PLAGL2 regulates actin cytoskeletal architecture and cell migration

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Introduction

The actin cytoskeleton plays a pivotal role in various fundamental biological processes, such as cell migration, cell–extracellular matrix adhesion and apoptosis. Extensive studies have identified a number of critical molecules for the organization of the actin cytoskeleton. One particular family of proteins, the Rho family of small guanosine triphosphatases (GTPases), is essential for the dynamic rearrangement of the actin cytoskeleton to promote cell–extracellular matrix adhesion and migration. The expression patterns of these diverse sets of GEFs and GAPs are factors that determine how Rho GTPases are activated in different cell lines and tissues. The elucidation of factors that control the expression of GEFs and GAPs will provide further insight into the regulatory mechanisms of Rho GTPase activation.

Pleomorphic adenoma gene like-2 (PLAGL2), a member of the PLAG gene family, is a C2H2 zinc finger transcriptional factor that is involved in cellular transformation and apoptosis. In this report, we show that PLAGL2 is associated with the organization of stress fibers and with small guanosine triphosphatase (GTPase) activity. Depletion of PLAGL2 in two different ovarian cancer cell lines, ES-2 and HEY, induced activation of RhoA, whereas activity of Rac1 was suppressed. Organization of actin stress fibers and focal adhesions was significantly promoted by PLAGL2 knockdown in a RhoA-dependent manner. Conversely, exogenous expression of PLAGL2 in MDA-MB-231 cells, a breast cancer cell line, resulted in the activation of Rac1 and the inactivation of RhoA. In addition, PLAGL2 expression induced lamellipodia formation and disruption of stress fiber formation. Finally, we show that CHN1 expression is essential for Rac1 inactivation in PLAGL2-depleted cells. Our results demonstrate a crucial role of PLAGL2 in actin dynamics and give further insight into the role of PLAGL2 in cellular transformation and apoptosis.

Materials and methods

Cells, antibodies and chemicals

ES-2 and HEY cells were propagated in RPMI supplemented with 10% fetal bovine serum (Equitech-BIO, Kerrville, TX) and antibiotics. MDA-MB-231 and 293T cells were maintained in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) with 10% fetal bovine serum (Equitech-BIO) and antibiotics. Antibodies for vinculin, Rac1, RhoA and Cdc42 were obtained from Abcam (Cambridge, MA). Anti-GFP antibody was obtained from Santa Cruz Biotechnology (Dallas, TX). Antibodies for p-Akt and p-ERK were obtained from Cell Signaling Technology (Danvers, MA). Anti-β-tubulin antibody was obtained from Sigma-Aldrich (St Louis, MO), and infected cells were selected by incubating with 1 μg/ml puromycin for 2 days. To produce ES-2 cells that constitutively expressed short hairpin RNAs (shRNAs), oligonucleotides encoding shRNA specific for human CHN1 (5'-GGGGAGATGATAATGTGCAAT-3') and luciferase (5'-CTTACGCGTATGCTTTGCA-3') were cloned into the pSIREN-RetroQ vector (TAKARA). 293T cells were transfected with the shRNA-encoding analysis has revealed that there are a large number of GEFs and GAPs in humans compared with the number of Rho GTPases (9). The expression patterns of these diverse sets of GEFs and GAPs are factors that determine how Rho GTPases are activated in different cell lines and tissues. The elucidation of factors that control the expression of GEFs and GAPs will provide further insight into the regulatory mechanisms of Rho GTPase activation.

Pleomorphic adenoma gene like-2 (PLAGL2) is a member of the PLAG family of C2H2 zinc finger transcriptional factors that includes PLAG1 and PLAGL1 (10,11). These genes share highly homologous successive C2H2 zinc finger motifs in the N-terminal and proline- and serine-rich regions in the C-terminal (12). PLAG1 is associated with the promotion of pleomorphic adenomas of the salivary gland as well as lipoblastoma and hepatoblastoma (13–17), whereas PLAG1 functions as a tumor suppressor (18,19). Interestingly, PLAGL2 has been reported to have both oncogenic and tumor suppressor functions. Experiments with mice demonstrated a role for PLAGL2 in the development of acute myeloid leukemia and lung cancer (20–22). Furthermore, exogenous expression of PLAGL2 in NIH3T3 cells promoted cellular transformation (23). A recent study also revealed that PLAGL2 activates the WNT/β-catenin pathway and contributes to the progression of human malignant glioblastoma (24). In contrast to these findings, other reports suggested tumor-suppressive functions for PLAGL2. Expression of PLAGL2 in promyelocytic U937 cells induces cell cycle block and apoptosis by promoting the expression of p73, a homolog of p53 (25). In addition, PLAGL2 promotes expression of Nip3, a proapoptotic protein, to induce apoptosis (26). These findings indicate that PLAGL2 is associated with various fundamental cellular processes, but the precise role of PLAGL2 in cells still remains largely unknown. In this report, we show a novel role for PLAGL2 in actin cytoskeleton organization and in the regulation of the activity of small GTPases, such as RhoA and Rac1.

