The level of cell surface β1,4-galactosyltransferase I influences the invasive potential of murine melanoma cells

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SUMMARY

β1,4-Galactosyltransferase I (GalT I) is localized on the leading lamellipodia of migrating cells, where it associates with the cytoskeleton and facilitates cell spreading and migration on basal lamina matrices. It has previously been reported that a variety of highly metastatic murine and human cell lines are characterized by elevated levels of cell surface GalT I, although the intracellular biosynthetic pool is similar between cells of high and low metastatic potential. In this study, we examined whether the elevated expression of surface GalT I characteristic of metastatic cells is instructive or incidental to their metastatic behavior by altering the expression of surface GalT I and by the use of GalT I-specific perturbants.

Surface GalT I levels were positively and negatively altered on murine melanoma cells by either overexpressing full-length GalT I or by homologous recombination, respectively. The consequences of altered surface GalT I expression on cell invasion in vitro and lung colonization in vivo were determined. Increasing surface GalT I expression on cells of low metastatic potential to levels characteristic of highly metastatic cells recapitulated the highly invasive phenotype in vitro. Alternatively, decreasing surface GalT I expression on highly metastatic cells to levels characteristic of low metastatic cells reduced their invasive behavior in vitro and metastatic activity in vivo. Within the physiological range of surface GalT I expression, the invasive potential of each clonal cell line correlated strongly with the level of surface GalT I expressed.

As an independent means to assess the involvement of surface GalT I in metastatic behavior, cells were pretreated with two different classes of surface GalT I perturbants, a competitive oligosaccharide substrate and a substrate modifier protein. Both perturbants inhibited metastatic colonization of the lung, whereas control reagents did not. Finally, as reported by others, surface GalT I on metastatic cells selectively interacted with one glycoprotein substrate, or ligand, of approximately 100 kDa, the identity of which remains obscure. These results show that the elevated expression of surface GalT I characteristic of highly metastatic cells contributes to their invasive phenotype in vitro and to their metastatic phenotype in vivo.

Key words: Metastasis, Cell surface, Galactosyltransferase

INTRODUCTION

Metastasis is a highly complex process in which neoplastic cells detach from the primary tumor, invade the extracellular matrix, enter the circulation, extravasate, and form secondary tumors at distant sites. Given the extreme complexity of the metastatic cascade, it is not surprising that only a very small proportion of cells from the primary tumor are able to complete all of the requisite steps. The well-accepted theory of clonal dominance recognizes that cells of many different metastatic potentials exist in the primary tumor (Liotta and Stetler-Stevenson, 1989; Fidler, 1990). The biological heterogeneity of cancer is well-described for other properties as well, such as sensitivity to drug therapy, and presumably results from random mutation and subsequent selection of individual cells (Liotta and Stetler-Stevenson, 1989; Ponta et al., 1994).

Since metastasis requires diverse cell-cell and cell-matrix interactions, it is not surprising that several different cell adhesion molecules have been implicated in this process (Edward, 1995). Cell adhesion molecules that recognize protein ligands on adjacent cells or in the extracellular matrix have received the most attention. In this regard, detachment from the primary tumor is accompanied by down regulation of the cell adhesion molecules E-cadherin and α4β1 integrin, among others (Takeichi, 1993; Fearon and Pierceall, 1995; Qing et al., 1994). Association and migration through the extracellular matrix are associated with enhanced expression of the integrins αvβ3, α3β1 and α5β1, immunoglobulin superfamily members ICAM-1 and MUC18, as well as degradative enzymes such as metalloproteinases (Edward, 1995; Seftor et al., 1992; Crawford and Matrisian, 1994). The significance of these cell surface components to the metastatic cascade is shown by the ability of several peptide competitive inhibitors to block experimental and/or spontaneous metastasis
in vivo (Iwamoto et al., 1987; Trikha et al., 1994; Saiki et al., 1989; Humphries et al., 1986).

Cell adhesion molecules that recognize carbohydrate ligands are some of the most poorly understood surface receptors, especially in the field of metastasis. Lately, they have attracted increased attention based upon their roles in a variety of cellular interactions, including lymphocyte homing (Lasky, 1992), cell migration, neurogenesis, and other aspects of development (Shur, 1994). The selectins and galectins are two families of cell surface lectins implicated in tumor cell-endothelial interactions and tumor emboli formation in the circulation, respectively (Vestweber, 1992; Raz and Lotan, 1987).

Glycosyltransferases are another class of carbohydrate-binding proteins that are expressed on the cell surface, where they are thought to mediate several cell-cell interactions during development (Roseman, 1971; Roth et al., 1971; Shur et al., 1998). Glycosyltransferases are traditionally viewed as intracellular enzymes responsible for oligosaccharide biosynthesis. However, a few specific glycosyltransferase are also expressed on the cell surface, the best studied being β1,4-galactosyltransferase I (GalT I).

