Elevated β1,4-Galactosyltransferase I in Highly Metastatic Human Lung Cancer Cells

IDENTIFICATION OF E1AF AS IMPORTANT TRANSCRIPTION ACTIVATOR*

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The elevated levels of β1,4-galactosyltransferase I (GalT I; EC 2.4.1.38) are detected in highly metastatic lung cancer PGLH7 cells compared with its less metastatic partner PGBE1 cells. Decreasing the GalT I surface expression by small interfering RNA or interfering with the surface of GalT I function by mutation inhibited cell adhesion on laminin, the invasive potential in vitro, and tyrosine phosphorylation of focal adhesion kinase. The mechanism by which GalT I activity is up-regulated in highly metastatic cells remains unclear. To investigate the regulation of GalT I expression, we cloned the 5′-region flanking the transcription start point of the GalT I gene (−1653 to +52). Cotransfection of the GalT I promoter/luciferase reporter and the Ets family protein E1AF expression plasmid increased the luciferase reporter activity in a dose-dependent manner. By deletion and mutation analyses, we identified an Ets-binding site between nucleotides −205 and −200 in the GalT I promoter that was critical for responsiveness to E1AF. It was identified that E1AF could bind to and activate the GalT I promoter by electrophoretic mobility shift assay in PGLH7 cells and COS1 cells. A stronger affinity of E1AF for DNA has contributed to the elevated expression of GalT I in PGBE1 cells. Stable transfection of the E1AF expression plasmid resulted in increased GalT I expression in PGLH7 cells, and stable transfecants migrated faster than control cells. Meanwhile, the content of the β1,4-Gal branch on the cell surface was increased in stably transfected PGLH7 cells. GalT I expression can also be induced by epidermal growth factor and dominant active Ras, JNK1, and ERK1. These data suggest an essential role for E1AF in the activation of the human GalT I gene in highly metastatic lung cancer cells.

The enzyme β1,4-galactosyltransferase I (GalT I; EC 2.4.1.38) is a constitutively expressed type II membrane-bound glycoprotein in vertebrates (1). It is unusual that it resides in two distinct subcellular compartments, the trans-Golgi network and the cell surface (2, 3). In the trans-Golgi complex, GalT I is one of the key enzymes involved in the sugar chain synthesis that catalyzes the transfer of galactose from UDP-Gal to terminal N-acetylgalcosamine, forming the Galβ1–4GlcNAc structure (4). Cell surface GalT I acts as a recognition molecule and participates in a number of cellular interactions, including neurite extension, cell growth, sperm-egg interaction, cell spreading, and migration (5–9).

Neoplasms undergo various changes in the carbohydrate moieties of their glycoconjugates, which indicate that the glycosyltransferases themselves may change in malignancies. Consistent with this hypothesis, the importance of specific sialyltransferases, fucosyltransferases, N-acetylgalcosaminyltransferase in tumorigenesis, and metastasis has been demonstrated (10–12).

Although the precise role of oligosaccharides in metastasis is presently unknown, accumulated evidence has shown that a number of highly metastatic murine and human cell lines are characterized by the elevated levels of cell surface GalT I (13, 14). In seven of eight human adrenal carcinoma cell lines, the levels of GalT I correlate with their relative degree of in vitro invasiveness. Additionally, in two B16 murine melanoma sublines with distinct in vivo metastatic abilities, cell surface GalT I activity is elevated in the highly metastatic variant. Moreover, the degree of metastasis is actually influenced by the relative expression of cell surface GalT I (15). Increasing cell surface GalT I expression in cells of low metastatic potential promoted their invasive potential in vitro, and decreasing the cell surface GalT I expression in highly metastatic cells reduced their invasive potential in vitro and metastatic potential in vivo. In a nude mouse model, the number of peritoneal dissemination foci of the antisense GalT I-transfected ovarian tumor cells was smaller than that of the control cells, which indicated that GalT I was involved in the invasive and metastatic potentials of ovarian cancer (16).

However, the mechanism by which GalT I activity is differentially up-regulated in highly metastatic cells is still unknown. Metastasis of cancer cells is a complex process involving multiple steps (17). Metastatic characteristics are partly derived from the deregulation of genes whose normal role is to...
control the division, differentiation, and migration of embryonic cells (18). The Ets transcription factor family has been reported to be involved in tumor metastasis through enhancement of angiogenesis and the expression of genes such as vascular endothelial growth factor, urokinase plasminogen activator, matrix metalloproteases, and integrins in a variety of cancer cell lines and tumor tissues (19–21). Recent studies demonstrated that Ets-1 played a significant role in regulating N-acetylglucosaminyltransferase V expression in a variety of cancer cells and might be involved in tumor metastasis via the up-regulation of N-acetylglucosaminyltransferase V (22). In this study, we sought to determine which transcription factor was preferentially involved in the human GaIT I gene up-regulation in highly metastatic lung cancer cells. Differential GaIT I expression was detected in PGLH7 and PGBE1 cells, two lung cancer cell sublines with different metastatic potentials. Our results indicated that the up-regulation of GaIT I in highly metastatic cells was mediated by E1AF on the GaIT I promoter.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes, bovine calf serum, RPMI 1640 medium, Trizol reagent, and the mammalian expression vector pcDNA3.0 was from Invitrogen. G418, PMSF, aprotinin, pepstatin, and epidermal growth factor (EGF) were from Sigma. Prime-A-Gene random primer labeling kit was from Promega. Hybond-CN+ nylon membrane, [α-32P]dATP, [γ-32P]dATP, and the enhanced chemiluminescence (ECL) assay kit were from Amersham Biosciences. Sialidase was from Roche Applied Science. Takara RNA PCR kit (AMV version 2.1) and Takara MutanBEST kit was from Takara. PEA3 antibody (sc-113 and sc-113X), anti-human FAK, and anti-human FAK-P antibody were from Santa Cruz Biotechnology. Anti-human β1 integrin antibody was from Pharmingen. Anti-GFP antibody was purchased from Roche Applied Science. Anti-human F-actin antibody was from Oncogene. Anti-mouse HRP secondary antibody and anti-rabbit HRP secondary antibody were purchased from New England Biolabs. Other reagents were commercially available in China.

