Silencing of D4-GDI Inhibits Growth and Invasive Behavior in MDA-MB-231 Cells by Activation of Rac-dependent p38 and JNK Signaling

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The Rho GDP dissociation inhibitor D4-GDI is overexpressed in some human breast cancer cell lines (Zhang, Y., and Zhang, B. (2006) Cancer Res. 66, 5592–5598). Here, we show that silencing of D4-GDI by RNA interference abrogates tumor growth and lung metastasis of otherwise highly invasive MDA-MB-231 breast cancer cells. Under anchorage-independent culture conditions, D4-GDI-depleted cells undergo rapid apoptosis (anoikis), which is known to hinder metastasis. We also found that D4-GDI associates with Rac1 and Rac3 in breast cancer cells, but not with other Rho GTPases tested (Cdc42, RhoA, RhoC, and TC10). Silencing of D4-GDI results in constitutive Rac1 activation and translocation from the cytosol to cellular membrane compartments and in sustained activation of p38 and JNK kinases. Rac1 blockade inhibits p38/JNK kinase activities and the spontaneous anoikis of D4-GDI knockdown cells. These results suggest that D4-GDI regulates cell function by interacting primarily with Rac GTPases and may play an integral role in breast cancer tumorigenesis. D4-GDI could prove to be a potential new target for therapeutic intervention.

Human breast cancer is a heterogeneous disease with diverse metastatic behavior and treatment responses (1). Attempts to classify this disease into clinically relevant subtypes have yielded multiple sets of gene expression signatures of noninvasive and invasive breast cancers (2–6). However, only a few genes overlap among the results from different laboratories, and most of the genes are not yet characterized as functional mediators of breast cancer progression. The molecular basis of breast tumorigenesis remains to be fully understood.

Rho GTPases, including Rac1, Rac3, Cdc42, and RhoA, are pivotal regulators of cell morphology, gene expression, cell proliferation, and apoptosis (7). The aberrant signaling through these molecules has been implicated in many aspects of tumorigenesis, including uncontrolled cell growth and metastatic phenotypes (8–12). In particular, Rac1 and its isoforms are key regulators of malignant transformation and invasive behavior of cancer cells (13–17). This is achieved at least partially by their ability to control cell growth under anchorage-independent conditions and resistance to anoikis, apoptosis induced by loss of adhesion (18–20).

As molecular switches, Rac/Rho GTPases cycle between inactive GDP-bound and active GTP-bound states (21). Their biological activity is tightly controlled by the Rho-GDP dissociation inhibitors (RhoGDIs),2 including RhoGDI (RhoGDI-1 or RhoGDI-α), D4-GDI (RhoGDI-2 or RhoGDI-β), and RhoGDI-3 (RhoGDI-γ). These proteins are thought to form stable complexes with individual Rho GTPases, thus keeping them in the cytosol. Upon growth factor stimulation, the GTPases are directed to effector sites, such as the plasma membrane, for activation (21–23). Thus, the expression levels of RhoGDIs relative to Rho GTPases must be precisely controlled to achieve normal cell function. RhoGDI binds most Rho GTPases in most types of cells (22). However, the Rho protein(s) regulated by D4-GDI in vivo are not clearly defined.

RhoGDIs are differentially expressed in human cancers, and this may contribute to the deregulation of Rho-dependent pathways in cancer cells. For instance, D4-GDI is widely expressed in hematopoietic tissues (24, 25) and is selectively down-regulated in Hodgkin lymphoma cells compared with non-Hodgkin lymphoma (26). Moreover, D4-GDI is reduced as a function of disease progression in bladder cancer (27–29). In contrast, D4-GDI is overexpressed in ovarian (30), colon (31), and breast (32) cancer cell lines as well as primary breast tumors (33–35). Notably, elevated D4-GDI expression correlates with the in vitro invasiveness of ovarian (30) and breast (32) cancer cells. In the latter, targeted disruption of D4-GDI prevents cells from invading through Matrigel (32), supporting the hypothesis that D4-GDI may be a promoter of tumorigenesis and metastasis in breast cancer.