One well defined system in which GalT I functions as a surface receptor is during cell migration on basal lamina matrices. After cells initially adhere to laminin via the integrins, surface GalT I levels increase severalfold, it associates with the cytoskeleton, and it localizes to the leading edge of the cell where it facilitates spreading and migration (Eckstein and Shur, 1989). GalT I must form stable associations with both its glycoside ligand in the extracellular matrix as well as with the cytoskeleton in order to mediate migration. If either interaction is perturbed, either by specific antagonists or by the expression of dominant negative truncated GalT I proteins, cellular interactions are compromised (Evans et al., 1993; Eckstein and Shur, 1992; Runyan et al., 1986, 1988; Romagnano and Babiarz, 1990; Appeddu and Shur, 1994a,b). Both the surface-localized and intracellular isoforms of GalT I recognize terminal N-acetylgalcosamine (GlcNAc) residues, although the substrate specificity of the surface enzyme is much more restricted than the Golgi enzyme, usually recognizing only one predominant substrate (Shur et al., 1998; Passaniti and Hart, 1990; Penno et al., 1989).

Since GalT I functions in diverse cellular interactions which contribute to metastasis, such as migration and/or cell adhesion, it is not unreasonable to propose that surface GalT I may participate in the metastatic cascade. In this regard, it is interesting that two reports have shown that GalT I is increased on the surface of metastatic cells (Passaniti and Hart, 1990; Penno et al., 1989). In seven of eight human adrenal carcinoma cell lines (Penno et al., 1989), the level of surface GalT activity correlates with their relative degree of in vitro invasiveness, a good predictor of in vivo metastatic ability (Albini et al., 1987). Additionally, in two B16 murine melanoma sublines with distinct in vivo metastatic abilities, surface GalT activity is elevated on the highly metastatic variant, whereas total cellular GalT I activity representing predominantly Golgi activity is similar in both cell types (Passaniti and Hart, 1990). Similar to that reported for surface GalT I in other systems, the surface enzyme on both human adrenal carcinoma and murine melanoma cells selectively interacts with one predominant glycoprotein substrate of ~100 kDa (Passaniti and Hart, 1990; Penno et al., 1989). It has been suggested that this surface GalT I substrate may be a lysosomal associated membrane protein (LAMP) (Passaniti and Hart, 1990; Penno et al., 1989); a particularly interesting possibility, since LAMP expression is characteristic on the surface of highly metastatic cells (Fukuda, 1991; Dennis and Breitman, 1989).

Although the above data show that GalT I expression is elevated on the surface of a variety of human and murine metastatic cells, there is currently no direct experimental evidence that addresses whether the elevated expression of surface GalT I is critical or incidental to the metastatic phenotype. The goal of this study was to examine the relationship between elevated GalT I expression and invasive behavior in vitro and metastasis in vivo. Furthermore, the suggestion that surface GalT I’s major substrate on these cells is a LAMP was also addressed using immunoprecipitation and immunoblotting analyses.

**MATERIALS AND METHODS**

**Cell lines**

Two sublines of the K1735 murine melanoma line with distinct metastatic abilities were provided by Dr I. J. Fidler, M. D. Anderson Cancer Center. The parental (P) cell line of low metastatic potential was developed by chemical carcinogenesis and UV irradiation of C/H/HeN mice (Kripke, 1979). The highly metastatic (M2) line was obtained from a lung metastasis after spontaneous metastasis of K1735 P cells (Talmadge and Fidler, 1982). Cells were grown under conventional conditions in minimal essential medium (MEM) supplemented with 5% fetal bovine serum, sodium pyruvate, non-essential amino acids, L-glutamine, MEM vitamin solution, 100 i.u./ml penicillin, 100 μg/ml streptomycin, and 0.1 mg/ml kanamycin (complete MEM; all reagents from Gibco).

**GalT I activity**

Cell surface and total GalT I activities were assayed as described (Runyan et al., 1986) on cells resuspended in medium B (140 mM NaCl, 4 mM KCl, 20 mM Hepes, pH 7.2) with 0.4% bovine serum albumin (BSA) and a mixture of protease inhibitors (PIC). Total cellular GalT I levels were measured after lysing cells in 30 mM N-octyl-β-D-glucopyranose in medium B for 30 minutes on ice. For surface-associated activity, only viable (>90% Trypan Blue exclusion), intact cells were used. In both cases, lysed or intact cells were incubated at 37°C in medium B/BSA/PIC for the indicated time in the presence of 0.1 mM UDP[3H]Gal, 10 mM MnCl2, 1 mM 5’AMP (to competitively inhibit UDPGal degradation), with or without the sugar acceptor substrate, 30 mM GlcNAc. 3H-Galactosylated product was separated from UDP[3H]Gal and other reaction products by high-voltage borate electrophoresis and quantified by liquid scintillation counting. Specific activity was calculated as cpm [3H]-galactosylated product per 10⁶ cells. Control cells (P, parental) averaged 2,933 cpm product per 10⁶ cells per hour.