**Cell Lines and Cell Transfections**—PGLH7 and PGBE1 cells, two cell sublines isolated from the metastatic human lung giant cell carcinoma (PG) with different spontaneous metastatic potentials (23), were obtained from the Department of Pathology, Peking University Health Science Center, and were cultured in RPMI 1640 medium containing 10% bovine calf serum, 100 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified CO2 incubator (5% CO2, 95% air). COS1 cell were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 50 μg/ml streptomycin). Cell transfections were performed by the Lipofectamine (Invitrogen) method according to the manufacturer's instructions. 48–72 h after transfection, cells were harvested. For stable transfection, 72 h after transfection the cells were selected in the RPMI 1640 medium containing G418 (400 μg/ml). After a 2–3-week growth in G418-containing medium, the individual G418-resistant clones were selected and expanded.

**Plasmids**—The 1.2-kb fragment containing LgGaIT I cDNA was cut out with HindIII and XhoI from pcDNA3.0-GaIT I (24) and was then subcloned into myc-pcDNA3.1 vector and EGFP/N1 vector. TLGT-GFP mutant was then derived from myc-pcDNA3.1-LGait I by PCR amplification. The primers were as follows: TLGT-GFP-S, 5'-AATCTCGAGATCCGCGGACC-3'; TLGT-GFP-AS, 5'-GTCGCGGCCACAAGTT-3'. The entire open reading frames of human E1AF, Ets-2s, and ETV5 genes were obtained from the PGBE1 cells total RNA by RT-PCR. Total RNAs were extracted from PGBE1 cells with Trizol reagent. Reverse transcription was performed according to the instructions included with the Takara RNA PCR kit (AMV version 2.1). The PCR amplifications employed 35 cycles with steps at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1.5 min with Primer 5'-ATCCTCGAGATCCGCGGACC-3' and E1AFas (5'-AATCTCGAGATCCGCGGACC-3') and E1AFas (5'-AATCTCGAGATCCGCGGACC-3') and E1AFas (5'-AATCTCGAGATCCGCGGACC-3') and E1AFas (5'-AATCTCGAGATCCGCGGACC-3'). The Myc-tagged E1AF expression vector was constructed by inserting E1AF cDNA sequence between the HindIII and Xhol sites of myc-pcDNA3.1. The primers used are listed as follows: E1AF-S, 5'-ATAAAGCTTATGGACGGGTTTTATGATCA-3'; E1AF-AS, 5'-ATAAAGCTTATGGACGGGTTTTATGATCA-3'. Amplification was carried out for 22–27 cycles under saturation, each at 94 °C, 45 s; 60 °C, 45 s; 72 °C, 1 min in a 50-μl reaction mixture containing 2 μl each cDNA, 0.2 μl each primer, 0.2 μl dNTP, and 2.5 units of TaqDNA polymerase. After amplification, 10 μl of each reaction mixture was analyzed by 1% agarose gel electrophoresis, and the bands were visualized by ethidium bromide staining. The PCR products for GaIT I were 1197 bp.

**Preparation of cDNA Probe and Northern Blot Analysis**—To prepare the GaIT I cDNA probe, RT-PCR products were separated and recovered from agarose electrophoresis. After purification and quantification, it was labeled with [32P]dATP, using a Prime-A-Gene random primer labeling kit (Promega) according to the manufacturer's instructions. PCR products were denatured and performed agarose gel electrophoresis (25). Briefly, 40 μg of total RNA was separated on formaldehyde gels and transferred to Hybond-N+ nylon membrane. The membranes were hybridized with a GaIT I fragment as the probe and glyceraldehyde-3-phosphate dehydrogenase fragment as an internal control. The blotted membranes were washed and exposed to x-ray film (Kodak) with an intensifying screen at −80 °C for 72 h.

**Preparation of Nuclear Extracts**—Nuclear proteins were isolated according to the method of Schreiber et al. (26). Briefly, cell pellets were resuspended in 400 μl of buфер A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.5 mM PMSF) on ice for 15 min, and then 25 μl of 10% Nonidet P-40 was added. After centrifugation, the nuclear supernatants were collected, and protein concentration was determined by the method of Lowry et al. (27). A total of 30 μg of protein from each sample was electrophoresed by 10% SDS-PAGE and transferred to a PVDF membrane. After blocking with TBS containing 5% nonfat milk and 0.1% Tween 20, the membrane was incubated with the primary antibody at 4 °C overnight. After washing with TBS containing 0.1% Tween 20 three times, each for 5 min, the membrane was then incubated with horseradish peroxidase (HRP)-labeled secondary antibody for 2 h at room temperature. The membrane was then developed by using the enhanced chemiluminescent (ECL) detection systems.

**Immunoprecipitation of β1 Integrin**—The cultured cells were washed with cold PBS and lysed by the addition of 200 μl of lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 100 mM NaF, 1 mM MgCl2, 1.5 mM EGTA, 1% Nonidet P-40, 10 μg/ml leupeptin and peptatin, and 1 mM PMSF). Cell lysate containing 500 μg of protein (determined by the method of Lowry) was incubated with 2 μg of monoclonal antibody to β1 integrin at 4 °C for 1 h. Then 20 μl of Protein G Plus-agarose suspension was added, and the sample was further incubated at 4 °C for 3 h to immunoprecipitate the integrin, followed by centrifugation and washing of the pellet. Finally, the protein of β1 integrin samples was adjusted to the same concentration (30 μg/ml). The immunoprecipitated integrin subunits were treated with neuraminidase to remove the terminal sialic acids of the N-glycans on the integrins by using a routine method in our laboratory. After washing, the 0.45-μg sample was subjected to 12% SDS-PAGE, and the bands were visualized with 1:300 HRP-BCA conjugate or 1:1000 diluted antibody to β1 integrin followed by a 1:500 HRP-labeled secondary antibody. Finally, the membrane was developed with ECL reagents, and the membrane was put under x-ray film for exposure.

