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A Role for Dystroglycan in Epithelial Polarization: Loss of Function in Breast Tumor Cells[1]

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ABSTRACT

Receptors mediating cell-basement membrane interactions are potent regulators of epithelial architecture and function, and alterations in signals from the basement membrane are implicated in the aberrant behavior of carcinoma cells. In this study, we have investigated the role of the basement membrane receptor dystroglycan (DG) in mammary epithelial cell function, and the significance of loss of DG function in breast tumor cell lines. Nonmalignant mammary epithelial cells express a functional DG. Analysis of multiple breast carcinoma cell lines revealed that DG is expressed in all of the cell lines examined, as evidenced by β-DG expression, but that α-DG is functionally diminished in the majority. High levels of α-DG correlated strongly with the ability of cells to polarize in the presence of the basement membrane. Overexpression of the DG cDNA in HMT-3522-T4-2 cells elevated α-DG levels and altered responsiveness to the basement membrane; DG overexpression restored the ability of the cells to undergo cytoskeletal changes, to polarize, and to restrict growth in response to basement membrane proteins. Moreover, restoration of DG function to these cells greatly reduced their tumorigenic potential in nude mice. These data point to DG as an important mediator of normal cell responses to the basement membrane, and as a significant variable in carcinoma cells, in which its frequent loss can contribute to aberrant cell behavior.

INTRODUCTION

Adhesion molecules that mediate both cell-cell and cell-ECM[4] interactions play a leading role in the organization and maintenance of tissue architecture. These adhesion molecules are multifunctional; they tether the cell to its extracellular environment, organize the cytoskeleton, and initiate signaling cascades that integrate with signals from receptors for soluble factors (1). Through their multiple functions, these receptors operate at the interface of cell structure and the regulation of growth and differentiation. Current models suggest that normal patterns of cell growth, differentiation, and development are achieved, in part, through the balance of competitive signals from multiple adhesion molecules (2, 3). Furthermore, mis-regulated expression of adhesion molecules is believed to promote the aberrant behavior of tumor cells; tumor cells characteristically display an abnormal complement of adhesion molecules, and exhibit loss of normal responsiveness to ECM proteins (4–6).

The distinct contributions of different adhesion molecules, particularly the ECM receptors, to tissue architecture and function are only partially understood. Moreover, although it is evident that the cellular machinery that senses the ECM is altered in tumor cells, it is not yet known where the critical changes that lead to loss of tissue architecture and growth regulation occur. Studies of cell-ECM interactions have largely focused on the integrins, an extensively characterized family of heterodimeric receptors. However, evidence has grown for the importance of non-integrin receptors, such as syndecans (7), Lutheran (8), and discoidin domain receptors (9). Particularly strong evidence has been established for the importance of DG, an ECM receptor first characterized in muscle cells, but subsequently found in neurons and epithelial cells (10). DG binds to multiple ECM components including laminins, perlecan, and agrin (11), and has been shown to have a role in BM assembly (12), neuromuscular junction formation (13), and kidney morphogenesis (14).

We have used functionally normal and tumorigenic mammary epithelial cells as model systems to investigate the contribution of ECM and specific adhesion molecules to tissue architecture and growth regulation. Epithelial structure and function is strongly regulated by the BM. Cell contact with the BM regulates cell growth, differentiation, cell shape, and gene expression (15). Tissue polarity, an essential aspect of all epithelial function, is also achieved through a collaboration of cell-cell and cell-BM interactions (16). Key signaling components of the BM include the laminin glycoproteins. Laminin-1 by itself can induce cell shape changes (17), growth arrest (4), functional differentiation (18), and polarization (19–21) in cultured mammary epithelial cells. Yet, despite extensive knowledge of laminin's structure and its influence on functional differentiation, some of the receptors mediating these responses have not been clearly identified.

In a previous study of normal mammary epithelial cell function, we divided laminin signaling functions among three different receptor systems, the β1 integrins, α6β4 integrins, and a yet-to-be-identified "E3 laminin receptor" (22). These results suggested that a non-integrin laminin receptor, binding to the globular "E3" domain (LG4 and LG5) of laminin, is a critical mediator of cell morphogenesis and growth control in mammary epithelial cells. This E3 laminin receptor mediated the rounding, aggregation, and growth arrest of cells cultured on plastic after exposure to laminin-1. The functions of α6 and β1 integrins were not required for this response (22). The properties of the E3 laminin receptor resembled those of DG, suggesting DG to be a critical player in normal mammary epithelial cell function.

In this report, we investigate the role of DG in the response of mammary epithelial cells to BM proteins, and in the altered response of mammary tumor cells to the BM.

MATERIALS AND METHODS

Reconstituted BM protein (Matrigel) was purchased from Collaborative Research (Waltham, MA) with endotoxin ratings below 1 unit/ml, and adjusted to a final protein concentration of 10 mg/ml using culture medium. The IIH6 anti-DG monoclonal antibody and polyclonal goat anti-DG antiserum were previously described (23). The anti-β-DG monoclonal antibody (clone NCL-b-DG) was purchased from Novacastra Laboratories (Newcastle upon Tyne,

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[4] The abbreviations used are: ECM, extracellular matrix; BM, basement membrane; DG, dystroglycan; T4-2 (cells), HMT-3522-T4-2 (cells); DAPI, 4′,6-diamidino-2-phenylindole; TBST, Tris-buffered saline-Tween 20; EGFR, epidermal growth factor receptor.

United Kingdom). The monoclonal anti-β1 integrin antibody (clone 18), β-catenin antibody (clone 14), and anti-E-cadherin monoclonal antibody (clone 36) were purchased from Transduction Laboratories (Lexington, KY). The PTEN antibody (MAB 4037) was purchased from Chemicon (Temecula, CA). Immunostaining antibodies: The anti-α6 integrin monoclonal antibody (clone J1B5) was a gift from Dr. Caroline Damsky (University of California-San Francisco, San Francisco, CA). Golgi staining was performed using the anti-GM130 monoclonal antibody (clone 35), purchased from Transduction Laboratories.

The anti-laminin-5 monoclonal antibody (clone BM-2) was a gift from Dr. Robert Burgeson (Harvard Medical School, Charlestown, MA). The pBA-TEM2 plasmid containing the E-cadherin cDNA was a gift from Bob Brackenbury (University of Cincinnati, Cincinnati, OH). Propidium iodide was purchased from PharMingen (San Diego, CA). DAPI and Soybean Trypsin Inhibitor were purchased from Sigma Chemical (St. Louis, MO). Rhodamine-conjugated phalloidin was purchased from Molecular Probes (Eugene, OR).