**Cell transfections**

The PDLGT construct, which includes the entire GalT I coding region, was co-transfected into K1735 P and M2 cells along with the neomycin resistance gene (neo) using calcium phosphate (Lopez et al., 1991). As controls, cells were transfected with neo alone. 5×10⁴ K1735 P or M2 cells were plated on tissue culture plastic 100 mm dishes 16 hours prior to transfection. Cells were co-transfected with 20 μg of DNA, washed twice with CMF-BSS, and then incubated for 24 hours with complete MEM medium. The cells were grown in complete MEM with G418 for 14 days and individual colonies of
resistant cell lines were selected, expanded, and screened. G418-resistant colonies were screened by both RNase protection assay and cell surface GaIT I assay to confirm transgene and protein expression, respectively.

**Ribbonuclease protection assay**

32P-labeled antisense RNA probe was synthesized from a –239 to +287 linearized fragment of GaIT I DNA template under the control of a T7 promoter using standard in vitro transcription (MAXIscript, Ambion) (Lopez et al., 1991). Radiolabeled probe (70,000 cpm) was hybridized with 10 µg cellular RNA. After RNase treatment, the remaining double-stranded RNA was precipitated, denatured, and analyzed on an 8 M urea, 5% polyacrylamide gel (RPA II kit, Ambion). The probe protects two endogenous long GaIT I fragments of approximately 490 and 327 nucleotides, and a 293 nucleotide fragment of the PDLGT transgene.

**Homologous recombination**

In order to reduce the level of GaIT I, an insertion type targeting vector was used to create K1735 M2 cell lines that have one mutated GaIT I allele. The insertion vector contains a homologous region that spans the first exon of GaIT I, but which has a point mutation in the first ATG from which translation of the long isoform of GaIT I is initiated (Lu and Shur, 1997). The vector also contains the neo and herpes simplex virus thymidine kinase (tk) genes outside of the homologous region for positive and negative selection. This vector was transfected into K1735 M2 cells using electroporation. Briefly, K1735 M2 cells were harvested, washed once in serum-free MEM, and 5x10^6 cells were resuspended in 1 ml of phosphate buffered saline (PBS) and 10 µg DNA. Cells were incubated for 5 minutes at room temperature and subjected to an electrical field of 230 V. The cells were incubated at room temperature for an additional 5 minutes and plated on tissue culture plastic 100 mm dishes at several dilutions to facilitate individual colony selection. Cells were grown in complete MEM with G418 for 14 days. Individual resistant colonies were selected, expanded, and screened by Southern analysis. Colonies with the proper insertion were grown in complete MEM without G418 for two days and then grown with FIAU for 14 days. Resistant colonies selected, expanded, and again screened by Southern analysis. Cells which underwent intrachromosomal recombination to produce one mutant GaIT I allele were expanded and assayed for GaIT I activity.

**Southern analysis**

DNA was isolated from the cells described above and 15 µg was digested overnight with HindIII. The digested DNA was subjected to agarose gel electrophoresis and blotted onto nitrocellulose. A 32P-labeled DNA probe was synthesized using random decamer oligonucleotide primers (DECAprime II, Ambion) and a 500 bp DNA template which spans the first GaIT I ATG codon (Lu and Shur, 1997). The nitrocellulose membrane was hybridized with the radiolabeled probe, washed to remove unbound probe, and exposed to film.

**In vitro invasion of basal lamina**

The invasive potential of cell lines was assessed using a modified Boyden chamber (Albini et al., 1987). These chambers consist of a T7 compartment. After overnight incubation at 37°C in a humidified, 5% CO2 incubator, cells on the upper surface of the filter were scraped off. The filter was fixed and stained and the remaining (invasive) cells counted by light microscopy.

**In vivo metastasis assays**

K1735 cells were harvested and resuspended in medium B. Cell suspensions of 1 or 2x10^4 cells/0.2 ml were injected into the lateral tail veins of unanesthetized C57/HeN mice (syngeneic with K1735 cells). The mice were monitored daily and sacrificed 6 weeks post injection. The lungs were removed and fixed in Bouin's solution. Tumors were counted under a dissecting microscope. Each experimental data point represents the average (± standard error) of 10-15 treated mice.

**GaIT I perturbation studies**

K1735 M2 cells were harvested as above and resuspended in medium B containing surface GaIT I perturbants. This suspension of cells and reagent was then injected into the lateral tail vein of C57/HeN mice and metastases assayed as described previously. Competitive substrates for GaIT I (Oxford Glycosystems) included an N-linked complex asialo-agalacto-tetraantennary oligosaccharide with four terminal GlcNAc residues. Two oligosaccharidase were used as controls; one has a structure identical to the competitive oligosaccharide except that the GlcNAc residues are masked by galactose, the second oligosaccharide is an oligomannose of similar molecular mass as the competitive oligosaccharide. In parallel studies, cells were pretreated with the substrate modifier a-lactalbumin (Sigma); carboxy-α-lactalbumin (Sigma) was used as control. Cells were incubated with either 16 µM oligosaccharide or 69 µM a-lactalbumin to yield a concentration of 1.6 µM and 6.9 µM after injection, respectively.