Abbreviations: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GTP, guanosine triphosphate; GTPase, guanosine triphosphatase; miRNA, messenger RNA; PBS, phosphate-buffered saline; PLAGL2, pleomorphic adenoma gene like-2; shRNA, short hairpin RNA; siRNA, small interfering RNA.

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siRNA transfection

The sequences of the small interfering RNAs (siRNAs) used to suppress PLAGL2 expression were 5′-GCU CUGUUAGGAGCC UUATT-3′ (siRNA1) and 5′-GUCUC UGAUAAGAAGAAUTT-3′ (siRNA2). The sequence of the control siRNA-targeting luciferase was 5′-CUACGG UAGGACUCU GAT-3′. Cells were transfected with 20nM of siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. The siRNAs were obtained from Sigma–Aldrich.

Immunofluorescence analysis

Cells were grown on glass coverslips coated with fibronectin, fixed with 4% parafomaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 3 min and blocked with phosphate-buffered saline (PBS) containing 7% fetal bovine serum for 30 min. The cells were incubated with primary antibody in PBS for 1 h, washed three times with PBS, incubated with fluorescein isothiocyanate-labeled secondary antibody in PBS for 1 h and then analyzed using a fluorescence microscope (BX60; Olympus, Tokyo, Japan). To visualize the actin cytoskeleton, the fixed cells were incubated with rhodamine-conjugated phalloidin for 30 min and washed.

Cell migration assay

Cell migration was quantified using Boyden chambers. Cells were transfected with siRNAs, and 72 h later, suspended cells (5.0 × 10⁵) were seeded onto the upper surface of the chamber. The lower surface of the filter was coated with fibronectin. Cells were fixed with 70% methanol at 2, 4 and 6 h later. Cells on the upper surface of the filter were removed using a cotton swab and stained with 0.5% crystal violet. Cells that migrated to the lower surface of the filters were counted in five randomly selected fields from three independent experiments.

Rho GTPase activity assay

Cells were lysed with pull-down lysis buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% NP40, 5 mM MgCl₂, protease inhibitor cocktail (Roche, Basel, Switzerland) and 1 mM phenylmethylsulfonyl fluoride) and incubated with GST-PAK-PBD or GST-Rho-kinase fusion protein bound to glutathione-agarose beads for 1 h at 4°C. The beads were washed with pull-down buffer four times and then subjected to western blotting analysis with each antibody to detect active Rho GTPases. Total protein was detected by immunoblotting of whole cell lysates.

Statistical analysis

Data are expressed as the means ± SD. Comparisons between the groups were performed using unpaired t-tests. P values of <0.05 were considered statistically significant.

Results

PLAGL2 depletion promotes stress fiber formation

Although we were searching for genes that regulate the cellular morphology of ovarian cancer cells using a limited library of siRNAs (27), we noticed that transfection of two different siRNAs-targeting PLAGL2 promoted the formation of actin stress fibers. Transfection of these siRNAs efficiently reduced levels of PLAGL2 mRNA in both ES-2 and HEY cells, which are ovarian cancer cells (Figure 1A). In addition, depletion of PLAGL2 by these siRNAs promoted clear organization of actin stress fibers in both cell types (Figure 1B).

Consistent with the changes in stress fiber formation, PLAGL2 depletion induced focal adhesion formation (Figure 1B). We counted the cells that showed stress fiber formation upon PLAGL2 depletion. PLAGL2 knockdown significantly increased the number of cells with organized stress fibers and focal adhesions in both cell lines (Figure 1C).