Cell Culture. The EpH4, ScP2, and T4-2 cell lines were described previously (24–26). All of the other cell lines were obtained from the American Type Culture Collection. All of the cells lines were cultured in DMEM/F12 medium (Invitrogen Corp., Carlsbad, CA). For EpH4, ScP2, BT474, T47D, MCF-7, Skbr-3, and MDA-MB-453, and MDA-MB-468 cells, the medium was supplemented with insulin (5 μg/ml; Sigma Chemical) and 5% (v/v) FCS (Atlanta Biologicals, Norcross, GA). For MDA-MB-435 and MDA-MB-231cells, the medium was supplemented with insulin and 2% FCS. T4-2 cells were cultured as described in Weaver *et al.* (3).

Cell Rounding and Three-Dimensional BM Assays. For three-dimensional BM assays, cells cultured on plastic were trypsinized, washed in serum-free DMEM/F12 medium, and pelleted by centrifugation. The cell pellet was treated with 30 μl of soybean trypsin inhibitor (10 mg/ml) and washed again in serum-free medium before counting. Cells (100,000–400,000) were thoroughly resuspended as single cells in 0.5 ml of Matrigel (kept cold on ice) and transferred to a Nunc 4-well dish (C no. 176740), which was immediately placed at 37°C to cause gelling of the Matrigel. After 30 min at 37°C, the gelled block of cells was overlayed with 0.5 ml of the given cell line's culture medium. This medium was subsequently changed at 2-day intervals until completion of the assay.

For cell-rounding assays, T4-2 cells were plated on Nunc tissue culture plastic or glass slides, and allowed to spread and grow to ~30% confluence. Subsequently, the populations were exposed to soluble BM proteins in the form of Matrigel added to fresh culture medium at a dilution of 2% (v/v). Cells were fixed after 48 h using 4% paraformaldehyde, stained with DAPI nuclear dye or rhodamine-conjugated phalloidin, and compared with cells grown in medium without Matrigel. For those cell lines routinely cultured in serum, cell-rounding assays were performed in serum-free medium with or without Matrigel.

Immunostaining. Three-dimensional BM cultures were washed with PBS fixed with 2% paraformaldehyde for 10 min at room temperature, and washed with PBS/glycine three times over 30 min. Subsequently, the cultures were treated with 18% sucrose in PBS for 15 min, then 30% sucrose for 15 min, followed by immersion in OCT solution (Tissue-TEK, Torrance, CA) and freezing on dry ice. Frozen cell blocks were sectioned at 4-to-8-μm sections, placed on gelatin-coated glass slides, and air-dried. For immunostaining, sections were blocked with 10% goat serum (v/v) in PBS buffer. Primary and secondary antibodies were diluted in blocking buffer.

Western Blots. Proteins were extracted from cells on plastic using radioimmunoprecipitation buffer [50 mM Tris (pH 7.4), 30 mM NaCl, 1% (v/v) NP40, 1% (wt/v) deoxycholate, 0.1% (wt/v) SDS, and protease inhibitor cocktail (Calbiochem, La Jolla, CA), and insoluble material was removed by centrifugation at 12,000 $\times$ *g* for 10 min. Protein electrophoresis was performed by standard SDS-PAGE methods, using nonreducing sample buffer. Proteins were transferred to Immobilon-P membranes (Amersham, Arlington Heights, IL), and blocked in 5% nonfat dried milk in TBST buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% (v/v) Tween 20]. For the IIH6 monoclonal antibody, the TBST buffer contained only 20 mM Tris and 100 mM NaCl. All of the antibodies were diluted in the same buffer used for blocking. Membranes were washed in TBST buffer alone. Antibody binding was detected using horseradish peroxidase-conjugated secondary antibodies and the SuperSignal chemiluminescent substrate (from Pierce, Rockford, IL), according to the manufacturer's instructions. Laminin overlays were performed as described previously (27) with the following variations: pure Laminin-1 (purchased from Invitrogen Corp.) was biotinylated using NHS-LC-Biotin (Pierce) according to the manufacturer's instructions. Biotinylated laminin was incubated on filters in the same manner as antibody incubations, using a laminin concentration of 1.0 μg/ml and TBST buffer containing 1 mM calcium chloride.

Tumorigenesis Assays. The tumorigenic potential of cell populations was assayed by s.c. injection into nude mice. Cell injections were administered twice, s.c., into both flanks of BALB/c nu/nu mice. Six injections were administered for each cell population, totaling 12 injections of each for the study. Cells (5×10^6) were injected at each site, in a 200-μl volume suspended in DMEM/F12 medium. The mice were retained for three months, then sacrificed before dissection and assessment of tumor formation. These experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Lawrence Berkeley National Laboratory.

Retroviral Cell Transfection. Retroviruses were produced using the "Retro-X System" of Clontech (Palo Alto, CA) according to the manufacturer's instructions. Specifically, the pLXSN viral vector was introduced into the PT67 packaging cell line by calcium phosphate transfection. The infected cells were selected by growth in the presence of G418 (Genetecin; Invitrogen Corp.). All of the cells were selected using 500 μg/ml G418, except T4 cells (using 100 μg/ml), MDA-MB-231 (using 1.0 mg/ml) and T47D (using 200 μg/ml). In all cases, infected populations were obtained by pooling more than 1000 independent colonies.

Cloning of cDNAs. The human DG cDNA was obtained by high fidelity reverse transcription-PCR from RNA of the BT474 cell line using the "Expand PCR System" (Roche Diagnostics, Indianapolis, IN) and the following primers: (5′-AGGAATTCGCTTGCGGGTTAAGGTG and 5′-GGTGAATTCTT-GGACCTGGGATGAGG). The PCR product was cut with *Eco*RI and cloned into the *Eco*RI site of the pLXSN retroviral expression vector (Clontech). The correct sequence of the cloned cDNA was verified by complete sequence analysis, and matched the reported sequence in GenBank accession no. XM_018223.1.

The E-cadherin cDNA was cloned into the LXSN expression vector by cutting the pBATEM2 plasmid with *Bgl*II and *Nru*I, polishing the cut ends with Klenow, and ligation into the *Hpa*I site of the pLXSN vector.