**Analysis of the surface galactosylated products**

To identify the surface GaIT I substrates, viable, intact cells were incubated at 37°C in medium B/PIC/BSA with 30 µM UDP[3H]Gal, 10 mM MnCl2, 1 mM 5'AMP, without GlcNAc, for 30 or 60 minutes. The cells were washed five times in medium B to remove any unused substrate, solubilized in sample buffer, and analyzed by SDS-PAGE and fluorography.

To metabolically label cells, 5x10^6 K1735 cells were labeled overnight with 0.01 mCi/ml [35S]methionine. The cells were solubilized on ice for 30 minutes in 100 µl lysis buffer (0.15 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.6, 0.5% NP40, 0.5% Triton X-100, PICS). The cell lysates were precleared with 1 µM normal rabbit sera and 10% activated Staphylococcus aureus (Sigma) to remove non-specific binding. The precleared lysate was then incubated overnight at 4°C with 10 µl anti-LAMP-1 or anti-LAMP-2 rabbit antibodies (provided by Dr Thomas August, The Johns Hopkins University School of Medicine) or normal rabbit serum. The resulting immunocomplexes were precipitated with Protein A-agarose (Pierce Chemicals), washed, solubilized in sample buffer, and analyzed by SDS-PAGE and fluorography (Maillet and Shur, 1993).

To compare the electrophoretic behavior of LAMP-1 and 2 with the GaIT I substrates, total cell lysates of both unlabeled cells and cells surface labeled as above with UDP[3H]Gal were solubilized in sample buffer and separated on SDS-PAGE. Molecular mass markers were run adjacent to both labeled and unlabeled lanes. The gel was transferred to nitrocellulose and the lanes containing the labeled and unlabeled material separated. The labeled lanes were immediately exposed to film. Those containing the unlabeled cell lysate were subjected to immunoblotting with the anti-LAMP-1 or 2 antisera (primary antibody), washed, and incubated with 125I-goat anti-rabbit antisera (secondary antibody). Alternatively, the ECL protocol was used (Amersham) in which the secondary antibody was labeled with horseradish peroxidase and detected via chemiluminescence. In both cases, the blot was washed after incubation with secondary antibody and exposed to film. Molecular mass markers were aligned to compare the relative migration of the GaIT I substrates with LAMP-1 and 2.
RESULTS

Previous studies have demonstrated a positive correlation between the level of cell surface β1,4-galactosyltransferase I (GalT I) activity and the invasive and metastatic abilities of murine melanoma and human adrenal carcinoma cells (Passaniti and Hart, 1990; Penno et al., 1989). In contrast, Golgi-associated GalT I is similar in cells of low and high metastatic potential. Given that surface GalT I functions during normal cell migration by serving as a receptor for extracellular glycoside ligands, the selective increase of surface GalT I on highly metastatic cells raises the possibility that it may contribute to the metastatic phenotype. To address this possibility, we examined the effects of molecularly increasing and decreasing cell surface GalT I levels on in vitro invasion and in vivo metastasis. As an independent means of assessing surface GalT I function, we examined the effects of GalT I-specific perturbants on metastatic behavior.

Studies by others suggested that the major surface-localized substrate for GalT I on metastatic cells is a 90-110 kDa protein and that the interaction of GalT I with this protein correlates with invasive and metastatic ability (Passaniti and Hart, 1990; Penno et al., 1989). These authors suggested that this protein may be a lysosomal associated membrane protein (LAMP). To address this in more detail, surface GalT I substrates were analyzed in untransfected murine melanoma cells as well as in transfected cells that overexpress surface GalT I.

**Highly metastatic K1735 cells have higher surface GalT I activity than low metastatic cells**

Two variants of the K1735 cell line were used (Kripke, 1979). The parental (P) cell line of low metastatic potential was derived from the original tumor, and the highly metastatic M2 line was derived from a lung metastasis (Talmadge and Fidler, 1982). Similar to that reported for other metastatic cells (Passaniti and Hart, 1990; Penno et al., 1989), surface GalT I levels were approximately 2.5-fold higher on the highly metastatic M2 variant, whereas total cellular GalT levels, which represent predominantly Golgi-associated enzyme, were approximately equal in both P and M2 cells (Fig. 1).

