10-30 µg) were also incubated with 25 µl of endo-β-galactosidase in 30 µl of 0.1 M sodium acetate buffer (pH 5.0) at 37°C for 18 hours. For sialidase digestion, 30 µg proteins were incubated in 20 µl containing 0.2 units/ml of enzyme, 0.1 M sodium acetate buffer, pH 5.0, 0.75% NP-40, 1 mM EDTA at

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37°C for 3 hours. For endoglycosidase-F (endo F) digestion, 30 µg proteins were suspended in a 20 µl reaction mixture containing 0.5% Triton X-100, 0.05% SDS, 50 mM β-mercaptoethanol, 50 mM EDTA, 0.2 mM PMSF, 10 mM sodium phosphate, pH 6.1, and 0.5 units of the enzyme, then incubated at 37°C for 18 hours. These enzyme-digested samples containing glycoproteins were resolved by electrophoresis and visualized by immunoblotting and lectin blotting, as described above.

**Cell culture with glycosylation inhibitors**

Proliferating cells cultured with DMEM and 5% FCS in 10 cm dishes were incubated for 20 hours with either deoxymannojirimycin (DMJ) at 0.05-0.2 mg/ml or swainsonine (SW) at 2.5-10 mM, lysed, then immunoblotting and lectin blotting were performed.

**Measurement of 2-deoxyglucose uptake**

Cells grown in 35 mm dishes containing DMEM and 5% FCS were washed with PBS and 2-deoxyglucose (2-DG) uptake was determined by incubation with 2-[3H]DG (0.5-1 µCi/ml) in the presence of 0.001-5 mM DG at 37°C for the indicated periods in 1 ml of glucose-free DMEM, as described (Kitagawa et al., 1989, 1991). The incorporation of the radioactivity into the cells for 5-10 minutes was linear. Kinetic constants for the 2-DG uptake were obtained by Lineweaver-Burk plotting.

**RESULTS**

**GLUT1 expression in human cell hybrids**

To investigate the role of glucose transport in human malignancy, GLUT1 expression in HeLa × fibroblast human cell hybrids was determined by immunoblotting with an antibody against C-terminal peptides specific for GLUT1 (Haspel et al., 1988; Masumi et al., 1993). Fig. 1B shows that a 50 kDa GLUT1 protein was detected in cell lysates from the non-tumorigenic CGL1 cell line, as described for GLUT1 in erythrocytes (Sogin and Hinkle, 1980; Haspel et al., 1985, 1988), mouse 3T3 fibroblasts (Masumi et al., 1993, 1994) and mouse 3T3L1 adipocytes (Schroer et al., 1986). In contrast, the molecular mass of GLUT1 was markedly increased to about 70 kDa in the tumorigenic hybrid, CGL4. Both CGL1 and CGL4 are subclonal derivatives of the parental non-tumorigenic hybrid cell, ESH5, as shown in Fig. 1A (Stanbridge et al., 1981, 1982).

These differences in the molecular mass of GLUT1 between the tumorigenic and non-tumorigenic human cell hybrids on SDS-PAGE gels were mainly due to modulation of glycosylation as we have found in TGF-β1-treated 3T3 cells (Masumi et al., 1993, 1994). Fig. 1C shows that both the 55 kDa and 70 kDa forms of GLUT1 were converted to the unglycosylated form with a molecular mass of 42 kDa (Haspel et al., 1985) when these cell lysates were directly digested with N-glycosidase F, which cleaves the N-linked oligosaccharides of glycoproteins at the Asn residue (Kornfeld and Kornfeld, 1985). The levels of GLUT1 expression were fundamentally similar among the hybrid cells, although there was some experimental variation (Fig. 1B and C).
Glycosylation changes in GLUT1 of human cell lines