In this study, we explored the roles of D4-GDI in breast tumor growth and metastasis by manipulating its protein expression in MDA-MB-231 cells, a highly invasive breast cancer cell line that expresses high levels of D4-GDI (32). Targeted disruption of D4-GDI abolishes tumor growth and experimental metastasis of MDA-MB-231 cells both in vitro and in vivo. We also show that D4-GDI regulates breast cancer cell growth through a signaling pathway that involves Rac GTPases and p38/JNK kinases. Thus, our results support a functional link

2 The abbreviations used are: RhoGDI, Rho-GDP dissociation inhibitor; siRNA, small interfering RNA; siD4-GDI, siRNA against D4-GDI; siLuc, siRNA against firefly luciferase; FBS, fetal bovine serum; GST, glutathione S-transferase; HA, hemagglutinin; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; polyHEMA, poly(2-hydroxyethyl methacrylate); FITC, fluorescein isothiocyanate; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-[hydroxymethyl]propane-1,3-diol; RFP, red fluorescence protein.

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between D4-GDI expression and enhanced breast cancer cell growth and invasion.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Antibodies**—The MDA-MB-231 human breast cancer cell line was obtained from the American Type Culture Collection (Manassas, VA). Its derivatives stably expressing small interfering RNA (siRNA) against human D4-GDI (siD4-GDI) or firefly luciferase (siLuc) transcripts were described previously (32). The expressed siD4-GDI sequence corresponds to nucleotides 305–324 of human D4-GDI. Clones 1-2-h and II-3-d as indicated were randomly chosen from 10 stable clones for these studies. Cells were grown in Dulbecco’s modified Eagle’s/F-12 medium (1:1; Mediatech, Herndon, VA) containing 5% fetal bovine serum (FBS), 4 mmol/liter glutamine, 50 mmol/liter β-mercaptoethanol, and 1 mmol/liter sodium pyruvate at 37 °C and 5% CO₂ in air. The stable cell lines were maintained in complete medium supplemented with hygromycin at 450 μg/ml. Antibodies specific to human D4-GDI, RhoGDI, Rac1, Cdc42, and RhoA were obtained from Thermo Scientific. Anti-glutathione S-transferase (GST) antibody (clone GST2) was from Sigma. Anti-hemagglutinin (HA) antibody (clone 12CA5) was from Roche Applied Science. Polyclonal antibodies to JNK, phospho-JNK (Thr183/Tyr185), p38, phospho-p38 (Thr180/Tyr182), ERK, and phospho-ERK (Thr202/Tyr204) were from Cell Signaling (Beverly, MA). The cell-permeable inhibitors of p38 (SB202190 and SB203580), JNK (SP600125), and ERK1/2 (PD98059) were from Alexis Biochemicals (San Diego, CA). siRNA oligonucleotide sequences specific to human D4-GDI, Rac1, and RhoA were designed using the BLOCK-iT RNAi Designer (Invitrogen). All Stealth RNA interference duplexes and their corresponding negative controls with similar GC content were purchased from Invitrogen. D4-GDI siRNA sequences were 5’-GAGCUGGAGACGCAAGCUAAUUA-3’ (designated siD4-GDI-I; positions 49–73), 5’-CAAAGAUGAAGAGGUCUAAUAG-3’ (designated siD4-GDI-II; positions 117–141), and 5’-GAAAGUGAUAAAGCAACUUAUUG-3’ (designated siD4-GDI-III; positions 402–426). Transient transfections of RNA interference duplexes were carried out using Lipofectamine RNAiMAX (Invitrogen).

**Animal Studies**—Female athymic nude mice (4–6 weeks old; NCI, National Institutes of Health) were used for all studies. Cells were grown on 70–80% confluence, harvested by trypsinization, and washed twice with phosphate-buffered saline. For xenografting studies, 5 × 10⁶ cells were suspended in 100 μl of Hanks’ balanced salt solution and injected subcutaneously into the backs of the mice (10 mice/group). Tumor growth was monitored at the indicated times by externally measuring tumor length (L) and width (W) with a caliper. Xenograft volume (V) was calculated by the following equation: \[ V = \frac{L \times W^2}{2} \times 0.5 \]. To assay metastasis, 2 × 10⁶ cells were suspended in 50 μl of Hanks’ balanced salt solution and injected into the lateral tail vein. After 6 weeks, the mice were killed, and the lungs were removed and kept in Bouin’s fixative. The tumor nodules on the lung surface appeared white, whereas the normal lung tissue appeared brown. The number of tumor colonies on the surface of each lung was counted under a dissecting microscope.