**Lectin Blotting**—Cells were harvested, rinsed with PBS, and lysed with 1% Triton X-100 in PBS. Cell lysates containing 30 μg of protein were boiled in SDS sample buffer with β-mercaptoethanol, loaded on
8% SDS-polyacrylamide gels, and then transferred onto a PVDF membrane. After being blocked with 5% BSA, the membrane was incubated with 1:100 dilution of HRP-RCA1 for 2 h at room temperature. The blots were washed and developed with the ECL detection system using x-ray film.

RNA Interference Assay—RNA interference was undertaken using the pSilencer2.0 vector (Ambion Inc.). RNA interference target sequences were selected from the human GalT I sequence (GenBank™/EBI accession number Y09723). Each candidate target sequence was analyzed by BLAST search to ensure that the hit would be unique to the GalT I mRNA. Target oligonucleotides were synthesized (AL1, 5'-AA-GGCCAGATCGAAAGTTCAAGAGACTTTGCTGATCTCGGCC-3'; and AL2, 5'-AATTAAAAAGGCGGATTCAGAAA-GTCTTATGACCTTGCTATCTCCGGTTGGC-3'), annealed, and cloned into pSilencer vector between the BamHI and HindIII sites. Recombinant plasmid DNA was prepared and tested for silencing activity against a GalT I-myc chimeric mRNA expressed from myc-pcDNA3.1 (Clontech) as an N-terminal fusion of GalT I with Myc. A negative control vector comprising a scrambled sequence was also prepared. The increasing amounts of siGalT constructs were cotransfected with myc-pcDNA3.1-GalT1 and EGFPN1 (Clontech) into COS1 cells or PGLH7 cells. 48 h later, lysates were prepared, and the levels of Myc and GFP were examined by immunoblotting. Specificity was assessed either by using the empty vector pSilencer plasmid, or by cotransfecting siGalT with myc-pcDNA3.1-HBO1 vector.

Promoter Deletion Constructs—A 1705-bp fragment (containing nucleotides 1653 to +52) of GalT I promoter was prepared by PCR amplification of human genomic DNA using a sense primer containing an XhoI restriction site and an antisense primer containing a HindIII restriction site. Primers were synthesized on the basis of the reported genomic sequence for human GalT I, forward 5'-GTCTCGAGGTGTGTAAGGAGTAGGTTGCTGAG-3' and reverse 5'-ATAACTGCTTATAGAAGGTGTTGCGCTAGAC-3'. Genomic DNA extracted from human peripheral blood was used as a PCR template. Following digestion with restriction enzymes, the GalT I promoter fragment was directionally cloned into the pGL2-Basic firefly luciferase expression vector (Promega) to generate a “full-length” GalT I reporter construct, and the

![FIG. 1. Increased GalT I mRNA level in highly metastatic PGBE1 cells.](image-url)

A, cell migration assay of PGLH7 (panels a, c, e, and g) and PGBE1 (panels b, d, f, and h) cells. Agarose drop explants assay and wound healing assay were prepared as described under “Experimental Procedures.” The data shown here are representative of four experiments. 18, 24, and 48 h later, migration of cells out of the agarose drop explants (left panel), or 24, 48, and 72 h later, migration of cells into the wound (right panel), in vitro was quantified in the graph. B, PGBE1 cells were more invasive than PGLH7 cells assayed in a modified Boyden chamber (p < 0.05, n = 3). C, Northern blot and RT-PCR analysis of GalT I in PGLH7 and PGBE1 cells. The lower band in RT-PCR is β-actin, which was amplified and used as an internal control. Data are representative of four experiments.
correct insertion was confirmed by sequencing. Reporter genes containing sequentially truncated fragments (−930/+/52, −571/+/52, −485/+/52, −318/+/52, −281/+/52, −215/+/52, −139/+/52, −25/+/52, and −26/135-bps) of the GaT I promoter region were prepared in a similar manner using sense primers containing XhoI restriction sites and the antisense primer that was used to generate the full-length GaT I reporter construct.

Site-directed Mutagenesis—To prepare mutated promoters, the putative Ets transcription binding factor site CTTCGCC containing nucleotide positions −205 and −200 was changed to CAACCC and named p-CAACCC. The mutation was created from p-215-4uc by PCR using Takara MutanBEST mutagenesis kit. Mutated constructs were sequenced, and the correct ones were selected for further experiments.

Luciferase and β-Galactosidase Assay—To demonstrate binding reactions, the avdin-biotin-peroxidase complex (ABC) technique was employed according to Hsu et al. (28). RCA-1 staining procedures were as described previously (29). Cells were plated on the dishes. To eliminate terminal acidic moieties, cells were digested with sialidase. Endogenous peroxidase activity was blocked with 0.3% H₂O₂ in methanol for 30 min. To minimize nonspecific binding reactions, specimens were covered for 15 min with 0.1% bovine calf serum and with a solution of avidin and then with a solution of biotin in PBS. The coated filters were washed with serum-free medium and dried immediately. Then cells were added to the upper compartment of the chamber (1 × 10⁵/100 μl of medium containing 0.1% BSA), and 800 μl of medium (containing 0.1% BSA) was added into the lower chamber. Cells were incubated and allowed to migrate for 24 h. After removal of nonmigrated cells, cells that had migrated through the coated filter were counted under a microscope in five fields at a magnification of ×400. Wound healing assays were performed as described (31). Briefly, subconfluent cells in 6-well plates were serum-starved overnight. Over 20 wounds were made on the cell monolayer by scratching with a 200-μl sterile tip. Cells were rinsed three times with PBS. Complete growth media were then added to the plates, and cells were allowed to migrate for 0, 24, 48, and 72 h. For cells migrating out of the agarose drop explants, 80% confluent cells were trypsinized and resuspended. 100 μl of agarose drop mixture was prepared (containing 1 × 10⁶ cell suspension and a final concentration of 0.3% agarose). Each agarose drop explants contained 1.5 μl of mixture. On each of the following hours, the distance of the leading edge of migrated cells from the edge of the agarose droplet was determined on eight sides of each droplet, and five drops were used for each point.