RESULTS

DG Is Expressed in Functionally Normal Mammary Epithelial Cells. DG is composed of two noncovalently linked subunits, α and β. β-DG spans the membrane and forms linkages with the cytoskeleton, whereas α-DG is extracellular and binds ECM components (27). Functionally normal murine mammary epithelial cell lines, Scp2 and EpH4, were tested for the expression of α and β-DG proteins. Immunoblots detected β-DG as a single band of M_r 43,000 (Fig. 1*A*). α-DG was detected as a broad band with molecular weight ranging from M_r 150,000–190,000, reflecting varied glycosylation. The ability of epithelial DG to bind laminin-1 was tested by laminin overlay, as described previously (27); similar to immunoblotting, biotinylated laminin-1 was overlayed on nylon membranes of proteins separated by electrophoresis. Bound laminin-1 was detected by horseradish peroxidase-conjugated streptavidin, and revealed strong binding of laminin-1 to bands corresponding to α-DG (Fig. 1*A*).

***α*-DG Levels Are Diminished in Many Breast Tumor Cell Lines, Indicating a Possible Loss of DG Function.** One method to investigate the role of DG in mammary epithelial cells is to assay cell behavior in the absence of DG function. Knockout of the *DG* gene in mice proved embryonic lethal around day 7 of development (28), which precluded studying DG−/− cells in the mammary gland. As an alternative, we investigated tumorigenic breast epithelial cells as a potential source of cells deficient in DG function. Breast tumor cells characteristically display abnormal responses to the BM (4), and alteration of DG function may be, in part, responsible. Therefore, we first attempted to determine whether DG was functionally compro-

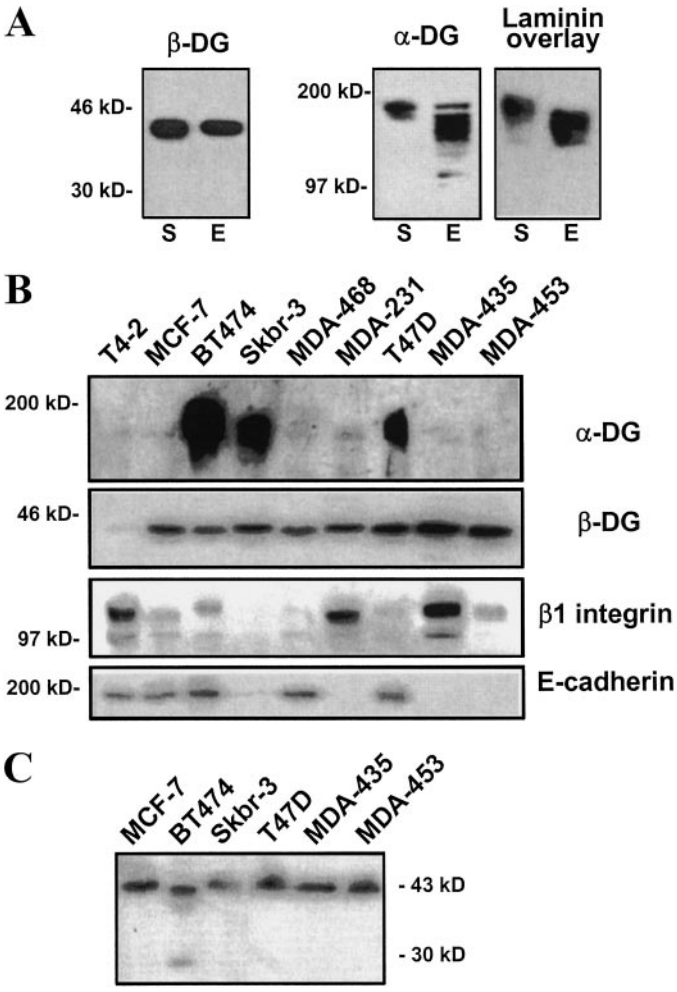


Fig. 1. DG expression in normal mammary epithelial cells and tumor cell lines. In *A*, functionally normal mammary epithelial cell lines Scp2 (*S*) and EpH4 (*E*) were extracted for total protein and subjected to immunoblot analysis for α-DG and β-DG expression, and assayed for laminin binding activity by laminin-overlay. Both α- and β-DG were detected, and α-DG bound laminin in both cell lines. In *B*, nine randomly selected human breast tumor cell lines were extracted for total cellular protein and subjected to immunoblot analysis of α-DG, β-DG, β1 integrins, and E-cadherin expression, loading equal total protein per lane. α- and β-DG and E-cadherin data were obtained from the same membrane, cut and reprobed, to avoid potential errors in loading. The immunoblot showed widely variable protein levels for α-DG, β1 integrins, and E-cadherin among the different cell lines. The ratios of α-DG:β-DG were variable in each cell line, indicating loss of functional α-DG in most, if not all, of the cell lines. BT474 cells displayed the highest α:β-DG ratio. In *C*, detection of the M_r 30,000 β-DG isoform showed that the presence of this isoform did not correlate with the loss of α-DG in breast tumor cell lines. *kD*, molecular weight in thousands.

mised in tumor cell lines, and, second, to determine whether such cells could be exploited to investigate DG function.

In a set of nine human breast tumor cell lines, we assayed the relative levels of α and β-DG, β1 integrins, and E-cadherin by immunoblot (Fig. 1*B*). Indeed, α-DG levels were highly variable among the different cell lines. Three of the nine tumor cell lines (BT474, Skbr-3, and T47D) showed relatively high levels of α-DG, whereas the others displayed relatively minor levels of the protein. The variable levels of α-DG observed by immunoblot were also evident using the laminin-overlay assay described in Fig. 1*A*; cell lines that displayed high α-DG levels displayed high laminin binding, and those with diminished α-DG levels showed diminished laminin binding, (data not shown). Thus, α-DG's laminin-binding function was diminished or absent from many of these tumor cells.

The β-DG immunoblot displayed a very distinct pattern from that of α-DG. β-DG levels were prominent in all of the cell lines except T4-2 cells, with relatively less variation among the cell lines than observed for α-DG. The DG subunits, α and β, are the product of a single gene, cleaved into two subunits by posttranslational processing (29). Therefore, it could be expected that the ratio of α-DG:β-DG protein would be constant in all cell lines. However, among the cell lines tested here, BT474 cells showed the highest α-DG:β-DG ratio, with all of the other cell lines displaying variable lower ratios. The loss of α-DG immunoreactivity could result from the loss of α-DG from the cell surface or from altered glycosylation that ablates both antibody and laminin binding. Because a similar immunoblot pattern was observed using a polyclonal α-DG antibody (not shown), it appeared that the α-DG protein was actually lost from the cell surface in those tumor cells lacking detectable α-DG. The one exception was the T4-2 cells, which displayed lower overall DG expression.