**Overexpressing surface GalT I on K1735 cells increases their invasiveness in vitro**

If, in fact, the elevated level of surface GalT I characteristic of highly metastatic M2 cells contributes to their invasive and metastatic phenotype, then increasing the expression of surface GalT I on low metastatic P cells should phenocopy the highly metastatic M2 variant. Surface GalT I was elevated in both P and M2 metastatic variants by transfection with cDNAs encoding the long GalT I cDNA (PDLGT). Cells were cotransfected with the neo selectable marker and and stable transfectants selected. Two independent P PDLGT cell lines (6-9 and 6-10) and two independent M2 PDLGT cell lines (5-8 and 5-11) were chosen. Clonal populations of K1735 P (2-9, 2-11, 2-14, 2-15) and M2 (1-2, 1-7, 1-10) cells transfected with neo alone were selected as controls.

RNase protection assays confirmed that PDLGT transfectants expressed the transgene (Fig. 2A). The probe used in the assay is able to distinguish by RNA produced by the endogenous long GalT I transcript (490 and 327 bp protected fragments) and that produced by the transgene (293 bp protected fragment). Cell surface GalT assays confirmed that the PDLGT transfectants had increased cell surface GalT I levels. The K1735 P PDLGT cell lines had 1.5- to 2-times the surface GalT I of P neo controls (Fig. 2B). K1735 M2 PDLGT cells had 2- to 4-times the surface GalT I of M2 neo controls (Fig. 2C).

The in vitro invasive ability of the PDLGT transfectants and neo controls was assayed using a modified Boyden chamber. Cells placed in the top compartment invade through a Matrigel-coated filter in response to a chemoattractant in the bottom compartment. The invasive cells that reach the underside of the filter are counted. The invasive ability measured by this assay has been shown to mimic the in vivo metastatic ability for several different cell lines (Albini et al., 1987).

As expected, K1735 M2 neo controls were more invasive than K1735 P neo controls (Fig. 3A). Importantly, both K1735 P and M2 PDLGT transfectants were more invasive than their respective neo controls (Fig. 3A). K1735 P PDLGT clones were approximately 3-times as invasive as P neo controls; K1735 M2 PDLGT clones were 3.5- and 8.3-times as invasive as P neo controls. Thus, increasing surface GalT I expression produced a concomitant increase in invasive behavior, and the degree of increased invasiveness correlated well (R=0.918) with the increase in surface GalT I expression (Fig. 3B).

The in vitro assays indicate that the level of surface GalT I facilitates the invasiveness of murine melanoma cells. We next determined whether this correlation also applied to metastatic potential in vivo. K1735 P neo and PDLGT transfectants were assayed for in vivo metastatic ability by ‘experimental’ metastasis assays in which tumor cells are intravenously injected into syngeneic mice. Due to extreme variability using these in vivo assays, additional P PDLGT (6-7 and 6-22) clones were isolated and characterized as above (Fig. 2B). When the P PDLGT cells were compared to their neo controls, their average metastatic ability (16±3.0 tumors/mouse) was greater than that of controls (10±2.3 tumors/mouse), similar in degree to the 1.5-2-fold increase in surface GalT I expression on the PDLGT transfectants (Fig. 2B). Although provocative, the variability intrinsic to these assays was such that the differences were too small to be significant (P=0.07).
Surface GalT I influences invasion

Fig. 2. RNase protection assays and GalT I enzyme activity confirm GalT I transgene expression in PDLGT clones. (A) RNase protection assay on total cellular RNA from K1735 M2 and P cells transfected with the PDLGT construct was done in order to confirm transgene expression. Protected RNA fragments corresponding to the endogenous long form of GalT I (490 and 327 bp) are found in all lanes, including neo-transfected controls. A protected RNA fragment corresponding to the transgene (293 bp) is found only in the PDLGT clones. (B) P cells transfected with PDLGT (6-7, 6-9, 6-10, 6-22) have 1.5- to 2-fold increased surface GalT activity as compared with P neo controls. (C) M2 cells transfected with PDLGT (5-8, 5-11, 5-67, 5-69, 5-71, 5-74, 5-80) have 1.8- to 4.2-fold increased surface GalT activity as compared with M2 neo controls.

Fig. 3. K1735 PDLGT clones that overexpress cell surface GalT I are more invasive than controls. (A) PDLGT clones of P (6-9 and 6-10) and M2 (5-8 and 5-11) and P and M2 neo controls were assayed for invasiveness in a modified Boyden chamber. All cell lines are compared to P neo control, defined as onefold. As expected, M2 neo controls were more invasive than P neo controls. P cells transfected with PDLGT were approximately 3-fold as invasive as their neo controls. M2 cells transfected with PDLGT were approximately 3.5- and 8.3-fold as invasive as the P neo controls. (B) There is a direct correlation between surface GalT I expression and invasive ability among multiple, independent PDLGT and neo transfectants. Surface GalT activity (from Fig. 2) and in vitro invasion (from Fig. 3) are plotted as fold P neo control. The correlation coefficient = 0.918. Bars = s.e.m.
When the metastatic potential of K1735 neo and P PDLGT cells was examined using ‘spontaneous’ metastasis assays, in which cells were injected into the flanks of mice to allow primary tumors to form, it was obvious that the initial growth rates of these cells, as assayed by daily measurement of tumor size, were significantly greater in PDLGT cell lines as compared to neo controls. Initial growth rates of the P PDLGT transfectants were almost twice that of the P neo controls, since P PDLGT transfectants required only 16 days to form a detectable tumor, whereas controls required 26 days ($P < 0.01$; Fig. 4A). However, the difference in growth rate diminished with increasing time, i.e., the time between detection of the primary tumor and resection of the tumor at 1.5 cm was similar in all cell lines: 21 days for P neo controls and 23 days for P PDLGT transfectants ($P = 0.84$; Fig. 4B). Analysis of GalT I expression in resected tumors showed that enzyme levels returned to control values (data not shown). Thus, although of interest, the increased growth rate in vivo and subsequent loss of elevated GalT I expression, precluded the use of the ‘spontaneous’ metastasis assays in this study.