Invasion and Migration Analysis—Boyden chamber invasion assay was performed basically as described previously by Albini et al. (29). Polycarbonate filters with 8-μm pores were coated with 500 μg/ml of Matrigel (BD Biosciences). The coated filters were coated with 0.1% BSA, and 800 μl of medium (containing 0.1% BSA) was added into the lower compartment of the chamber. Subsequently, the samples were rinsed three times in PBS and incubated for 60 min with the ABC reagent and again washed in PBS. The peroxidase-binding sites were visualized by incubation with a fresh solution of 0.02% hydrogen peroxide and 0.1% diaminobenzidine hydrochloride for 5 min. The cells were rinsed in tap water, followed by distilled water. Finally, the samples were dehydrated, cleared, and mounted. The mean density of RCA-I-positive labeling was from six different regions within the transfected PGL7 cells and the controls. The values are expressed as the mean labeling density ± S.D. from three independent experiments using image cytometry.

RESULTS

Highly Metastatic PGBE1 Cells Have Higher GaT I mRNA Level Than Low Metastatic PGLH7 Cells—PGLH7 and PGBE1 cells, isolated from metastatic human lung giant cell carcinoma (PG), were two cell sublines with different spontaneous metastatic potentials. We analyzed the cell behavior of PGLH7 and PGBE1 cells in a wound healing test, agarose drop explants and Southern blot analysis and semi-quantitative RT-PCR. As shown in Fig. 1A, PGBE1 cells readily migrate out of the agarose drop explants or into the wound in vitro (mechanical scratch made on the surface of growing cell culture) relative to PGLH7 cells. The difference in their invasive potentials was confirmed by Boyden chamber assay. PGBE1 cells showed higher ability to migrate through Matrigel-coated 8-μm pore-size membranes (Fig. 1B). We next analyzed GaT I mRNA expression in PGLH7 and PGBE1 cells by Northern blot analysis and semi-quantitative RT-PCR. As shown in Fig. 1C, GaT I mRNA expression was higher in highly metastatic lung cancer PGBE1 cells than in low metastatic PGLH7 cells.

PGLH7 and PGBE1 Cells Have Similar Galactosylation Levels of Glycoproteins—To determine whether the levels of GaT I at the cell surface or global alteration in galactosylation of glycoprotein might be associated with metastatic potentials of PGLH7 and PGBE1 cells, we examined total galactosylated glycoprotein using RCA lectin blotting. Total cell lysates from PGLH7 and PGBE1 cells were separated by electrophoresis and labeled with biotinylated RCA lectin, which interacts specifically with oligosaccharides terminating with the Galβ1→4
GlcNAc group (32). The results showed no significant differences in the galactosylation profiles of PGLH7 and PGBE1 cells (Fig. 2A), although the possibility exists that differences in one or a small number of glycoproteins would not be detected in this assay. Tumor cell binding to components of the basement membrane triggers intracellular signaling pathways, which...
results in the modulation of gene expression, facilitating metastasis. β1 integrin plays an important role in regulating tumor cell migration and metastasis (33). β1 integrin expression and galactosylation were then examined in PGLH7 and PGBE1 cells. The galactosylation of the β1 integrin immunoprecipitated from PGBE1 and PGLH7 was observed (Fig. 2C). But the levels of expression and galactosylation of β1 integrin were not changed in PGBE1 and PGLH7 cells (Fig. 2, B and C).

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**Fig. 4.** Human GalT I upstream genomic sequence and putative transcription factor binding sites. Presented above is the sequence of 1653-bp genomic region immediately upstream of the human GalT I transcription start site (11001) and 52 bp sequence downstream the position 11001. Potential transcription factor binding sites identified by searching TRANSFAC transcription factor data base are underlined and labeled.
Decreasing Surface GalT I Expression by siRNA or Interfering Surface GalT I Function by Mutation Inhibited Cell Adhesion on Laminin, Invasive Potential of PGBE1 Cells In Vitro, and Tyrosine Phosphorylation of Focal Adhesion Kinase—To test the metastatic potential associated with GalT I functioning as cell surface receptor, we constructed a dominant negative form of GalT I (TLGT), which only contains N-terminal cytoplasmic and transmembrane domains of the long form of the GalT I protein (Fig. 3A) (9). In order to visualize GalT I fusion constructs on the surface of live cells, we fused the GalT I and TL-GalT I with GFP. Transient transfection showed that both GalT I-GFP and TL-GalT I-GFP were readily detected in the plasma membrane in COS1 cells and PGLH7 cells (data not shown) as reported previously (6). To evaluate precisely the relationship between GalT I expression and invasive behavior in vitro, we designed and synthesized three different duplex siRNAs complementary to human GalT I mRNA. GalT I siRNA specifically suppressed GalT I-myc expression (Fig. 3B), whereas they had no effect on HBO1-myc expression (Fig. 3C).