Alterations of β-DG have been proposed to cause a loss of α-DG from the cell surface (30, 31). These alterations are evident by a smaller (M_r 30,000) β-DG isoform, which may be formed by proteolytic cleavage. In our cell extracts the M_r 30,000 β-DG isoform was sometimes detected, but always comprised a small fraction of the total β-DG protein (Fig. 1*C*). In addition, the presence of this smaller isoform did not correlate with cell lines lacking α-DG. Therefore, loss of α-DG in these breast tumor cell lines does not appear to result from alterations in β-DG.

Higher α-DG Levels Correlates with Epithelial Polarization in Response to BM Proteins. α-DG is the subunit of DG that binds BM components and is, therefore, required for receptor function. Seeing the widely variable levels of α-DG protein among the nine cell lines in Fig. 1, we next asked whether the relative levels of α-DG predicted the ability of these cells to respond correctly to BM proteins. For this purpose we used the "three-dimensional BM assay." In this assay cells are cultured within a three-dimensional gel of reconstituted BM proteins (Matrigel). Functionally normal mammary cells grow from single cells to form polarized multicellular epithelial structures, whereas tumorigenic mammary epithelial cells grow as disorganized cell masses, failing to polarize or arrest growth (4).

Each of the tumor cell lines analyzed in Fig. 1 was cultured within the three-dimensional BM assay. The resulting structures were examined for the formation of a polarized epithelial cell layer (Fig. 2). Polarity was defined by basal α6 integrin localization, apical localization of the Golgi, and basal localization of nuclei. Among the nine cell lines tested, only two cell lines were observed to form polarized structures when cultured in the three-dimensional BM assay; T47D and BT474 each showed basal polarization of nuclei and α6 integrin, and apical polarization of the Golgi (Fig. 2, *f* and *e*, and not shown). T47D cells formed large colonies with clearly polarized cells contacting the BM proteins. The BT474 cells, having the highest surface levels of α-DG, were more growth restricted in the three-dimensional-BM assay; even then, colonies of four cells or more were polarized (Fig. 2*e*). T4-2 cells, MDA-MB-468 cells, and MDA-MB-453 cells were apolar, as determined by α6 integrin localization and random nuclei distribution (Fig. 2, *a*, *b*, and *c*). MCF-7 and Skbr-3, which did not display detectable α6 integrin staining, were judged apolar by random Golgi and nuclei distribution (Figs. 2*c* and 3*a*). MDA-MB-231 and MDA-MB-435 cells, which are highly invasive *in vivo* (32), displayed invasive behavior also within the Matrigel (Fig. 2, *g* and *h*), and were clearly apolar.

Among the cells in Fig. 2, those that possessed the ability to polarize in response to the BM (BT474 and T47D cells) each displayed relatively high levels of α-DG, whereas those that lacked the ability to polarize displayed low α-DG levels. Therefore, the levels of α-DG accurately predicted the behavior of these eight cell lines. The one exception was Skbr-3 cells, which failed to polarize (Fig. 3*a*), despite displaying relatively high levels of α-DG. These cells instead

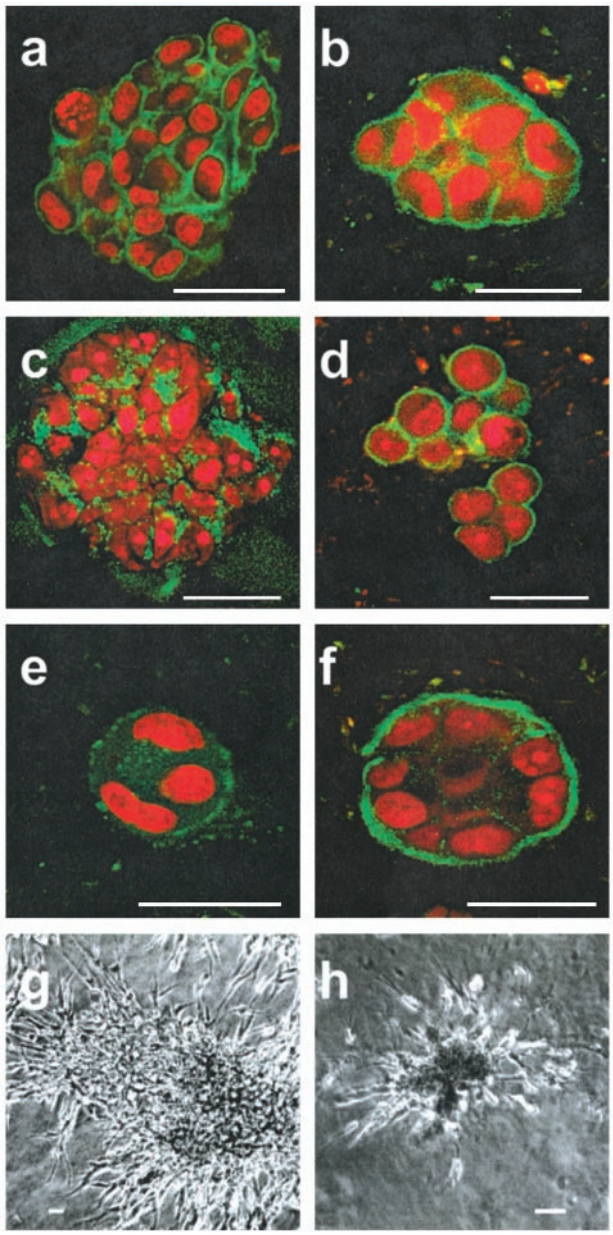


Fig. 2. Response of breast tumor cell lines to growth in the three-dimensional BM assay. Each of the tumor cell lines analyzed in Fig. 1 were seeded as single cells into a three-dimensional gel of reconstituted BM proteins (Matrigel) and allowed to grow for 6–10 days: *a*, T4-2; *b*, MDA-MB-453; *c*, MCF-7; d, MDA-MB-468; *e*, BT474; *f*, T47D; *g*, MDA-MB-231; and *h*, MDA-MB-453. The resulting structures were immunostained and assessed for polarization, as determined by α6 integrin localization (*green*: *a*, *b*, *d*, *e*, *f*) to the basal cell surface, nuclei localization (*red*) to the basal side, and Golgi localization (*green*, *c*) to the apical side, relative to the nuclei. Two cell lines, BT474 (*e*) and T47D (*f*), formed polarized structures in response to the BM proteins. All of the others were apolar, and MDA-MB-231 and MDA-MB-435 also grew invasively into the Matrigel as shown by phase imaging (*g* and *h*, respectively). *Bar* (*a–f*), 30 μm; *g* and *h* are larger scale.

grew as loose groups of individual cells with minimal cell-cell interaction, attributable in part to a lack of E-cadherin expression (see Fig. 1*B*; Ref. 33). Epithelial polarity requires both cell-BM and cell-cell interactions (16). We hypothesized, therefore, that the inability of Skbr-3 cells to polarize resulted not from an inability to sense the BM, but instead from a lack of adherens junctions. This prediction was tested by reexpressing the E-cadherin cDNA in Skbr-3 cells by retroviral infection and selection for the infected cell population. As seen in Fig. 3, the population of Skbr-3 cells expressing E-cadherin regained the ability to form polarized structures. Polarity was observed in >80% of Skbr-3 cells infected with the E-cadherin-expressing virus, compared with no polarized colonies observed in cells infected within an empty virus as control. In contrast, expression of E-cadherin in MDA-MB-231 cells, which possessed relatively low α-DG levels, did not permit polarization of any cells in the population (not shown).