To further examine the glycosylation changes in GLUT1 and their possible correlation with the tumorigenic phenotype, the form of GLUT1 expressed in several human cell lines, including parental cells from which the hybrid cells were derived, was determined. As in the tumorigenic hybrid CGL4, GLUT1 in HeLa-S3 migrated as the 70 kDa form (Fig. 2). The HeLa variant, D98/AH2, which was the parental cell line of the hybrids (Stanbridge et al., 1981) also had a 70 kDa GLUT1 (data not shown). In contrast, in the normal diploid fibroblast WI38, the molecular mass of GLUT1 was about 50 kDa, as found in the non-tumorigenic CGL1 hybrid. The molecular mass of GLUT1 in SV40-transformed WI38 cells (WI38VA13), which is transformed in vitro (Girardi et al., 1966), was also 50 kDa. These differences in size of GLUT1 were due to changes in glycosylation, since digestion with N-glycosidase F produced a 40 kDa-GLUT1 in all cases (Fig. 2). These results further support the hypothesis that the glycosylation change in GLUT1 is associated with the tumorigenic state of the cervical carcinoma HeLa cell.

Glycosidase digestion on GLUT1 in human hybrid cells

To characterize the altered oligosaccharide structures of the GLUT1 protein, digestion by several types of glycosidases was performed. Digestion of whole cell lysates containing GLUT1 with endo-β-galactosidase (endo-β-gal), which cleaves poly-lactosamine sequences at the Gal-GlcNAc linkage (Gross et al., 1983), reduced the molecular masses of the 50 kDa and 70 kDa GLUT1 in human cell hybrids similarly to about 45 kDa (Fig. 3), which may correspond to the Asn-linked mannose core (GlcNAc)n-Man3GlcNAc2- (n=2-4).

Endoglycosidase F (endo F) cleaves Asn-linked GlcNAc-GlcNAc bonds of biantennary complex type oligosaccharides, but is inactive to tri- or tetra-antennary chains (Tarentino et al., 1985). We showed that GLUT1 in human RBC, which is mainly composed of a biantennary complex oligosaccharide (Endo et al., 1990), was sensitive to this enzyme (Masumi et al., 1994). However, both GLUT1 proteins in CGL1 and CGL4 were resistant to endo F-digestion (Fig. 3), suggesting that the N-linked complex oligosaccharides of GLUT1 in human cell hybrids are mainly composed of tri- or tetra-antennary sugar chains containing poly-lactosamine sequences. We also found that the glycosylation change in GLUT1 in the tumorigenic human cells was not due to an increase in the sialic acid content, since sialidase digestion did not modulate the size of GLUT1 in either hybrid (data not shown).

Characterization of the N-linked oligosaccharide structures of GLUT1 using immobilized lectins

We examined the ability of GLUT1 to bind various immobilized lectins. Whole membrane proteins isolated from CGL1 and CGL4 were solubilized with 2% Triton X-100, and fractionated by lectin-agarose affinity chromatography. Binding was detected by means of immunoblotting as well as lectin
blotting. GLUT1 protein from both cell types did not bind to concanavalin A (Con A)-agarose (data not shown). These results indicated that there was little or no contribution of high-mannose type as well as biantennary oligosaccharides to GLUT1 glycosylation in the HeLa-derived cell hybrids, consistent with the results of endo F resistant digestion described above.

*Datura stramonium* agglutinin (DSA) binds with high affinity to tri- or tetra-antennary polylactosamine sequences in complex type N-linked oligosaccharides (Cummings and Kornfeld, 1984; Yamashita et al., 1987). The 50 kDa GLUT1 in non-tumorigenic CGL1 membranes partially (20%) bound to a DSA-agarose (Fig. 4B, lanes 1-4). In contrast, all of the 70 kDa GLUT1 from the tumorigenic CGL4 cells bound to this lectin (lanes 5-8), suggesting that the complex polylactosamine repeats are present to a higher extent.