Because cell surface GalT I mediates fibroblast spreading and migration on laminin but does not participate during cell interactions with fibronectin (34), PGBE1 cells transfected transiently with TL-GalT I-GFP and siGalT I were plated on laminin (15 μg/ml). The differences in adhesion and spreading of pSilencer2, myc pcDNA3.1 vector-transfected cells were indistinguishable, whereas fewer siGalT I-transfected and TL-GalT I cells showed cell spreading (Fig. 3D). We next reduced surface GalT I expression by siRNA or introduced the dominant negative mutant GalT I in highly metastatic PGBE1 cells, and we tested the invasion behavior using a modified Boyden chamber (15). As expected, PGBE1 cells transfected with siGalT1 or

![Figure 5](image)

**Fig. 5.** Ets family transcription factors involved in activation of GalT I promoter. **A,** 267-bp GalT I regulatory region carries the majority of the basal promoter activity. GalT-Luc constructs containing various length of GalT I promoter regions were transiently transfected into PGLH7 cells. Luciferase activity was normalized to β-galactosidase activity and standardized to the normalized activity from pGL2-Basic. Each value is the mean ± S.D. of at least three independent experiments. **B,** activation of GalT I promoter by Ets transcription factor E1AF. p-1653-luc construct and vectors containing Ets-1, Ets-2, E1AF, ETV1, ETV5, Elk-1, Net, or the empty control vector were cotransfected into the PGLH7 cells. Normalized luciferase activity was standardized to p-1653-luc with vector alone. Each value is the mean ± S.D. of at least three independent experiments.

![Figure 6](image)

**Fig. 6.** Activation of GalT I promoter by E1AF. **A,** elevated expression of E1AF protein in nuclear extracts from PGBE1 cells. 30 μg of nuclear extracts from each cell type was loaded onto a 10% denatured polyacrylamide gel, and E1AF protein levels were determined by Western blotting using the anti-PEA3 antibody. The size of E1AF protein was 60 kDa. **B,** E1AF dose dependence of GalT I promoter activation. Increasing amounts of E1AF expression plasmid were cotransfected into PGLH7 cells along with p-930-luc constructs. Results shown are the means ± S.D. of at least three independent experiments.
TL-GFP were less invasive than PGBE1 controls (Fig. 3E). Clustering of cell surface GalT I induces transient tyrosine phosphorylation of focal adhesion kinase in NIH3T3 (36). To address the effects of decreasing surface GalT I expression or targeted mutation in surface GalT I on laminin-mediated signaling, the levels of FAK expression and FAK phosphorylation in PGBE1 cells transfected with siGalT1 or TL-GFP were analyzed. The results showed that FAK phosphorylation was decreased in PGBE1 cells transfected with siGalT1 or TL-GFP. But the level of FAK expression was not altered (Figs. 3F and 4G). All these results suggested that cell surface GalT I was involved in the invasion and metastasis of PGBE1 cells.

Cloning of Human GalT I Gene 5'-Flanking Region and Identification of Major Regulatory Region—We next investigated the transcriptional regulation of GalT I gene in highly metastatic PGBE1 cells. A search of the GenBankTM human genomic sequences resulted in the identification of genomic sequences upstream of the GalT I transcriptional start site (designated as +1). To determine whether this sequence (GenBankTM accession number NT_008421, at nt 732212–742549) included the GalT I promoter region, a fragment extending from −1653 to +52 was amplified by PCR from human genomic DNA and cloned into a promoterless pGL2-Basic, creating the reporter plasmid p-1653-luc. Transient transfection of the PGLH7 cells with this plasmid resulted in luciferase levels some 110-fold higher than the promoterless control plasmid pGL2-Basic.

Computer analysis of the human GalT I promoter revealed a highly GC-rich content in its promoter region. The GalT I promoter lacks a typical TATA box, as seen with many GC-rich promoters. The TRANSFAC search program predicted a number of potential transcription factor-binding sites near or upstream of the putative transcription initiation site, including Sp1, AP4, C/EBP, Ets-1, E1AF, and GATA-1 (Fig. 4).

To examine the promoter region for GalT I basal transcription, luciferase reporter constructs containing progressive deletions of the 1705-bp genomic DNA fragment were generated. Each construct as well as the control vector pGL2-Basic were transiently transfected into PGLH7 cells and assayed for reporter activity. Our results showed that deletion of sequences from nt −1704 to −215 did not appreciably reduce promoter activity (Fig. 5A). In contrast, the p-139-luc construct had much lower activity than the p-215-luc construct, indicating that sequences between nt −215 and −139 were critical for basal GalT I transcription. The deletion analysis in transiently transfected HeLa and SMMC-7721 cells also demonstrated that construct p-215-luc had minimal luciferase activity (data not shown).

E1AF Can Induce the GalT I Promoter Activity—To assess the importance of the members of the Ets transcription factor family in the regulation of the GalT I promoter activity, we cotransfected PGLH7 cells with the plasmid p-1653-luc and vectors containing Ets family members, such as Ets-1, Ets-2, E1AF, ETV1, ETV5, Elk-1, Net, or the empty control vector. The fold stimulation of luciferase was calculated as normalized luciferase activity obtained in cells expressing Ets family members divided by the luciferase activity of samples originating from vector-transfected control cells (Fig. 5B). The highest activation of the GalT I promoter was obtained by E1AF. Expression of Ets-1 also stimulated the luciferase reporter gene 2.4-fold compared with the control vector, whereas Ets-2, ETV1, ETV5, and Elk did not show significant activation of the GalT I promoter. On the contrary, Net reduced the GalT I promoter activity. In conclusion, these results demonstrate that the Ets family member E1AF can mediate regulation of the GalT I gene.

**E1AF Trans-activates Human GalT I Gene**

![Fig. 7. Identification of cis-element in GalT I promoter for Ets responsiveness. A, mapping the regions of the GalT I promoter necessary for E1AF responsiveness. PGLH7 cells were transfected with p-1653-luc construct or with the truncated GalT I promoter constructs shown above and with or without E1AF expression vector. Luciferase activity was normalized to β-galactosidase activity and standardized to the normalized activity from p-1653-luc with control vector alone. Data shown are the means ± S.D. of at least three independent experiments. B, site-directed mutation analysis of GalT I promoter. p-215-luc and mutated promoter construct p-215M-luc were transfected into PGLH7 cells, together with or without E1AF expression vector. Luciferase activity is compared with the wild-type p-215-luc without E1AF.