**Plasmids and Transfection**—The pcDNA3.1 constructs (Invitrogen) encoding HA-tagged human D4-GDI, RhoGDI, Rac1, and Cdc42 were obtained from Guthrie Research Institute (Sayre, PA). The inserts were amplified by PCR and subcloned into a pCEFL-GST vector to generate constructs encoding GST-D4-GDI and GST-RhoGDI. The pEFGP-C3 vectors (Clontech) for green fluorescent protein (GFP)-Rac1, GFP-Cdc42, and GFP-RhoA were generated in a similar manner. The pGEX-KG-PAK1 and pGEX-riptokin constructs, encoding a GST-fused effector-binding domain specific to Rac/Cdc42 and RhoA, respectively, have been described previously (36). The rescue plasmid for restoring D4-GDI expression, pEFGP-C3-D4-GDI-re, was as described (32). The insert was subcloned into a pDSSRed-C1 vector (Clontech), yielding a construct for expression of a red fluorescence protein-tagged D4-GDI-re protein. All constructs were verified by automated DNA sequencing. Transfections were carried out using FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s instructions.

**Cell Viability and Apoptosis Assays**—Wild-type, siLuc-expressing, or siD4-GDI-expressing MDA-MB-231 cells were cultured in complete medium supplemented with 5% FBS on plastic dishes (anchorage-dependent growth) or 0.6% agarose (anchorage-independent condition). To assess colony formation in soft agar, cells (1 × 10⁵/ml) were mixed with 0.3% agarose and 0.6% agarose base layer as described (37). To determine anoikis, cells were grown on soft agar or poly(2-hydroxyethyl methacrylate) (polyHEMA; Sigma)-coated 6-well plates, which prevents cells from adhering to the plastic wells. The polyHEMA coating was performed as described (38). Briefly, polyHEMA was dissolved in 95% ethanol (120 mg/ml), and aliquots were added to each well, yielding a 1-mm coating layer. After culture for the indicated times, cells were harvested and analyzed using a FACSscan after staining with fluorescein isothiocyanate (FITC)-annexin V and propidium iodide (39). Cell growth on regular plastic dishes was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (40).

**In Vitro Invasion Assay**—The in vitro invasion activity was assayed using BioCoat Matrigel invasion chambers (BD Biosciences) as described (32). Cells (1 × 10⁵/ml) in serum-free medium were loaded into the upper chamber of Transwells. Invasion across a 1-mm Matrigel layer was initiated by adding 20% FBS (chemoattractant) to the lower chamber. Control chambers contained the same pore size membrane (8.0 μm) but without the Matrigel coating. The chambers were incubated for 20 h at 37 °C in a 5% CO₂ atmosphere. Cells remaining on the upper surface of the membranes were removed by scraping with a cotton swab, and those on the lower surface of the membranes were counted after staining with toluidine blue (Fluka). The percent invasion was calculated as follows: the number of cells that traversed the Matrigel-coated membrane divided by the number of cells that traverse the uncoated membrane. Statistical analyses were performed using an unpaired, two-tailed t test.
D4-GDI Regulates Anoikis through Rac1

Endogenous Rho GTPase Activity—The assay is based on the high affinity binding of GTP-bound GTPase to its specific effector protein (36). RhoA-GTP was detected using the GST-rhotekin Rho-binding domain, whereas the GST-PAK1-binding domain was used to quantify Rac and Cdc42 activities. Briefly, cells were grown to 80% confluence on plastic dishes or 0.6% soft agar in the presence of 5% FBS, harvested, and lysed in buffer containing 50 mM HEPES, 150 mM NaCl (pH 7.5), 1 mM EGTA, 1% Triton X-100, 10% glycerol, 10 mM MgCl2, and protease inhibitors. Equal amounts of cell lysates were incubated with agarose-immobilized GST-rhotekin or GST-PAK1 at 4 °C for 30 min. The coprecipitates were subjected to immunoassays with assay buffer. The presence of Rho GTPases in the immunoprecipitates was detected by Western blotting. To facilitate affinity precipitation assays, stable MDA-MB-231 cell lines expressing GST-D4-GDI or GST-RhoGDI were generated. GDI complexes were isolated with glutathione-agarose beads.