**Decreasing surface GalT I on K1735 M2 cells reduces their invasive ability in vitro**

The above results demonstrate that elevating surface GalT I on metastatic cells leads to a concomitant increase in their invasive potential as assayed in vitro. Lung colonization following tail vein injection suggested a similar increase in metastatic potential, but the variability in these assays precluded any significant observation.

A more compelling approach to assessing surface GalT I function in invasive and metastatic behavior is to determine
whether decreasing surface GalT I expression leads to reduced invasive activity in vitro and/or metastasis in vivo. We tested this possibility by reducing surface GalT I expression on highly metastatic K1735 M2 cells to levels characteristic of low metastatic K1735 P cells. Homologous recombination was used to mutate one GalT I allele in K1735 cells so that it could no longer make RNA corresponding to the long GalT I isoform. K1735 M2 cells were transfected with an insertion type targeting vector, selected with G418, and screened by Southern analysis for insertion of the vector (Fig. 5A). Those cells which had the proper insertion were selected for spontaneous intrachromosomal recombination with FIAU and again screened by Southern analysis. Three cell lines, derived from two independent insertion events, were identified by Southern analysis that contained the proper mutation. When these cells were assayed for GalT I activity, the levels for mutant cells were 0.5- to 0.7-fold that of controls ($P<0.02$ in all cases) (Fig. 5B).

K1735 cells with decreased surface GalT I levels were assayed for in vitro invasiveness as described earlier. All three K1735 M2 cell lines with decreased surface GalT I were less invasive than K1735 M2 neo controls, ranging from 0.35- to 0.62-fold M2 neo control (Fig. 6). The invasive ability of all of these cell lines were significantly lower than M2 neo controls ($P<0.05$ in all cases), and were not significantly different from each other. Thus, reducing surface GalT I levels on highly invasive M2 cells produced a concomitant decrease in their in vitro invasiveness. Furthermore, the relative decrease in K1735 M2 cell invasive behavior (~50%) was remarkably similar to the relative decrease in surface GalT I expression (~50%).

Reducing cell surface GalT I on K1735 cells reduces their metastatic ability in vivo

K1735 M2 cells with reduced surface GalT I levels were injected intravenously and lung metastases determined. All three M2 clones characterized by reduced surface GalT I expression showed reduced metastasis, relative to M2 neo control clones (Fig. 7); two of these (B10-2, B10-4) had highly significant reduced metastatic potential ($P<0.005$). When data from all three cell lines were averaged, metastasis of K1735 M2 cells with reduced surface GalT I levels was reduced by 50% ($P=0.032$), similar to the metastatic potential characteristic of K1735 P controls. The proportionality of metastatic potential to the expression of surface GalT I is again illustrated by the fact that decreasing surface GalT I expression on K1735 cells to levels characteristic of K1735 P cells (i.e. a 50% reduction) leads to a 50% reduction in metastatic behavior.

Surface GalT I perturbants reduces in vivo metastasis

The ability to reduce the the in vitro invasive activity and the in vivo metastatic activity of melanoma cells by reducing their expression of surface GalT I lends support to the hypothesis that surface GalT I facilitates the invasive/metastatic phenotype. As an independent means to assess surface GalT I function, the effects of two different classes of GalT I-specific perturbants were determined on the metastatic potential of highly metastatic K1735 M2 cells.
An oligosaccharide with terminal GlcNAc residues inhibits in vivo metastasis. A complex asialo-agalacto-tetraantennary oligosaccharide with four terminal GlcNAc residues (A, oligoGlcNAc) and two control oligosaccharides, one with an identical structure but with the GlcNAc residues masked by galactose (B, oligoGal) and an oligomannose of similar molecular mass (C, oligoMan), were used to treat cells as described in the text. The control oligosaccharides did not affect metastasis levels, but the oligoMan, were used to treat cells as described in the text. The control, carboxymethyl-α-lactalbumin, did not affect metastasis levels, but α-lactalbumin inhibited metastasis by 75% (P<0.01). The metastatic potential of each cell line was assayed in 10-15 mice; bars = standard error.