Highly Metastatic PGBE1 Cells Have Higher E1AF Levels Than Low Metastatic PGLH7 Cells—To test further the hypothesis that E1AF activates the GalT I expression in highly metastatic lung cancer cells, we analyzed the expression of E1AF in PGLH7 and PGBE1 cells. Western blot analysis showed that nuclear extracts from PGBE1 cells had increased expression of E1AF protein (Fig. 6A). The elevation of E1AF protein correlated with an increased level of mRNA in PGBE1 cells, as assayed by semi-quantitative RT-PCR (data not shown). To determine trans-activating effects of E1AF on the GalT I gene, transfection studies using the GalT I reporter construct p-930-luc and increasing amounts of E1AF expression plasmid were performed. The forced expression of E1AF potently stimulated the GalT I promoter in a dose-dependent manner in PGLH7 cells, with a maximum activation of 7.8-fold (Fig. 6B).

Identification of the Cis-elements Responsible for the Effect of E1AF—Deletion analysis was then performed to define functionally important cis-elements in this 1705-nt region. Luciferase assays showed that a deletion from −215 to −139 resulted in a drastic decrease in the promoter activity and loss of E1AF activation as compared with that of the p-261/−138-luc construct (Fig. 7A). The minimal inducible promoter activity is located within the −215/−139 region of the GalT I promoter.

**FIG. 7. Identification of cis-element in GalT I promoter for Ets responsiveness. A, mapping the regions of the GalT I promoter necessary for E1AF responsiveness. PGLH7 cells were transfected with p-1653-luc construct or with the truncated GalT I promoter constructs shown above and with or without E1AF expression vector. Luciferase activity was normalized to β-galactosidase activity and standardized to the normalized activity from p-1653-luc with control vector alone. Data shown are the means ± S.D. of at least three independent experiments. B, site-directed mutation analysis of GalT I promoter. p-215-luc and mutated promoter construct p-215M-luc were transfected into PGLH7 cells, together with or without E1AF expression vector. Luciferase activity is compared with the wild-type p-215-luc without E1AF.**
Inspection of this 76-nt region revealed potential Ets protein-binding sites. To determine whether this potential binding site was necessary for GalT I transcription, we introduced site-directed mutagenesis into this Ets element (H11002205 to H11002200). It was found that the luciferase reporter activity was decreased to almost the same level as pGL2-basic. Mutation of the consensus Ets site deprived E1AF of responsiveness (Fig. 7B). These results indicate that the Ets element is an important cis-element for the transcriptional activation of the human GalT I.

Table I

| Name                | Sequence                                      | Position       |
|---------------------|-----------------------------------------------|----------------|
| Et-B                | 5'-gatctcgagaggaagttcga-3'                    | Consensus Ets-1/E1AF binding |
| Ets(GalT0.2)        | 5'-tgcccgacttcccctgctagaaa-3'                | 212 to 184     |
| Ets(GalT0.2)/M1     | 5'-tgcccgacttcccctgctagaaa-3'                |                |
| Ets(GalT0.2)/M2     | 5'-tgcccgacttcccctgctagaaa-3'                |                |
| Ets(GalT0.2)/M3     | 5'-tgcccgacttcccctgctagaaa-3'                |                |

Fig. 8. Gel mobility shift assays with nuclear extracts from PGBE1 and PGLH7 in the regulation regions of the GalT I gene. A, EMSA of PGBE1 nuclear protein extracts (5 μg) incubated with 32P-labeled Ets element probe, mutations in the ETS site (M1), mutations in the SP1 site (M2), and mutations in the SP1–2 site (M3). B, EMSA was performed using nuclear proteins of PGE1 cells and human GalT I promoter sequence −212 to −184 double-stranded radiolabeled probe and supershift by E1AF antibody. C, EMSA of the same amounts nuclear extracts from PGLH7 and PGBE1 incubated with 32P-labeled Ets element probe.
Identification and Characterization of Transcription Factors Binding to the Ets-binding Element by EMSA—By having shown that the Ets-binding site upstream of the GalT I transcription start site is necessary for E1AF responsiveness, it was imperative to identify the protein interacting with the site. Incubation of the double-stranded 28-mer oligonucleotide probe (Table I) containing Sp1-binding sites and one Ets-binding site between nt −212 and −184 with nuclear extracts and analysis by EMSA revealed at least three specific protein-DNA complexes (Fig. 8A, 1st lane). The bands with * were markedly reduced by incubation with the labeled Ets mutation probe M1, which contains mutations in the Ets-binding sites (Fig. 8A, 2nd lane). But the bands with * were not reduced by incubation with the labeled Sp1 mutation probes M2 and M3 (Fig. 8A, 3rd and 4th lanes). Thus, bands with * represented proteins binding to the Ets site.

To identify specific proteins that bind to the Ets-binding site, we used antibodies against E1AF. It was found that antibody against E1AF supershifted protein-DNA complexes (Fig. 8B, 6th lane), consistent with our competition experiments. The formation of these complexes was inhibited by the addition of a 50- and 100-fold excess amount of the unlabeled oligonucleotide (Fig. 8B, 7th and 8th lanes).

We then asked whether elevated E1AF binding to the Ets-binding site contributes to the increased promoter activity in PGBE1 cells. To address this question, we examined the binding capability of the same amount of nuclear extract from PGBE1 and PGLH7 to the GalT I promoter in EMSA. Our results shown in Fig. 9C indicated that nuclear proteins of PGBE1 formed much stronger bands than PGLH7. It is concluded that E1AF, the Ets family member, binds to the Ets-binding site between nt −205 and −200 in GalT I promoter, promotes GalT I transcription, and contributes to the different expression of GalT I in PGBE1 and PGLH7 cells.