Immunoprecipitation and Affinity Precipitation—For immunoprecipitation assays, cells were lysed in assay buffer containing 50 mM HEPES, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 10% glycerol, and protease inhibitors (Calbiochem). The lysate was cleared by centrifugation at 4 °C for 10 min at 14,000 × g. Cell lysate was then precleared with protein A-agarose beads for 30 min, and the proteins were immunoprecipitated with anti-D4-GDI antibodies overnight at 4 °C, followed by incubation with protein A-agarose for 1 h at 4 °C. The beads were then collected by centrifugation and washed three times with assay buffer. The presence of Rho GTPases in the immunoprecipitates was detected by Western blotting. To facilitate affinity precipitation assays, stable MDA-MB-231 cell lines expressing GST-D4-GDI or GST-RhoGDI were generated. GDI complexes were isolated with glutathione-agarose beads.

Fluorescence Microscopy—Cells were cultured on glass chamber slides at 70–80% confluence and transiently transfected with plasmids for GFP-tagged Rho proteins. After fixation in 3% paraformaldehyde for 30 min, the slides were mounted with Antifade reagent (Invitrogen). Confocal microcopy acquisitions were performed on a Zeiss LSM 5 PASCAL confocal laser scanning microscope. All images are representative of three to five independent experiments.

Western Blotting—Cell lysates in 2% SDS lysis buffer or immunoprecipitates were resolved by SDS-PAGE on 4–12% BisTris gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore Corp.). Immunoreactive proteins were visualized by ECL reagent (Santa Cruz Biotechnology). When necessary, the membranes were stripped using Restore Western blot stripping buffer (Pierce) and reprobed with appropriate antibodies.

RESULTS

D4-GDI Down-regulation by siRNA Abrogates Tumor Formation and Experimental Metastasis—Our previous work showed that D4-GDI is highly expressed in a number of breast cancer cell lines, with the greatest abundance in MDA-MB-231 cells (32). We also showed that down-regulation of D4-GDI by RNA interference results in a decrease in cell migration and invasion through Matrigel, which can be rescued by restoration of D4-GDI protein expression (32). Because MDA-MB-231 cells have been shown to be highly invasive (41), we wanted to determine whether lowering D4-GDI expression in these cells might inhibit tumor formation and metastasis in vivo. To this end, we established stable MDA-MB-231 cell lines that express siD4-GDI. Silencing of firefly luciferase (siLuc) was used as a control. siD4-GDI specifically suppressed D4-GDI protein expression without changing the levels of RhoGDI, a homolog sharing 75% sequence identity (Fig. 1A). Two stable clones lacking D4-GDI, clones I-2-h and II-3-d, were randomly chosen for further studies. The number of subclones was limited to five.

The effect of D4-GDI expression on tumor growth was assessed by subcutaneous injection into nude mice. Parental MDA-MB-231 cells developed into large tumors, whereas D4-GDI knockdown cells (clone I-2-h) showed only minimal growth in vivo (Fig. 1B), even at 3 months post-inoculation (data not shown).

To determine whether down-regulation of D4-GDI inhibits metastasis of MDA-MB-231 cells, cells were injected intravenously through the tail veins of nude mice. Consistent with published results (42, 43), wild-type MDA-MB-231 cells formed 250–300 metastatic nodules per lung in 80% of the animals analyzed (23 of 29) 6 weeks post-injection (four groups of n = 10 mice, 11 dropouts due to critical conditions, a total of 29 mice analyzed) (Fig. 1C). Similar results were obtained for cells expressing siLuc. In contrast, no nodules were visible in the lungs of animals injected with D4-GDI knockdown cells (clone I-2-h; four groups of n = 10 mice, a total of 40 mice analyzed). Metastatic nodules were not observed in the livers of animals from both groups. D4-GDI knockdown clone II-3-d behaved similarly to clone I-2-h in tumor growth and lung colony formation (data not shown). These results show that targeted disruption of D4-GDI effectively abrogated both tumor growth and experimental metastasis of MDA-MB-231 cells in nude mice.