A similar binding profile was also obtained with wheat germ agglutinin (WGA)-agarose, which binds oligosaccharides containing lactosamine structures as well as sialic acids (Gross et al., 1983). Most of the 70 kDa GLUT1 in CGL4 cells bound to this lectin (Fig. 4D, lanes 7-10), whereas a much smaller fraction of the 50 kDa GLUT1 from CGL1 bound (lanes 2-5). Lectin blotting confirmed that the DSA- and WGA-binding glycoproteins were quantitatively retained by these immobilized lectins under our experimental conditions (Fig. 4A and C). These results suggested that the increased polylactosamine content of GLUT1 from CGL4 cells is due to an increase in lactosamine repeating units and/or numbers of sugar chains containing lactosamine.

**Effects of mannosidase inhibitors on the GLUT1 synthesis**

Biosynthesis of Asn-linked oligosaccharides starts with the addition of a Glc₃Man₉GlcNAc₂ core to an Asn residue of the protein, followed by sequential digestion by glucosidases I and II to generate the common high-mannose intermediate Man₅GlcNAc₂-Asn. Subsequently, this intermediate is processed by mannosidases I and II as well as N-acetylglucosamine transferase I, forming Man₅-(Man)-ManGlcNAc₂-Asn and (Man)(GlcNAcMan)-ManGlcNAc₂-Asn, respectively.

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Fig. 4. Separation of GLUT1 protein by lectin affinity chromatography. (A) Triton X-100-solubilized membrane proteins from CGL1 (lanes 1-4) and CGL4 cells (lanes 5-8) were separated on a DSA-agarose column, then DSA-blotted. Lanes 1, 5 (S), solubilized membrane proteins (15 µg); lanes 2, 6 (U), unbound proteins (12 µg); lanes 3, 7 (B₁) and 4, 8 (B₂), DSA-bound fractions (1-2 µg). (B) GLUT1 in these fractions was determined by immunoblotting. In (C) and (D), the Triton X-100-solubilized membranes from CGL1 (lanes 2-5) and CGL4 (lanes 7-10) were also fractionated by a WGA-agarose column, followed by WGA-blotting (C) and immunoblotting for GLUT1 (D). Lanes 1, 6 (L), cell lysates (15 µg); lanes 2, 7 (S), solubilized membranes (15 µg); lanes 3, 8 (U), unbound proteins (12 µg); lanes 4, 9 (B₁) and 5, 10 (B₂), WGA-bound fractions (1-2 µg). Bound materials to DSA and WGA were eluted with 1% N,N'-diacetylchitobiose and 0.5 M N-acetylglucosamine, and recovered in two fractions (B₁ and B₂), respectively. Arrowheads indicate molecular masses of GLUT1 in kDa.
These oligosaccharides are further modified by several specific glycosyl transferases for GlcNAc, Gal, Fuc, and sialic acid to complete the complex types of oligosaccharides (reviewed by Kornfeld and Kornfeld, 1985; Rademacher et al., 1988).

Our studies of the oligosaccharide structures of GLUT1 in human cell hybrids suggest that the differences might be due to processing after digestion by α-mannosidases. We therefore examined the effects of inhibitors of mannosidases I and II on the biosynthesis of GLUT1. CGL1 and CGL4 cells were cultured with these inhibitors at the indicated concentrations. The inhibitor of mannosidase I in the Golgi apparatus, DMJ, which blocks conversion of Man₉GlcNAc₂-Asn to Man₅GlcNAc₂-Asn (Buck et al., 1974), prevented maturation of the 50 kDa and 70 kDa GLUT1 forms in the CGL1 and CGL4 cells. A 43 kDa protein, which may be a precursor containing a high-mannose oligosaccharide (Man₉GlcNAc₂-), was recognized by GLUT1 antibody (Fig. 5A). The 43 kDa GLUT1 proteins in DMJ-treated CGL1 and CGL4 cells were resistant to endo-β-gal (Fig. 5C, lanes 5, 6 and 11, 12), whereas the mature GLUT1 proteins were sensitive to this enzyme (lanes 1, 2 and 7, 8, and Fig. 3), further supporting the identity of these proteins as high-mannose intermediates.