Overexpression of DG in T4-2 Cells Restores Correct Responsiveness to BM Proteins and Inhibits Tumorigenicity. The results presented above suggested, by strong correlation, that α-DG plays a role in the ability of cells to form a polarized epithelial cell layer in response to the BM, in alliance with E-cadherin. This model would thus predict that augmenting the levels of α-DG would alter the response to BM proteins in those cells in which levels are low, and could restore polarity to cells that also express E-cadherin. Overexpression of the DG cDNA by cell transfection, would be a straightforward method to augment the levels of α-DG in a tumor cell, if the subsequent loss of α-DG function were not a factor.

To test the potential effects of DG overexpression on cell behavior, we introduced the DG cDNA in those cell lines that displayed low α-DG levels and failed to polarize in the presence of BM. Cells of each cell line were infected with a retrovirus expressing the full-length human DG cDNA, or infected with an empty vector as a control. After infection, cells were selected for neomycin resistance (also encoded by the virus) and populations of infected cells were obtained. Immunoblots of both α- and β-DG from the infected cells showed that whereas β-DG levels were elevated in each cell line overexpressing the DG cDNA, α-DG levels were elevated only in the T4-2 cells (Fig. 4). This result was not surprising in light of the data presented in Fig. 1*B*, showing the loss of detectable α-DG in many cell lines despite high overall DG expression. Overexpression of the DG cDNA was not sufficient to override the loss of α-DG, which appears to be the dominant regulator of α-DG levels in MCF-7 and MDA-MB-468 cells, and in MDA-MB-231, MDA-MB-453, and MDA-MB-435 cells. In contrast, loss of α-DG was relatively minor in T4-2 cells; therefore, overexpression of the DG cDNA resulted in significantly higher levels of α-DG in these cells.

Cell populations overexpressing DG were tested for their ability to polarize and restrict growth in the three-dimensional BM assay. As expected, overexpression of the DG cDNA did not change the behavior of those cells wherein α-DG levels were not changed (not shown). In T4-2 cells; however, elevation of α-DG proteins levels by cDNA overexpression restored the ability of the cells to form polarized epithelial structures when grown within the three-dimensional BM assay (Fig. 5*A*). Cells infected with the empty virus (control cells) did

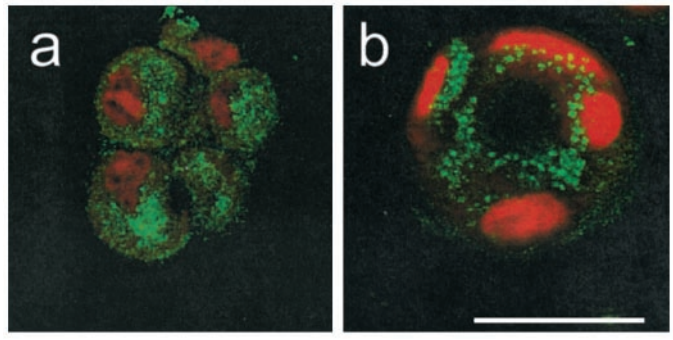


Fig. 3. Restoration of polarity in Skbr-3 cells by E-cadherin expression. Skbr-3 cells infected with an empty virus control (*a*) or a virus expressing the E-cadherin gene (*b*) were seeded as single cells into a three-dimensional gel of reconstituted BM proteins (Matrigel) and allowed to grow for 10 days. Golgi (*green*) and nuclei (*red*) were randomly distributed in control cells (*a*) but were polarized in cells expressing E-cadherin (*b*). Other basal polarity markers, such as α6 integrins, were not detected in the Skbr-3 cells. *Bar*, 30 μm.

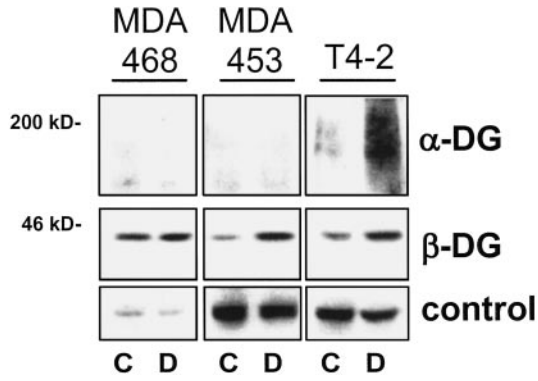


Fig. 4. Overexpression of DG increases α-DG levels in T4-2 cells. Tumor cell lines were infected with a retrovirus expressing the human DG cDNA (*D*) or empty virus control (*C*), and selected for the infected cell population. Immunoblots of α-DG and β-DG from the infected cell populations showed that β-DG levels were elevated in each of the cell lines by overexpression of the DG cDNA. However, α-DG levels were elevated only in T4-2 cells, in which the loss of α-DG was less significant. Equal protein loading was controlled by detection of β-catenin (*control*). To best reveal changes in protein expression, the immunoblots of T4-2 cell extracts were exposed to film longer than were those of the other cell lines, accommodating lower endogenous α- and β-DG levels in the T4-2 cells.

not polarize, and continued to divide, as did the uninfected T4-2 cells (see Fig. 2*a*). In contrast, T4-2 cells overexpressing the human *DG* gene organized into polarized and growth-restricted epithelial structures. Polarity was observed by basal α6 integrin localization, by polarized deposition of BM proteins (laminin-5), and by apical polarization of the Golgi apparatus. Within the infected population a total of 89 ± 5% possessed the ability to polarize, and reduced cell proliferation in this population was evidenced by the smaller colony size (Fig. 5, *A* and *B*).