Loss of D4-GDI Induces Anoikis in MDA-MB-231 Cells—To determine the mechanism through which D4-GDI knockdown blocks tumor growth, we studied the growth properties of stable clones under different conditions in vitro. In monolayer culture (anchorage-dependent growth), D4-GDI-depleted cells retained the epithelial morphology of parental MDA-MB-231 cells. The growth rate was only slightly lower (~2-fold) than that of the parental cells and cells expressing siLuc (Fig. 2A). When cultured on soft agar (anchorage-independent growth), the parental MDA-MB-231 cells formed over 150 colonies >50 μm in size per well at day 10. A similar number of colonies were found for cells expressing siLuc. However, D4-GDI-deficient cells formed only zero to five colonies per well (Fig. 2B). Cells were then grown on Matrigel, mimicking in vivo growing conditions. D4-GDI knockdown cells lost the unpolarized and branching morphology of parental cells and showed at least 10-fold lower cell viability at day 10 (Fig. 2C), consistent with our previous findings that down-regulation of D4-GDI results in the loss of cell-matrix adhesion (32). These results show that D4-GDI knockdown had a minimal effect on anchorage-dependent growth of MDA-MB-231 cells but significantly inhibited cell growth under anchorage-independent conditions.

To determine whether the observed growth inhibition was due to anoikis, cells were cultured on polyHEMA-coated plates, which completely prevents cell adhesion (38). To rule out the possible off-target effects of siRNA, we designed three siRNA duplexes targeting different regions on the D4-GDI gene. At 24 h post-transfection, D4-GDI protein was decreased by ~60%, whereas
RhoGDI was not altered (Fig. 3A). Cells were then cultured on polyHEMA-coated wells for an additional 24 h and analyzed for apoptosis by fluorescence-activated cell sorting. Strikingly, D4-GDI knockdown cells underwent massive cell death (30–50%) as indicated by FITC-annexin V and/or propidium iodide staining (Fig. 3B and C). In contrast, cells transfected with a control siRNA had only basal (≤5%) apoptosis. Collectively, these results demonstrate that D4-GDI knockdown induced anoikis of MDA-MB-231 cells grown under anchorage-independent conditions. Given that D4-GDI knockdown only slightly reduced cell proliferation (Fig. 3A), it is therefore reasonable to propose that the observed growth inhibition on soft agar or even in nude mice is at least in part due to induction of anoikis in the target cells.
D4-GDI Regulates Anoikis through Rac1

Consistent with our previous data (32), knockdown of D4-GDI with different siRNA sequences uniformly inhibited invasion of MDA-MB-231 cells in vitro (Fig. 3D). Similar effects were obtained with the three siD4-GDI duplexes, supporting the specificity of siRNA effects.

D4-GDI Associates Primarily with Rac GTPases in Breast Cancer Cells—To delineate the signaling pathways by which D4-GDI controls cell survival and invasion, we set out to identify the Rho GTPases that associate with D4-GDI in breast cancer cells. To this end, whole cell extracts from MDA-MB-231 cells were immunoprecipitated using anti-D4-GDI antibodies and probed for Rac1, Cdc42, and RhoA, which represent the three major subfamilies of Rho GTPases. Endogenous Rac1 was strongly detected in D4-GDI immunocomplexes from parental cells, whereas Cdc42 and RhoA showed little or no detectable binding to D4-GDI (Fig. 4A). None of these GTPases was detected in the immunoprecipitates from D4-GDI knockdown cells.

To confirm these results, we generated stable MDA-MB-231 cell lines that express GST-D4-GDI or GST-RhoGDI fusion protein or GST alone and performed affinity precipitation assays. Consistent with the above results, Rac1, but not Cdc42 or RhoA, was precipitated with GST-D4-GDI (Fig. 4B). As expected (22), GST-RhoGDI formed stable complexes with all three GTPases.