The inhibitor of mannosidase II, SW, blocks digestion of GlcNAc-Man₅GlcNAc₂-Asn to the precursor containing GlcNAcMan₃GlcNAc₂-Asn, allowing further processing to generate hybrid oligosaccharides containing (Gal-GlcNAc)(lactosamine) repeats in SW-treated GLUT1 in both cell hybrids (Gross et al., 1983). When the cells were treated with SW for 20 hours, a broad GLUT1 band, slightly reduced in molecular mass, was found in CGL1 and CGL4 cells (Fig. 5B). These small reductions in the molecular mass of GLUT1 indicated that processing of the mannosyl residues derived from the tri-mannosyl core digestion of the Man α₁-6 arm did not contribute significantly to GLUT1 molecular mass on SDS-PAGE gels.

However, the molecular mass of GLUT1 in the SW-treated CGL4 cells remained larger than that in the drug-treated CGL1 cells (Fig. 5B). Furthermore, the presence of the Gal-GlcNAc (lactosamine) repeats in SW-treated GLUT1 in both cell hybrids was confirmed by a reduction in their mass to 43 kDa after digestion of endo-β-gal (Fig. 5C, lanes 3, 4 and 9, 10). These results revealed that the tumor-associated glycosylation change in GLUT1 was mainly due to increases in the number of N-acetyl-lactosamine repeats at least in one mannosyl sugar chain, which could be acted upon by GlcNAc- and Gal-transferases, even in the presence of SW.

### Glucose transport activity with the modulated GLUT1

To examine the functional relevance of the altered glycosylation of GLUT1 in human tumor cells, transport activity in the hybrid cells was determined. As shown in Fig. 6A, twice as much 2-deoxyglucose was taken up at 1 μM by tumorigenic CGL4 cells than by non-tumorigenic CGL1 cells. Further kinetic studies on 2-deoxyglucose uptake by the hybrid cells demonstrated that the affinity for this glucose analogue in the tumorigenic CGL4 cells expressing the larger GLUT1 was about 2-fold higher than that of the non-tumorigenic hybrid CGL1, but there was little change in Vₘₐₓ values (Fig. 6B).

![Fig. 5. Effects of glycosidase inhibitors on the GLUT1 synthesis.](Image)

(A) Proliferating CGL1 (lanes 1-4) or CGL4 cells (lanes 5-8) were incubated with deoxymannojirimycin (DMJ) at zero (lanes 1, 5), 0.05 (lanes 2, 6), 0.1 (lanes 3, 7) and 0.2 mg/ml (lanes 4, 8), then the GLUT1 expressed in the cell lysates was determined by immunoblotting. (B) A similar experiment was performed with swainsonine (SW), at the following concentrations: lanes 1, 5, zero; lanes 2, 6, 2.5 μg/ml; lanes 3, 7, 5 μg/ml; lanes 4, 8, 10 μg/ml. (C) Cell lysates from CGL1 (lanes 1-6) or CGL4 (lanes 7-12) which had been exposed to one of the inhibitors were incubated with lanes 2, 4, 6, 8, 10, 12) or without (lanes 1, 3, 5, 7, 9, 11) endo-β-gal, then GLUT1 expression was determined by immunoblotting. The exposed inhibitors are as indicated: lanes (1, 2, 7, 8), no inhibitor (−); lanes (3, 4, 9, 10), 10 μg/ml SW (S); lanes (5, 6, 11, 12), 0.2 mg/ml DMJ (D). Arrowheads indicate molecular masses of GLUT1 in kDa.
This decrease in the $K_m$ for 2-DG uptake may account for the increased glucose transport in tumorigenic CGL4 cells.