The ability of mammary epithelial cells to form polarized structures in the three-dimensional BM assay correlates strongly with a nontumorigenic phenotype (3, 4). Therefore, T4-2 cells overexpressing the *DG* gene may have reduced tumorigenic potential. To test the tumorigenic potential of these cells, uninfected and infected T4-2 cells were injected s.c. into nude mice in each hind flank. Among the mice injected with uninfected T4-2 cells and infected T4-2 control cells, tumors were formed at the majority of injection sites. In contrast to the frequent tumor production by the control cells, T4-2 cells infected with the human DG gene produced no tumors at any injection sites ($n = 12$), demonstrating a clear reduction or elimination of tumorigenic potential in these cells in which polarity was restored (Fig. 5*B*).

One signaling molecule found to be regulated by DG overexpression was the PTEN phosphatase, a known tumor suppressor (34). Immunoblots of total protein extracts showed a 3-fold up-regulation of PTEN protein levels in the cells overexpressing DG and cultured within the three-dimensional-BM assay (Fig. 5*C*). This up-regulation was not evident when comparing cells cultured on plastic; therefore, up-regulation depended on cell contact with BM proteins.

DG Signaling Mediates Cytoskeletal Reorganization. Our previous studies of signaling from laminin in functionally normal mammary epithelial cells had implicated a non-integrin laminin receptor in the induction of cell shape and cytoskeletal changes (cell rounding), and this receptor had properties that resembled those of DG (22). To directly test the involvement of DG in shape changes induced by BM proteins, we tested whether the restoration of DG function in the T4-2 cells by cDNA overexpression, also enhanced or restored the ability of these cells to undergo cell rounding in response to laminin.

In the "cell-rounding" assay, cells are cultured as monolayers on tissue culture plastic and exposed to laminin (using pure laminin or Matrigel) placed in the culture medium. Under these conditions, functionally normal cells respond to laminin by changing cell shape (rounding) and aggregating (17). In testing the response of tumor cells in the cell-rounding assay, T4-2 cells, and T4-2 cells infected with the empty virus, were found not to respond significantly, and were, therefore, deficient in the response or cell-rounding and aggregation (Fig. 6, *a*, *c*, *e*, *g*, *i*). However, the T4-2 cells overexpressing the human *DG* gene changed shape by rounding and aggregating in the presence of Matrigel (Fig. 6, *b*, *d*, *f*, *h*, *j*), similar to the response of functionally normal cells. The same result was obtained using pure laminin in place of Matrigel (not shown). As observed previously in nonmalignant cells (22), the cell-rounding response in T4-2 cells overexpressing DG was not inhibited by α6 or β1 integrin blocking antibodies (separately or in combination), but it was inhibited by heparin (not shown), a known inhibitor of DG binding to laminin (27). Imaging of actin in these cells revealed obvious changes to the cytoskeleton in cells overexpressing DG but only in the presence of BM proteins; actin stress fibers were reduced, and the actin was recruited to cortical structures that were concentrated at cell-cell boundaries (Fig. 6*j*). Cell rounding was accompanied by an increase in cell height and enhanced cell-cell interactions when E-cadherin was present (Fig. 6*j*). Therefore, the augmentation of DG levels in T4-2 cells led to a change in their response to BM proteins in the cell rounding assay, as it did in the three-dimensional BM assay, and the ability to undergo cell rounding accompanied the ability to polarize in response to BM proteins.

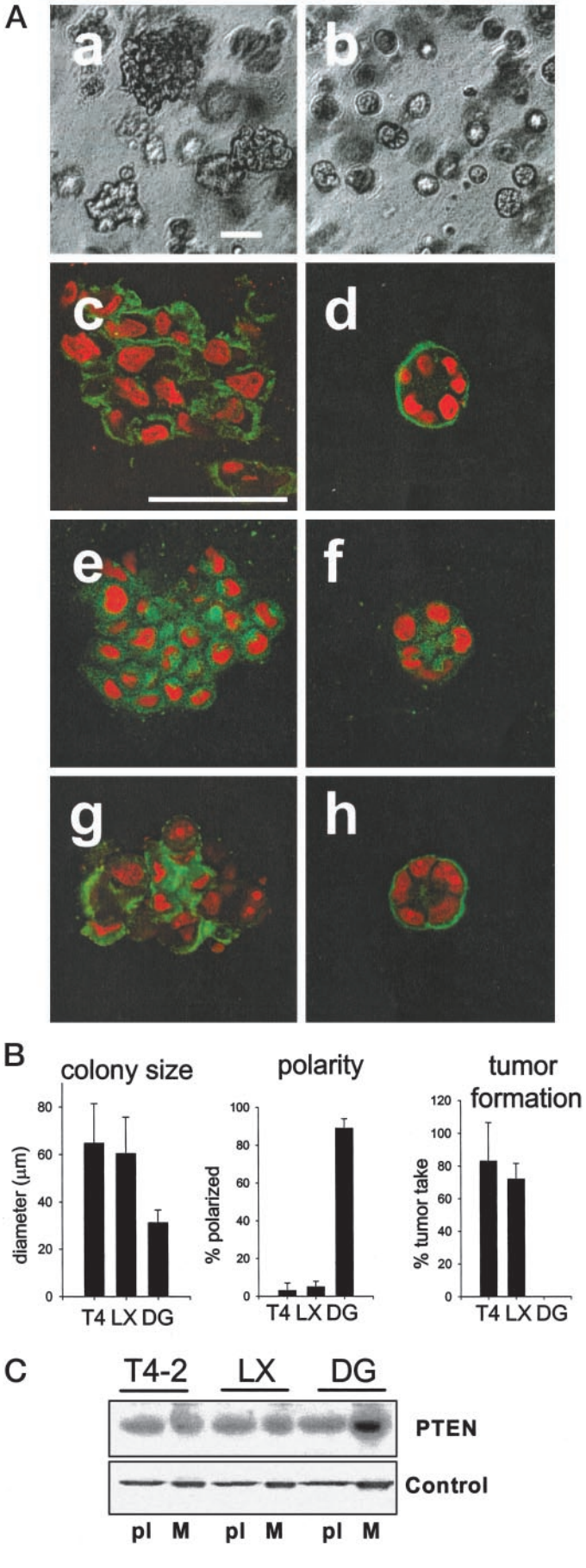
The observed correlation between cell rounding and polarization responses in T4-2 cells, was observed in each of the cell lines in Fig. 1. Those tumor cell lines expressing functional α-DG levels (BT474s, T47Ds, and Skbr-3s) demonstrated clear cell-rounding in response to the BM when grown on plastic (not shown). The other cell lines responded little, or not at all. Cell rounding induced by laminin was independent of E-cadherin function as observed in Skbr-3 cells, which lack E-cadherin expression yet "rounded-up" as single cells when exposed to BM proteins.