To determine whether D4-GDI might also associate with other Rac GTPases that are expressed in breast cancer cells, several HA-tagged GTPases were transiently expressed in the stable cell lines containing the GST fusion proteins. GST-D4-GDI was shown to be as effective as GST-RhoGDI in pulling down HA-Rac3 (Fig. 4C), as determined by anti-HA Western blotting. In contrast, RhoC and TC10, two other distinct members of the Rho family, were both found in the immunocomplexes of GST-RhoGDI, but not those of GST-D4-GDI. Similar results were obtained with respect to the selectivity of D4-GDI for Rac1 in studies of MDA-MB-468 cells, in which D4-GDI is also highly expressed (Fig. 4D). As a control, Rac1 was not detected in the immunocomplexes of anti-D4-GDI antibody from MCF-7 cells (Fig. 4D), which were shown to be deficient in D4-GDI expression (32). Taken together, these results suggest that D4-GDI associates primarily with Rac1 and Rac3 GTPases in human breast cancer cells.

Rac1 Is Constitutively Activated and Translocated to Membranes following D4-GDI Depletion—Like other Rho GTPases, Rac1 exists predominately in the cytosol in unstimulated cells (44). To determine whether D4-GDI affects the subcellular localization and activity of Rac1, cell lysates were fractionated into cytosolic and membrane fractions and assessed for the presence of each GTPase by immunoblotting. As shown in Fig. 5A, Rac1 was enriched in the membrane fraction of D4-GDI-depleted cells compared with parental cells and cells expressing siLuc, whereas no change in subcellular localization was detected for Cdc42 or RhoA. Confocal microscopy analysis of
the transiently expressed GFP-tagged Rho proteins revealed that GFP-Rac1 was localized in the cytosol of wild-type cells, but it was found prominently in the plasma membrane and internal membranes upon D4-GDI depletion (Fig. 5B). In contrast, GFP-Cdc42 localized in a pattern similar to that of GFP-RhoA, with prominent fluorescence in the cytosol in both wild-type and D4-GDI knockdown cells. We then examined the effect of D4-GDI restoration on Rac1 subcellular distribution by transfection of a rescue plasmid expressing red fluorescence protein (RFP)-tagged D4-GDI-re. As shown in Fig. 5C, expression of RFP-D4-GDI-re induced a reversion of Rac1 subcellular localization, i.e. from the plasma membrane to the cytoplasm. RFP alone had no apparent effect on Rac1 subcellular localization. These results indicate that loss of D4-GDI resulted in translocation of Rac1 from the cytosol to membrane compartments in the cells without significantly affecting Cdc42 or RhoA subcellular distribution.

Next, we determined the activation state of the endogenous GTPases using an effector pulldown assay (36). Consistent with the binding patterns, loss of D4-GDI caused a significant increase in the proportion of active Rac1 under normal culture conditions (~3-fold higher compared with control cells), whereas Cdc42 activity remained unchanged in the cell lines. The level of RhoA-GTP was also increased in D4-GDI-depleted cells, maybe due to a sequential activation by Rac1 (56). To rule out the possible off-target effects of siRNA, we restored D4-GDI protein expression in cells expressing siD4-GDI by transfection of a rescue plasmid in which the siRNA target sequence was mutated (32). GFP-D4-GDI-re expression was confirmed by Western blotting (Fig. 5E). Interestingly, ectopic expression of D4-GDI suppressed Rac1 activation without affecting Cdc42 activity. Taken together, these results demonstrate not only the specificity of RNA interference but also the selective interaction between D4-GDI and the Rac subfamily of Rho GTPases.