In one series, K1735 M2 cells were pretreated with 16 μM asialo-agalacto-tetraantennary oligosaccharide with four terminal GlcNAc residues (oligoGlcNAc) that serve as competitive substrates for GalT I. Two oligosaccharides were used as controls; one with an identical structure but with the GlcNAc residues masked by galactose (oligoGal) and an oligomannose of similar molecular mass (oligoMan) (Fig. 8). The suspension of cells and oligosaccharides was injected intravenously to produce a final concentration of ~1.6 μM oligosaccharide (assuming a serum volume of 2 ml), and lung metastases measured as previously described. Cells without any oligosaccharide treatment were also injected as a control.

Seven independent experiments were conducted, using 5-20,000 cells treated with either nothing, control oligosaccharides, or oligoGlcNAc, each one of which was injected into 10-15 mice. In all instances, mice injected with cells treated with the control oligosaccharides had similar number of metastases as mice injected with untreated cells. However, mice injected with cells treated with the GlcNAc-bearing oligosaccharide had 55% less metastases than the control animals (P<0.01) (Fig. 8).

In a second experimental series, K1735 M2 cells were treated with the GalT substrate modifier protein, α-lactalbumin (69 μM). Control cells were either untreated or treated with carboxymethyl-α-lactalbumin, which no longer has GalT substrate modifier activity. The cell suspensions were injected intravenously into mice to produce a final concentration of ~6.9 μM reagent. Carboxymethyl-α-lactalbumin treatment had no effect on the degree of lung metastasis, but α-lactalbumin treatment reduced metastasis by 75% (P<0.01) (Fig. 9).

Collectively, these results show the following. First, elevating the expression of surface GalT I on cells of low metastatic potential to levels characteristic of highly metastatic cells leads to a consequent increase in their in vitro invasive ability. Second, decreasing surface GalT I expression on highly metastatic cells to levels characteristic of low metastatic cells produces a low metastatic phenotype, as assayed by both in vitro invasion assays and in vivo metastasis assays. Third, perturbing surface GalT I function with either a competitive glycoside substrate or with a substrate modifier protein decreases metastatic potential in vivo.

**Surface galactosylation of a 90-110 kDa protein correlates with in vitro invasive ability**

Previous studies (Passaniti and Hart, 1990; Penno et al., 1989) have reported that the major substrate for surface GalT in both murine melanoma and human adrenal carcinoma cells is a 90-110 kDa protein. The ability of adrenal carcinoma cells to invade through Matrigel in vitro and murine melanoma cells to metastasize in vivo after subcutaneous injection correlated with GalT’s interaction with this protein. The identity of the 90-110 kDa protein is unknown; however, preliminary data reported by others suggested that it may be a lysosomal membrane protein (LAMP) (Passaniti and Hart, 1990).
M2 cells were either unlabeled or labeled with ability of these cell lines (see Fig. 3). (B) K1735 surface GalT I and the ~100 kDa substrate another substrate of ~50 kDa is detected, but it is primarily with a 90-110 kDa protein. (In this gel, analyzed by SDS-PAGE. Surface GalT I interacts with a doublet at ~100 kDa, similar to that reported by others (Passaniti and Hart, 1990; Penno et al., 1989). (Subsequent experiments showed that this protein(s) runs between 90 and 110 kDa and behaves as either a doublet or single band depending on the percentage of acrylamide in the gel.)

PDLGT-transfected and neo control K1735 M2 and P cells were assayed as above for in vitro galactosylation (Fig. 10A). There are several galactosylated proteins, but the predominant protein(s) runs as a doublet at ~100 kDa, similar to that reported by others (Passaniti and Hart, 1990; Penno et al., 1989). (Subsequent experiments showed that this protein(s) runs between 90 and 110 kDa and behaves as either a doublet or single band depending on the percentage of acrylamide in the gel.)

To determine if the 90-110 kDa protein(s) is a LAMP, cells were either surface-labeled with UDP[3H]Gal, or metabolically-labeled with [35S]methionine as a positive control, and immunoprecipitated with anti-LAMP-1 or anti-LAMP-2 antibodies. Although the antibodies precipitated the appropriate size proteins in [35S]methionine-labeled cells (116 and 112 kDa) (not shown), there were no immunoprecipitated proteins in the UDP[3H]Gal-labeled cells. Furthermore, unlabeled K1735 cell lysates and cells labeled with UDP[3H]Gal were electrophoresed on the same gel to directly compare the electrophoretic mobility of the 90-110 kDa 3H-galactosylated protein(s) to LAMP-1 and LAMP-2. The gel was cut and the lanes with unlabeled proteins were immunoblotted with anti-LAMP-1 antibody, stripped, and then subjected to another immunoblot with anti-LAMP-2 antibody. The side of the gel with 3 H-galactosylated proteins was subjected to fluorography and the electrophoretic mobility compared directly by aligning molecular mass markers resolved on both sides of the gel (Fig. 10B). LAMP-1 and the 90-110 kDa GalT I substrates behave as distinct proteins. There is partial overlap of the GalT I substrate(s) with LAMP-2 suggesting that it could be a subpopulation of LAMP-2, but this seems unlikely given the discrepancy between their electrophoretic characteristics.