E1AF Can Induce the GalT I Promoter Activity in COS1 Cells—Our results demonstrated that E1AF can promote GalT I transcription in PGLH7 and PGBE1 cells. To ensure that the observed response is not limited to PGLH7 and PGBE1 cells, we used COS1 cells that expresses relativity low levels of PEA3 (37). Myc-tagged E1AF plasmids were expressed in COS1 cells (Fig. 9A), and E1AF protein was located exactly in the nucleus (data not shown). E1AF increased GalT I promoter activity 5–6-fold compared with mock-transfected cells (Fig. 9B).

We then asked whether E1AF can bind to the Ets-binding site between nt −205 and −200 in the GalT I promoter in COS1 cells, EMSAs were performed by using nuclear extracts from COS1 cells transfected with WT-E1AF-myc. The nuclear extracts from WT-E1AF-myc-transfected COS1 cells formed a complex with the probe (Fig. 9C, 2nd lane). Anti-Myc antibody added to the EMSA reaction mixture resulted in supershift of the band (Fig. 9C, 4th lane), whereas anti-actin antibody did not shift any bands (Fig. 9C, 3rd lane). E1AF was demonstrated to bind to and activate GalT I promoter in COS1 cells.

Expression of GalT I in E1AF-transfected PGLH7 Cells—pcDNA3.0-E1AF was stably transfected into PGLH7 cells, and its effect on GalT I expression and its biological activities were assessed. The results of Fig. 10A showed that there is an increase in the GalT I mRNA following transfection with the pcDNA3.0-E1AF vector. We further compared the GalT I promoter activity in PGLH7 cells and E1AF-transfected PGLH7 cells (Fig. 10B). E1AF-transfected PGLH7 cells showed about three times higher GalT I promoter activity than PGLH7 cells. Because the gene expression of GalT I was altered, whether the galactosylation of proteins was also changed was further investigated. In order to determine whether Galβ1→4GlcNAc was expressed differently on N-glycans in E1AF-transfected PGLH7 cells, cell samples were subjected to RCA-I lectin staining analysis. It was found that E1AF-transfected PGLH7 cells could enhance the content of β1,4-Gal branch in the cell surface glycoconjugates (Fig. 10C).

Overexpression of E1AF-promoted Cell Migration—We next examined the differences of cell migration ability between PGLH7 cells and E1AF-transfected PGLH7 cells. It was found that E1AF-transfected PGLH7 cells migrated faster out of the agarose drop explants than PGLH7 cells (Fig. 10D). The PGLH7 cells were still not ready to migrate out of the explants 18 h after agarose drop explants were prepared, whereas E1AF transfectedants had already migrated outside.

**Fig. 9.** E1AF regulates the activity of GalT I promoter in COS1 cells. A, COS1 cells were transfected with either myc-pcDNA3.1 or myc-E1AF expression vector. The total cell lysates were separated on SDS-PAGE, electroblotted, and incubated with anti-Myc antibody. Six representative immunoblots illustrated the abundance of Myc-tagged E1AF in COS1 cells. B, comparative specific activity of the deletion mutants. The data were derived from three independent experiments. C, EMSA of nuclear protein extracts (5 µg) from COS1-transfected E1AF incubated with 32P-labeled Ets element probe.
GalT I Expression Can be Induced by EGF and Dominant Active Ras—Because Ets transcription factors have been well defined as nuclear effectors of a central signal transduction pathway, the Ras/MAPK signaling pathway (38), we next explored the possible relationship between Ras/MAPK and E1AF in GalT I induction. GalT I mRNA levels in serum-starved and EGF-stimulated HeLa cells were assessed by Northern blot analysis. Fig. 11A shows the GalT I mRNA induction by EGF (10 ng/ml). GalT I mRNA increased gradually following the addition of EGF. Analysis of time-response relationships demonstrated maximal GalT I mRNA activation after 4 h of EGF exposure, which corresponds well with the results obtained in GalT I promoter studies (Fig. 11B). Transient transfection of reporter plasmids containing GalT I reporter construct p-215-luc into PGBR1 cells showed dose-dependent reporter gene activity in response to serum stimulation (3.1-fold increase, Fig. 11C). To determine whether RAS signaling pathways were involved in serum-induced GalT I transcriptional activation, we transiently cotransfected PGLH7 with reporter plasmids containing the GalT I p-215-luc promoter and either the dominant negative expression construct RAS-DN or a constitutively activated RAS-DA expression construct. As expected, expression of RAS-DN decreased the GalT I promoter activity in a dose-dependent manner, whereas expression of RAS-DA caused a similarly dependent activation (Fig. 11D), indicating a role for RAS in GalT I induction. Ras signal can alter gene expression by three distinct MAPK cascades (39–41). To investigate further the importance of MAPK in mediating the activity of GalT I p-215-luc promoter, a series of transient transfections were performed (Fig. 11E). Transient overexpression of ERK1 or JNK1 in PGLH7 cells led to a significant increase in GalT I p-215-luc promoter activity. Site-directed mutagenesis of the putative Ets site at position −205 to −200 abolished the activation of GalT I promoter by RAS-DA (Fig. 11F).
Cancer metastasis is a complex process. It requires the coordinated expression or the activation of multiple genes so that cells migrate from the primary site, enter the circulatory system, arrest, and proliferate at a secondary site. In this study, we have provided evidence that E1AF-induced GalT I expression.