**DISCUSSION**

Human cancers develop as a consequence of genetic changes that perturb normal cellular growth control mechanisms (reviewed by Marshall, 1991; Weinberg, 1991). One such alteration is the loss or inactivation of tumor suppressor genes. Currently, human tumor suppressor genes have been isolated from chromosomes 5 (APC), 11 (WT-1), 13 (Rb), 17 (p53 and NF-1) and 18 (DCC), and their functions in tumor growth and progression have been extensively characterized. In addition, studies utilizing somatic cell fusion and microcell transfer indicate that tumorigenicity of the HeLa cervical carcinoma cell line is controlled by a putative tumor suppressor gene on chromosome 11 (Stanbridge et al., 1981; Saxon et al., 1986). Although recent studies on loss of heterozygosity (LOH) in human tissues from cervical carcinoma suggest that the likely chromosomal position of this gene(s) is 11q22-24 (Hampton et al., 1994), the function and nature of the gene(s) remains unknown.

The present study suggests that the glycosylation change in the membrane protein GLUT1 with altered affinity to 2-deoxyglucose is associated with a putative tumor suppressor gene involved in the tumorigenicity of a cervical carcinoma. This is based upon the following evidence: (1) a normal human lung fibroblast W138 and the tumor-suppressed cell hybrid CGL1 expressed a 50 kDa GLUT1 similar to that described in other cell systems (Haspel et al., 1985; Birnbaum et al., 1986; Schroer et al., 1986; Masumi et al., 1993, 1994). (2) In contrast, the tumorigenic cell hybrid CGL4, as well as a parental HeLa cell line expressed a 70 kDa GLUT1 due to alterations in N-linked glycosylation (Figs 1 and 2). (3) This glycosylation-dependent increase in the molecular mass of GLUT1 was not evident in SV40-transformed WI38 (Fig. 2) or human hepatoma HepG2 cells (Haspel et al., 1985; Birnbaum et al., 1986), suggesting that the glycosylation change in GLUT1 is not a general alteration found in human tumors or transformed cells, although more studies are needed to establish a direct correlation between the glycosylation change in GLUT1 and a tumor suppressor function on chromosome 11.

The oligosaccharide structures of many glycoproteins may play important roles in cell-cell recognition, protein secretion and targeting of proteins into cellular organelles (reviewed by Rademacher et al., 1988). Changes in the N-linked oligosaccharides on glycoproteins are associated with malignant transformation and metastasis (Yamashita et al., 1985; Dennis et al., 1987; Rademacher et al., 1988). One consistently observed alteration in neoplastic transformation is the expression of larger N-linked oligosaccharides, probably due to the induction of some specific glycosyltransferase(s). However, most of these studies have been carried out with whole glycoproteins in various types of transformed cells. The present study indicated a marked structural change in the N-linked sugar chains of GLUT1 protein in a tumorigenic human cervical carcinoma. Der and Stanbridge (1981) have also reported the tumor-specific expression of a 75 kDa glycoprotein at the cell surface of CGL4 cells. Recently, this glycoprotein has been identified as an intestinal alkaline phosphatase, and its expression in CGL4 cells is, however, regulated at the level of transcription (Latham and Stanbridge, 1990, 1992).

The 70 kDa GLUT1 in the tumorigenic hybrid cells had a higher affinity for DSA and WGA (Fig. 4), and was sensitive to endo-β-galactosidase, which also cleaved polylysosome sequences present in control 50 kDa GLUT1 (Fig. 3). Both GLUT1 proteins in the human cell hybrids probably contain tri- or tetra-antenary oligosaccharide chains with N-acetyl-lactosamine repeating units, since these glycoproteins were resistant to endo F-digestion (Fig. 3). Based upon the results of glycosidase digestions and lectin binding, we speculate that the induction of a higher molecular mass GLUT1 (70 kDa) in the human tumorigenic hybrid cells is due to the modulation of complex oligosaccharides containing the N-acetyl-lactosamine repeating units. The results of α-mannosidase inhibitor studies indicated that extension of the N-acetyl-lactosamine repeating units is, at least in part, responsible for the tumor-associated glycosylation change in GLUT1 (Fig. 5), which may involve modulation of galactosyl- and GlcNAc-
transfereases. However, it appears that the glycosylation change may exhibit substrate specificity, since the expression of total glycoproteins, examined by binding of various lectins, was not drastically changed in these HeLa-derived cell hybrids (our unpublished data). Further analysis of the modulated oligosaccharide structures, as well as identification of the glycosyltransferase(s) involved in the GLUT1 glycosylation in human cell hybrids, will be an important subject for future studies.