**Rac1-dependent Activation of p38 and JNK Kinases Is Required for Anoikis of D4-GDI-depleted Cells**

Rac1 has been shown to induce apoptosis through activation of the p38 and JNK stress-activated protein kinase pathways (45, 46). These kinases are also implicated in the execution of apoptosis by different cytotoxic agents (47, 48). Consequently, we studied p38 and JNK activation following the loss of D4-GDI by assessing their phosphorylation states using antibodies specific to the phosphorylated species of each enzyme. Compared with control cells, D4-GDI knockdown cells showed a significant increase in the phosphorylation levels of both JNK and p38 kinases under normal (data not shown) as well as anoikis (Fig. 6A) culture conditions. ERK phosphorylation was not changed in the cell lines. The phosphorylation of JNK and p38 returned to basal levels after restoration of D4-GDI expression, further demonstrating the specificity of D4-GDI siRNA.
We then examined the relationship between Rac1 and the sustained activation of p38 and JNK by gene silencing. As shown in Fig. 6B, knockdown of Rac1 suppressed the phosphorylation of both p38 and JNK kinases and markedly reduced apoptosis of D4-GDI-depleted cells when grown on soft agar. In contrast, knockdown of RhoA did not alter the phosphorylation status of p38 or JNK. These results suggest that Rac1, but not RhoA, is primarily responsible for the observed kinase activation and spontaneous apoptosis. In line with these results, pretreatment with the p38 inhibitor SB202190 (48) or SB203580 (49) (data not shown) significantly inhibited apoptosis of D4-GDI-depleted cells (Fig. 6C). The inhibition effect was also observed with the JNK inhibitor SP600125 (50). However, incubation with the ERK inhibitor PD98059 (48) had no effect on cell survival under the same conditions. Thus, D4-GDI deficiency appears to induce anoikis at least partially through Rac1-dependent activation of the JNK and p38 pathways. Collectively, the results establish a causal link between D4-GDI depletion and the described molecular changes, including the activation of Rac1 and p38/JNK kinases and increased anoikis.

**DISCUSSION**

A major challenge to breast cancer treatment has been the identification of therapeutic targets for preventing metastasis. In this study, we have shown that silencing of D4-GDI blocks tumor growth and lung nodule formation of MDA-MB-231 cells in nude mice by inducing anoikis, in part through Rac-mediated activation of the JNK and p38 pathways. Collectively, the results establish a causal link between D4-GDI depletion and the described molecular changes, including the activation of Rac1 and p38/JNK kinases and increased anoikis.
tumor, survive in the blood or lymphatic circulation, invade distant tissues, and establish a secondary tumor in a new environment. Anoikis of tumor cells serves as a physiological barrier to metastasis (18, 19). Notably, silencing of D4-GDI had only a minimal effect on cell proliferation (Fig. 2A), but it significantly inhibited anchorage-independent growth (Fig. 2, B and C). When cultured on polyHEMA-coated wells or soft agar, D4-GDI knockdown cells underwent rapid and substantial apoptosis (Fig. 3), known as anoikis. These results suggest that the growth inhibition by D4-GDI knockdown is at least in part through induction of anoikis in the target cells. In addition, D4-GDI knockdown cells were found to be hypersensitive to apoptosis by TRAIL (tumor necrosis factor-related apoptosis-inducing ligand),3 a cytokine that has been implicated in immunosurveillance of developing and metastatic tumors (51). These effects may cooperate to suppress the growth and invasion of MDA-MB-231 cells both in vitro and in vivo. Our results are distinguished from those of Schunke et al. (33), who showed an increase in the migratory activity of MDA-MB-231 cells after D4-GDI knockdown. The reason for the discrepancy is unclear but may be related to a difference in experimental approaches. Their study employed a transient knockdown of the D4-GDI gene and measured cell migration across an 8-μm filter without coating. We measured the invasion of stable clones through Matrigel (1 mm) coated with basement membrane complexes. As shown (Fig. 2) (33), D4-GDI-depleted cells behave quite differently under the two different growth conditions. Schunke et al. also reported that D4-GDI expression levels were not associated with breast cancer progression. However, Hu et al. (35) revealed a biphasic expression of D4-GDI (i.e. increase and then decrease) along the progression of mammary epithelial proliferation and carcinogenesis. In agreement with Hu et al., our data suggest that D4-GDI may be a metastasis-related protein in breast cancer. Interestingly, D4-GDI is also overexpressed as a function of ovarian cancer progression (30). By contrast, D4-GDI is reduced in bladder cancer compared with benign tissue (27). In addition, D4-GDI (LyGDI) is highly expressed in non-Hodgkin lymphoma, but not in Hodgkin lymphoma (26). The difference in D4-GDI expression patterns may reflect the diversity of genetic backgrounds and/or tumor stages as well as activation versus inhibition of Rac or other Rho GTPases in the course of cancer progression (21).