**DISCUSSION**

Cancer metastasis is a complex process. It requires the coordinated expression or the activation of multiple genes so that...
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The mechanism of how GalT I influences the tumor cell invasive potential is still unknown. In this study, we found highly metastatic PGBE1 cells had a higher GalT I mRNA level than low metastatic PGLH7 cells. The invasive capacity was significantly reduced by decreasing surface GalT I or introducing dominant negative GalT I in PGBE1 cells. GalT I has been shown to exert various functions other than a catalytic enzyme (5–9). We then asked whether cell surface GalT I acts catalytically or in a lectin-like fashion. By using RCA lectin blotting, we found no difference between PGBE1 cells and PGLH7 cells. The \( \beta \) subunit-containing integrins are receptors mainly for extracellular matrix proteins such as laminin and fibronectin and are responsible for cell anchorage and motility. We also found \( \beta \) integrin could be modified by galactosyltransferase. But there was no difference in \( \beta \) integrin expression between PGBE1 and PGLH7 cells. Notably, the highly metastatic PGBE1 cells could interact with the extracellular matrix protein laminin and induce FAK phosphorylation. Decreasing surface GalT I expression or targeted mutation of surface GalT I in PGBE1 cells resulted in decreased FAK phosphorylation, but the level of FAK expression was not altered. Taken together, these observations raise the intriguing possibility that galactosyltransferase promote tumor cell invasion by inducing transient tyrosine phosphorylation of focal adhesion kinase.

Cell surface GalT I has been implicated in tumor invasion and metastasis. But the mechanisms regulating its expression in highly metastatic cancer cells have not been defined. The 5'-flanking region of the mouse GalT I gene has been studied. We compared the promoters of the human and mouse GalT I gene and did not find a high homology (data not shown). In this report, we investigated the involvement of Ets factors in the transcriptional regulation of GalT I in highly metastatic human lung cancer cells. Sequence analysis revealed that the human GalT I promoter is a TATA-less, GC-rich promoter, which is consistent with the notion that GalT I belongs to the family of housekeeping genes.

The ets genes, which currently comprise nearly 30 members, encode transcription factors bearing conserved DNA binding domains (the ETS domain) (42). E1AF is believed to play important roles in tumor invasiveness and metastasis through transcriptions of metastasis-related genes (43, 44). Expression of E1AF is correlated with the metastasis phenotype of breast cancer (45–47) and invasive phenotype of neuroblastoma (48), oral squamous cell carcinoma (49, 50), and non-small-cell lung cancers (51–54). It was found in this study that expression of E1AF was increased in highly metastatic lung cancer cells compared with its low metastatic counterpart cells, which suggested that E1AF might be involved in lung cancer cell metastasis phenotype.

Ets proteins are capable of regulating transcription by binding to the Ets-binding site (EBS) in the promoters of their target genes, and EBS comprises the highly conserved core sequence 5'-GGAA/T(+)A3' (42). The GalT I promoter region was analyzed by using transient transfection experiments. Cotransfection with E1AF resulted in a 7.8-fold increase in luciferase activity as compared with vector alone, whereas the transfection with Ets-2, ETV1, ETV5, Elk, and Net, other members of Ets transcription factors, failed to increase luciferase activities, indicating a specific effect of E1AF on the GalT I promoter. It was found by deletion analysis that the region between nt 215 to 139 in the GalT I promoter is critical for activation by E1AF. Mutation of the consensus EBS in this region (position −205 to −200) led to a complete loss of responsiveness to E1AF. EMSA analysis showed specific binding of E1AF to this EBS in PGLH7 cells and COS1 cells. Nuclear extract from PGBE1 cells formed stronger band with the GalT I promoter than PGLH7 cells. All these results suggested that E1AF bound to DNA with specificity and activated transcription of GalT I promoter bearing Ets-responsive element, accounting for the increased GalT I mRNA levels found in highly metastatic PGBE1 cells. To the best of our knowledge, this is the first evidence associating Ets transcription factors and galactosyltransferase in human tumor metastasis.

There are several potential Sp1 sites near the putative Ets site in GalT I promoter. The involvement of juxtaposed PEA3/ SP-1 sites has been reported for other genes, such as HTLV1 long terminal repeat, caspase-8, and parathyroid hormone-related protein (55, 56). Additionally, Sp1 plays an essential role in the transcriptional activity of the GalT I gene in cancer cells (57). In this study, mutation of the Sp1 sites adjacent to the EBS site (−205 to −200) did not affect the binding capacity of E1AF to GalT I promoter. Thus the possible involvement of Sp1 in up-regulation of GalT I in metastatic cells is excluded by EMSA analysis.

To elucidate the role of the Ets protein in lung cancer cells, we stably transfected E1AF into the PGLH7 cells. It is important to emphasize that in the present study the expression levels of GalT I mRNA in the E1AF-transfected cell lines were higher than that in control cells. RCA-I staining intensities of membrane glycoproteins in the E1AF-transfected cells changed, suggesting E1AF enhanced expression of Galβ1→4GlcNAc on N-glycans. At the same time, these cells migrated faster than control PGLH7 cells. Indeed, all these results suggest that E1AF induces GalT I expression in stably transfected PGLH7 cells, which may contribute to the highly metastatic potential of lung cancer cells.

The activity of E1AF has been reported to be activated by Ras-MAP kinase signaling (20, 38). The importance of Ets factor activity for Ras function has been shown by the finding that dominant negative Ets block Ras mediated cell transformation (58). It was found in this study that constitutively activated Ras is capable of enhancing the promoter activity of GalT I by 8.3-fold, whereas dominant negative Ras decreased GalT I promoter activity. Additionally, the transient overexpression of ERK1 or JNK1 in PGLH7 cells led to a significant increase in GalT I promoter activity. Whereas site-directed mutagenesis of the putative Ets sites at position −205 to −200 abolished activation of GalT I promoter by Ras. All these results indicated the involvement of MAPK and E1AF in GalT I activation in highly metastatic lung cancer cells.

GalT I is one of the seven known β1,4-galactosyltransferase polypeptides (35). The expression of β1,4-galactosyltransferase II–VII was also analyzed in PGLH7 and PGBE1 cells. It was found that β1,4-galactosyltransferase IV was increased in highly metastatic PGBE1 cells, whereas other family members remained unchanged. The possible involvement of β1,4-galactosyltransferase IV has yet to be investigated. Additional studies on the relationship between glycosylation and metastasis should provide important insights into mechanisms of cell-cell interactions and tumor progression to the metastatic stage. It is likely that rapid progress will be made toward understanding the connections between GalT I and tumor metastasis.

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