In order to investigate this further, GalT I substrates in untransfected and transfected K1735 P and M2 cells were identified by in vitro galactosylation. K1735 M2 and P cells were harvested and incubated with UDP[3H]Gal to radiolabel proteins that interact with GalT I. The cells are washed, lysed, and analyzed by SDS-PAGE (Fig. 10A). There are several galactosylated proteins, but the predominant protein(s) runs as a doublet at ~100 kDa, similar to that reported by others (Passaniti and Hart, 1990; Penno et al., 1989). (Subsequent experiments showed that this protein(s) runs between 90 and 110 kDa and behaves as either a doublet or single band depending on the percentage of acrylamide in the gel.)

PDLGT-transfected and neo control K1735 M2 and P cells were assayed as above for in vitro galactosylation (Fig. 10A). There is a general correlation between 3 H-galactosylation of the 90-110 kDa protein(s) and in vitro invasive ability (see Fig. 3A).

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**DISCUSSION**

Metastasis is a multifaceted process characterized by complex phenotypic changes, some of which are mediated by alterations in cell surface components. Previous studies have shown that the level of cell surface β1,4-galactosyltransferase I (GalT I) activity correlates with in vitro invasive ability of human adrenal carcinoma cells and with in vivo spontaneous metastatic ability of B16 murine melanoma cells (Passaniti and Hart, 1990; Penno et al., 1989). Similarly, in this study we found that surface GalT I activity is higher on more invasive and metastatic K1735 M2 murine melanoma cells, relative to low metastatic K1735 P variants. Importantly, in all cases, the activity of the Golgi enzyme remains unchanged.

In order to determine whether the elevated surface GalT I characteristic of highly metastatic cells is critical or incidental to their invasive and/or metastatic behavior, we examined the effects of molecularly manipulating surface GalT I expression and of GalT I perturbants on invasive and metastatic behavior. Surface GalT I expression was elevated on cells of low metastatic potential to levels characteristic of highly metastatic cells by overexpression of full-length GalT I (PDLGT). Similarly, surface GalT I levels were reduced on highly metastatic cells to levels characteristic of low metastatic cells by homologous recombination. In all instances, the level of surface GalT I dictated the cell’s invasive behavior in vitro. K1735 P cells expressing the PDLGT construct had elevated GalT I activity and increased invasive ability in vitro, whereas M2 cells with reduced GalT I activity had invasive activity similar to low metastatic P cells.

In contrast to that seen using in vitro invasion assays, elevating surface GalT I expression on cells of low metastatic potential only slightly increased their in vivo metastatic potential following intravenous injection. These results are not unexpected given the complexity of metastasis, which requires the function of many different molecules, any one of which could be rate-limiting. In marked contrast, however, reducing surface GalT I expression on highly metastatic M2 cells decreased their metastatic behavior in vivo. As an independent means to assess surface GalT I function during metastatic
The interaction between surface GalT I and its major substrate, a 90-110 kDa protein(s), has been shown to correlate with invasion in vitro and spontaneous metastatic ability in vivo (Passaniti and Hart, 1990; Penno et al., 1989). Similar to that reported by others, the predominant substrate for surface GalT I on K1735 cells is a 90-110 kDa protein(s), and galactosylation of this protein(s) was greater in the highly metastatic M2 cells as compared to the low metastatic P cells. The identity of the 90-110 kDa GalT I substrates(s) remains unclear, but the suggestion that it may be a LAMP is inconsistent with the present studies.

Surface GalT I is but one of many surface components that participate in the metastatic cascade (Edward, 1995; Takeichi, 1993; Fearon and Pierceall, 1995; Qing et al., 1994; Seftor et al., 1992; Iwamoto et al., 1987; Trikha et al., 1994; Saiki et al., 1989; Humphries et al., 1986; Raz and Lotan, 1987). Furthermore, during the past year it has become apparent that GalT I is but one member of a rapidly expanding family of β,1,4-galactosyltransferases (GalT II-VI) (Almeida et al., 1997; Lo et al., 1998; Sato et al., 1998). Nevertheless, previous results have shown that GalT I activity is upregulated on highly metastatic cells (Passaniti and Hart, 1990; Penno et al., 1989), and results presented here show that the degree of metastasis is, in fact, influenced by the relative expression of surface GalT I. This then raises the possibility that strategies to block surface GalT I function with competitive inhibitors, or to reduce its expression through gene targeting, may provide one avenue to inhibit metastatic progression. Furthermore, the realization that many other β,1,4-galactosyltransferases exist raises the intriguing possibility that they may also contribute to the metastatic cascade. This awaits direct testing.

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