DISCUSSION

Polarity and Epithelial Function. Epithelial cells contacting a BM are polarized along the apical-basal axis. This polarized architecture is essential to tissue function. In addition, evidence from diverse organisms has demonstrated that correct tissue architecture in epithelia not only facilitates function but also places restrictions on critical aspects of cell behavior, such as cell growth; disruption of epithelial polarity leads to hyperproliferation of epithelial cells that mimic the cancerous or precancerous state (35–37), and the restoration of polarity to a carcinoma cell line can reinstate growth control (3, 38). How epithelial polarity is established and how this polarity regulates cell growth are open and important questions in cell and cancer biology.

Current models of tissue morphogenesis assert that epithelial architecture is determined by the collaboration of cell-cell and cell-ECM adhesion molecules (16). A number of studies have shown that polarity is influenced by BM proteins and have placed particular emphasis on the importance of laminins (19–21, 39–41). Yet, the BM receptors that regulate polarity have not been defined. The current paradigm supposes that the integrins are the major players in cell-ECM interactions and are likely to direct signals for polarity. But, as yet, no single BM receptor has been shown to have a dominant role in establishing polarity. Deletion of integrin subunits, including α3, α6, β1, and β4, from various epithelial tissues did not produce a gross loss of tissue polarity (42–49). Perturbation of integrins by function-blocking antibodies or dominant-negative gene products have given conflicting results on the role of integrins in the establishment of polarity (3, 41, 50, 51), signifying a complexity in receptor functions that is poorly understood.

DG Signaling. Results presented here indicate that DG plays a role in cytoskeletal organization, growth control, and in the determination of cell polarity in mammary epithelial cells. Each of these functions correlates



with the presence of α-DG in the cell, and each of these functions is enhanced through DG cDNA overexpression in T4-2 cells. As yet, restoration of DG function by cDNA overexpression was achieved in only one cell line. How DG exerts its influence on epithelial function remains an open question. Evidence is strong for a role of DG in BM assembly (12, 52). Therefore, as an organizer of the BM, DG might regulate the function of other receptors that depend on a correctly assembled BM; one candidate is the $\alpha 6\beta 4$ integrin, which relocalized in these studies to the basal membrane domain when polarity was restored by DG overexpression. Similar to the integrins, DG binds both the ECM and the cytoskeleton and possesses potential signaling functions of its own (10). Therefore, analogous to the integrins, DG is likely to also act through both cytoskeletal organization and signaling pathways. With respect to cell shape changes, DG appears to function independently. The cell shape changes may be achieved by simple competition for the actin cytoskeleton between $\beta 1$ integrins and DG. These effects on the cytoskeleton may or may not participate in signals for polarity, although, thus far, the cell-rounding response correlates strongly with the ability to polarize.

Interestingly, the known tumor suppressor PTEN was one molecule found to be regulated by DG overexpression. PTEN up-regulation in DG-overexpressing cells was dependent on cell contact with the BM, which is consistent with a signaling response mediated by DG. However, it remains to be determined whether PTEN regulation is a direct or an indirect consequence of DG signaling. It also remains to be determined whether PTEN up-regulation is a critical mediator of events induced by DG including cell shape changes, polarity, and decreased tumorigenic potential.

The Balance of Receptors. Proliferation and differentiation are regulated by the competing influences of multiple signals in the cellular microenvironment. As seen above, augmenting DG function restricted the growth potential of T4-2 cells, coincident with the reestablishment of polarity. This indicates that DG is part of the machinery designed to limit the growth potential of cells in the presence of BM. In previous studies, we have found that the tumorigenic phenotype of the T4-2 cell line is caused, in part, by increased signaling from $\beta 1$ integrin and EGFR pathways (3, 38). The T4-2 cells express high levels of $\beta 1$ integrin and EGFR, and were able to polarize through the inhibition of $\beta 1$ integrin, EGFR, or mitogen-activated protein kinase functions (3, 38). One model to explain these observations is that DG was functionally undermined in T4-2 cells by competing signals resulting from $\beta 1$ integrin and EGFR overexpression. Inhibition of these competing signals could unmask DG function, resulting in reversion of the tumorigenic phenotype. This model proposes that the response of cells to the BM is regulated by a competitive balance between DG, $\beta 1$ integrins, and growth factor signaling. In this context, the absolute levels of DG are less important than the

Fig. 5. Response of T4-2 cells overexpressing the DG cDNA. In *A*, T4-2 cell populations infected with the empty virus (*a, c, e, g*) and virus expressing the DG cDNA (*b, d, f, h,*) were cultured within the three-dimensional BM assay and were assessed for growth characteristics and polarity. Phase photographs (*a* and *b*) show the clear difference in size between the control (*a*) and DG-expressing cells (*b*). $\alpha 6$ integrin localization (*green, c* and *d*); Golgi (*green, e* and *f*); laminin-5 (*green, g* and *h*), and nuclei (*red*) each show polarization only in the cells overexpressing DG. *Bar*, 50 μm. *B*, comparison of size, polarity and tumorigenic potential between populations of control T4-2 cells, and T4-2 cells expressing the DG cDNA. Uninfected T4-2 cells (*T4*), T4-2 cells infected with an empty virus (*LX*), and T4-2 cells overexpressing DG (*DG*) were compared for size and polarity after growth for 10 days in the three-dimensional BM assay. Size was determined by colony diameter, and polarity by basal $\alpha 6$ integrin localization. Tumorigenic potential was determined by growth of tumors after s.c. injection in nude mice ($n = 12$, $n = 18$, $n = 12$, respectively). Enhanced DG expression in T4-2 cells reduced final colony size, increased polarity, and reduced tumorigenic potential. *C*, detection of PTEN protein levels in uninfected T4-2 cells (*T4-2*), T4-2 cells infected with an empty virus (*LXSN*), and T4-2 cells overexpressing DG (*DG*), cultured on plastic (*pl*) and within Matrigel (*M*). PTEN levels were enhanced in T4-2 cells overexpressing DG, but only when exposed to Matrigel, consistent with signaling in response to the BM. E-cadherin detection (*Control*) was used to control for protein loading. A 3-fold increase in PTEN levels was observed relative to E-cadherin levels.