The mechanism by which D4-GDI regulates breast cancer cell invasion is not clear but may be related to Rac-dependent survival pathways. We have provided several lines of evidence indicating that D4-GDI selectively interacts with Rac1 and Rac3 GTPases in breast cancer cells, but not with Cdc42, RhoA, RhoC, or TC10. However, these data do not rule out the possibility that D4-GDI binds Rac1 or Rac3 indirectly via other proteins in the cells. Additional studies are necessary to determine the selectivity of D4-GDI for Rac GTPases. Interestingly, both Rac1 and Rac3 are often overexpressed in breast cancer cells (52–54). Consistent with these results, D4-GDI depletion resulted in a release and constitutive activation of Rac1 in the target cells. This process is associated with a sustained activation of the mitogen-activated protein kinase (MAPK) family kinases JNK and p38, but not ERK. Restoration of D4-GDI expression suppressed both Rac1 activation and p38/JNK phosphorylation, demonstrating the specificity of D4-GDI silencing (Figs. 5E and 6A). Furthermore, knockdown of Rac1 or inhibition of the JNK and p38 kinases with small drug inhibitors blocked anoikis in soft agar (Fig. 6). These results suggest that D4-GDI depletion induces anoikis at least partially through

3 T. Khan and B. L. Zhang, unpublished data.
activation of JNK and p38. However, its precise mechanism of action remains to be elucidated. Nevertheless, the results presented here support Rac1 as a potent activator of the JNK and p38 pathways (45, 46, 55) and are also consistent with a pro-apoptotic role for Rac GTPases in non-hematopoietic cells (45, 56, 57). Many studies have described the link between induction of JNK and p38 activation and apoptosis in response to chemotherapies (19, 47, 48). Silencing of D4-GDI appears to activate Rac-dependent apoptosis pathways, mimicking stressful stimuli. Surprisingly, the level of GTP-bound RhoA was also increased in D4-GDI-depleted cells despite their undetectable interaction. This may be a sequential event of Rac1 activation, as seen in NIH3T3 cells following growth factor stimulation (58). In support of this idea, knockdown of Rac1 by siRNA reduced RhoA-GTP levels in the target cells (data not shown). Furthermore, RhoA knockdown had no significant effect on D4-GDI depletion-induced apoptosis (Fig. 6), suggesting that RhoA activation might be a bystander effect of D4-GDI depletion.

As molecular switches, Rac GTPases have been reported to elicit apparently conflicting roles in cancer cell invasion and apoptosis, giving either stimulatory (52–54) or inhibitory (13–15, 17) effects. The results suggest that Rac may play dual roles in cancer progression depending on cell types and cellular environment (reviewed in Ref. 16). In studies using dominant-active (Rac1V12) or dominant-negative (Rac1N17) Rac1 mutants, Rac activation appeared to protect cells from apoptosis or anoikis and to promote cell invasion in fibroblast cells (20), T-lymphoma cells (59), and Madin-Darby canine kidney epithelial cells (18, 60). However, ectopic expression of Rac1V12 or Tiam1 (a Rac-specific activator) inhibited invasion of Ras-transformed Madin-Darby canine kidney cells (13). A recent study showed that Rac1 inhibition by the pharmacological inhibitor NSC23766 suppressed cell migration in non-metastatic breast cancer cell lines (MCF-7 and T47D) or a moderately metastatic line (Hs578T) but that the inhibitor increased cell migration in highly invasive cell lines (MDA-MB-231 and MDA-MB-435) (17). Our data are consistent with the latter, showing that Rac activation following D4-GDI depletion is beneficial in that it blocks MDA-MB-231 cell invasion by inducing anoikis. The results also suggest that Rac activities are suppressed in the parental breast cancer cells, which may be responsible for the onset of invasive phenotypes of breast cancer. Given the selective binding of D4-GDI to Rac1 and Rac3, it will be interesting to determine the expression levels of D4-GDI relative to Rac GTPases in breast cancers and to analyze its prognostic relevance in aggressive breast carcinomas.

In summary, we have shown that D4-GDI regulates MDA-MB-231 cell growth and invasion through Rac-dependent pathways. The data presented warrant additional studies with other breast cancer cell lines and primary tumor samples. If it is confirmed in clinic, D4-GDI could prove to be a potential new target for therapeutic intervention of aggressive breast cancers.

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