Although the functional relevance of the modulated glycosylation of GLUT1 to the transport activity and tumor growth remains unknown, these and other results support the notion that the glycosylation change in GLUT1 may influence the affinity for D-glucose. White et al. (1981, 1983) have previously reported kinetic studies of hexose uptake in various cell lines including isogenic hybrids between normal and tumorigenic cells, both human and murine. In every case, including the HeLa-fibroblast cell hybrids, tumorigenicity, as defined by the ability of cells to grow in vivo, is linked to a decrease in the Michaelis constant for hexose uptake. The molecular mass of GLUT1 in these tumorigenic cell hybrids and their parental tumor cells is more heterogeneously distributed probably due to a glycosylation change (Bramwell et al., 1990). Consistent with these results, we found in this study that the affinity for 2-DG of a tumorigenic human cell hybrid (CGL4), which expressed a larger form of GLUT1, was increased by 2-fold compared to that in a non-tumorigenic cell hybrid (CGL1) (Fig. 6). We previously reported a reduction in $K_m$ for 2-DG in TGF-$\beta$-treated 3T3 cells producing highly glycosylated GLUT1 (Masumi et al., 1994). Moreover, a mutated GLUT1 protein lacking N-linked oligosaccharides has reduced affinity for 2-DG (Asano et al., 1991). The modulation of GLUT1 glycosylation does not interfere with the distribution of the protein inside the cells and it is exclusively localized at the plasma membrane (Masumi et al., 1994).

These results suggest that the modulation of GLUT1 glycosylation at the cell surface may play a functional role in tumor growth and metastasis by increasing the availability of glucose as a local environmental nutrient. However, the possibility that other members of the glucose transporter family are also involved in promoting HeLa cell growth cannot be ruled out, since some mammalian cells express several transporters (Rhoads et al., 1988; Kaestner et al., 1989; Charron et al., 1989; Yamamoto et al., 1990; Bell et al., 1993). In addition, the increased amounts of GLUT1 regulated at the transcriptional level, as described in the oncogene-induced transformation of rodent fibroblasts (Flier et al., 1987; Birnbaum et al., 1987; Hiraki et al., 1989), would also support tumor growth and maintenance.

In this context, it is notable that TGF-$\beta_1$ induces the enhanced expression of GLUT1 mRNA as well as the modulation of GLUT1 glycosylation in mouse 3T3 fibroblasts, and stimulates their growth (Kitagawa et al., 1991; Masumi et al., 1993, 1994). Since the TGF-$\beta_1$-induced glycosylation change in GLUT1 resembled that seen in the human cell hybrids in terms of sensitivity to glycosidases and lectin-affinity, a common cellular protein(s) such as a glycosyl-transferase(s) and its modulator molecule(s) could be involved in these different systems.

TGF-$\beta_1$ is a potent modulator of cell growth and differentiation in a number of diverse systems (reviewed by Massagué, 1990). There may be a direct interaction between TGF-$\beta_1$ signals and cyclin-dependent cell cycle (CDCC) control (reviewed by Marshall, 1991; Sherr, 1994; Hunter and Pines, 1994). Considering these points, speculation of a functional linkage between a putative tumor suppressor gene on chromosome 11 and CDCC control is attractive. Further studies based upon the glycosylation change in GLUT1 protein of the human cell hybrids will complement the search for identification of the putative tumor suppressor and its function in the control of human tumor cell growth.

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