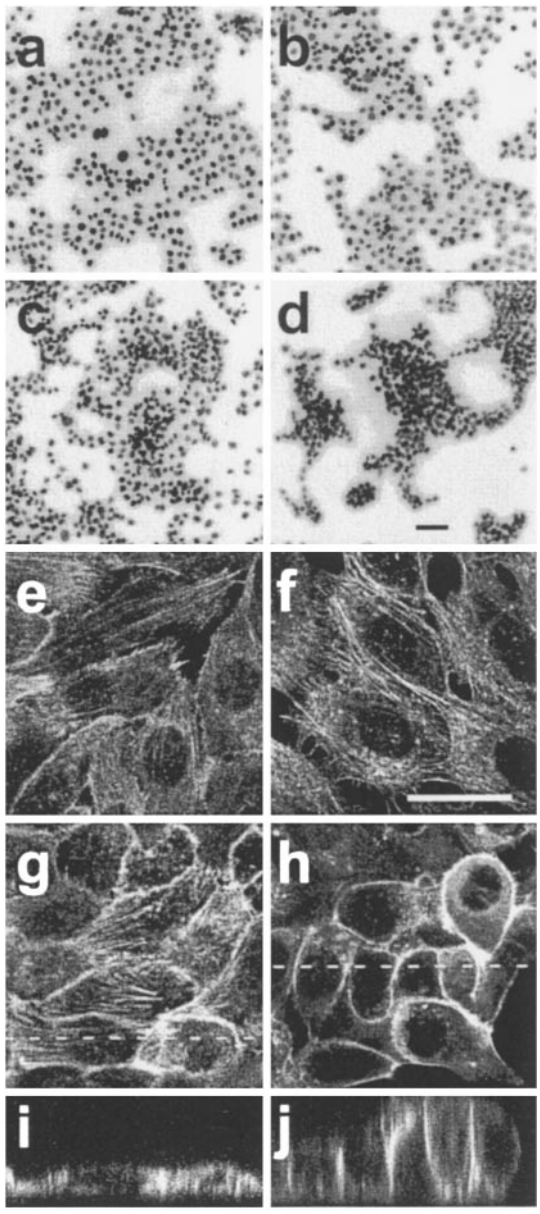


Fig. 6. Enhanced cell aggregation and rounding in T4-2 cells overexpressing DG. The T4-2 cells infected with an empty virus (*a, c, e, g, i*) and cells overexpressing DG (*b, d, f, h, j*) were placed on tissue culture plastic and allowed to spread and grow to ~30% confluence. Subsequently, the populations were either untreated (*a, b, e, f*) or exposed to soluble BM proteins in the form of pure laminin (not shown) or Matrigel added to the culture medium (*c, d, g, h, i, j*). Cell distribution, shown by DAPI staining of nuclei (*a–d*), shows the degree of cell aggregation, and reveals enhanced cell aggregation in response to BM proteins in T4-2 cells overexpressing DG (*d*). Actin staining was imaged by confocal microscopy (*e–j*). Loss of stress fibers and increased cortical actin is observed in cells overexpressing DG after treatment with BM proteins (*h*). Z-scans, vertical sections achieved using confocal microscopy (*i* and *j*), measure cell height and illustrate the degree of cell rounding. Z-scans of Matrigel-treated cells (*g* and *h, dashed lines*) showed enhanced cell rounding in cells overexpressing DG (*j*), compared with control cells (*i*). *Black bar*, 100 μm; *white bar*, 30 μm.

integrated signaling from DG, other BM receptors, and growth factor receptors.

DG as a Possible Tumor Suppressor. Cancer is characterized by a loss of tissue architecture, loss of differentiation, and loss of growth control, and ultimately by invasion of surrounding tissues. Although loss of tissue architecture is generally seen as a trait of cancer cells, it is increasingly evident that correct tissue architecture itself is an integral component of the regulatory machinery that governs cell growth and function (3, 4, 35–37, 53).

Results presented here could place DG among the regulators of cell architecture that function as tumor suppressors; as shown here, DG function is frequently lost in carcinoma cell lines, and restoration of this receptor function can reduce a cell's tumorigenic potential. Examination of breast and prostate cancer tissue specimens has also revealed a general loss of DG expression (54). We have not yet defined a causal link between DG and tumorigenicity. The effects of DG could be direct, for example, through effects on cell architecture, or indirect, through regulation of molecules like PTEN, as discussed above. In either case, DG function represents an important variable that has been omitted in previous investigations of tumor cell biology, and adding DG to the list of variables could lead to better predictive models for tumor cell behavior. Because DG operates in BM assembly, loss of DG function may explain some BM defects associated with tumor formation. In addition, the role of DG as a coreceptor may explain divergent conclusions about other receptors, such as the $\alpha 6\beta 4$ integrin (55, 56).

Evidence presented here indicates that the loss of DG function can occur by several mechanisms: diminished receptor expression; up-regulation of competing signaling pathways; or loss of functional α-DG from the cell surface. Among the tumor cells tested here, the balance between DG and $\beta 1$ integrins is most strongly regulated by the loss of α-DG function. As yet, the mechanism for loss of α-DG is unknown. Loss cannot be explained by genetic alteration of the *DG* gene because overexpression of the native DG cDNA did not restore α-DG to the surface of most tumor cells lacking the molecule. In our study, α-DG appears to be shed from the cell surface, and not simply masked by altered glycosylation, based on immunoblots using polyclonal antibodies. Release of α-DG from the cell surface has been observed in other cell models and is not unique to cancer cells (57, 58). α-DG may be shed by protease action or by a loss of DG-associated molecules that stabilizes α-DG at the cell surface. One laboratory has reported "anomolous" DG in carcinoma cells, including the loss of α-DG immunoreactivity (30). This report implicated a M_r 31,000 variant of β-DG. Whereas a M_r 30,000–31,000 variant was detected in our samples also (Fig. 1*C*), it represented only a small fraction of the total β-DG, and we observed no correlation between the presence of the smaller β-DG isoform and the loss of α-DG. A more recent report has pointed to proteolysis of β-DG as a mechanism for loss of DG function (31), but significant cleavage of β-DG is not evident in our samples and could not explain the predominant and selective loss of α-DG.

Work described here indicates an important role for DG in multiple responses of epithelial cells to the BM, including growth control, cytoskeletal organization and polarity, all of which impact strongly on tissue function. Because DG might function as a coreceptor, it could form the foundation of multiple normal signaling responses in epithelial cells through other receptors as well. DG is found to be an important variable in tumor cells, being functionally compromised in many breast tumor cell lines. Evidence presented here indicates that the status of DG function in tumor cells may provide a diagnostic measure of cancer progression, and the restoration of DG function may be therapeutic in many forms of breast cancers.

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