To exclude the possibility that the stress fiber formation was induced by the off-target effect of the siRNAs, we performed a rescue experiment using ES-2 cells. We expressed green fluorescent protein (GFP) or N-terminally GFP-tagged PLAGL2 (GFP-PLAGL2) in ES-2 cells by retrovirus infection. Cells were then transfected with control siRNA or PLAGL2 siRNA1 that targeted the 3′-untranslated region of PLAGL2 mRNA. Similar to parental ES-2 cells, GFP-expressing ES-2 cells showed robust organization of stress fibers after PLAGL2 siRNA1 transfection (Figure 1D). In contrast, PLAGL2 siRNA1 transfection did not promote stress fiber formation in GFP-PLAGL2-expressing ES-2 cells (Figure 1D). These results show that stress fiber formation was specifically promoted by PLAGL2 depletion.

PLAGL2 depletion affects the activity of small GTPases

We next examined whether the activity of the small GTPases, RhoA, Rac1 and Cdc42, was affected by PLAGL2 depletion using a pull-down assay. ES-2 and HEY cells were transfected with control or PLAGL2 siRNA1; 72 h later, cells were lysed and active small GTPases were affinity precipitated by GST-Rho-kinase-RBD or GST-PK-PBD bound to glutathione-agarose beads. The precipitates were then subjected to immunoblot analysis with antibodies against each small GTPase. Consistent with the changes in stress fiber formation, RhoA was activated by PLAGL2 knockdown (Figure 2A). Interestingly, the activity of Rac1 was reduced in cells transfected with PLAGL2 siRNA1, whereas PLAGL2 knockdown did not induce clear changes in Cdc42 activity (Figure 2A). To determine whether the promotion of stress fiber formation by PLAGL2 depletion was due to excess RhoA signaling, we used Y27632, a Rho kinase inhibitor. Addition of Y27632 abolished the enhanced formation of stress fibers induced by PLAGL2 knockdown (Figure 2B). We also used dominant-negative RhoA (T19N) to suppress RhoA activity in cells (28). ES-2 cells were infected with a recombinant retrovirus that encoded either GFP or GFP-RhoA (T19N), and cells were then transfected with control or PLAGL2 siRNA1. As shown in Figure 2C, PLAGL2 depletion did not promote stress fiber formation in GFP-RhoA (T19N)-expressing cells, whereas GFP-expressing cells showed clear stress fiber formation after PLAGL2 siRNA1 transfection. These results show that the stress fiber formation induced by PLAGL2 depletion is mediated by RhoA activation.

PLAGL2 depletion suppresses cell migration

Small GTPases are one of the critical factors for the promotion of cell migration; thus, we examined the effects of PLAGL2 knockdown on the migration of ES-2 and HEY cells using Boyden chambers. Cells transfected with siRNAs were placed on the upper surface of the filter and allowed to migrate to the bottom surface, which was coated with fibronectin. We counted cells that migrated to the bottom surface at 2, 4 and 6 h after the seeding. As shown in Figure 3A, cell migration of PLAGL2-depleted cells was delayed compared with control siRNA-transfected cells. We next examined whether changes in small GTPase activities were involved in the delayed migration of PLAGL2 knockdown cells. Since RhoA was activated by PLAGL2 depletion, we examined migration of PLAGL2-depleted cells in the presence of Y27632. Addition of Y27632 promoted migration of control siRNA-transfected cells (Figure 3B). In addition, delayed cell migration by PLAGL2 knockdown was partially restored by the addition of Y27632 (Figure 3B). We also tested if activation of Rac1 can restore the migration defect induced by PLAGL2 knockdown. We established ES-2 cells that constitutively expressed wild-type or active form of Rac1 and examined cell migration. Similar to the addition of Y27632, expression of active form of Rac1 partially restored migration of PLAGL2 knockdown cells (Figure 3C). These results suggest that the delayed migration by PLAGL2 depletion is mediated by the changes in small GTPase activities.

Exogenous expression of PLAGL2 disrupts stress fiber formation and promotes lamellipodia production

We next examined the effects of PLAGL2 overexpression in cells. ES-2 and HEY cells were infected with a GFP-PLAGL2-encoding retrovirus, and infected cells were treated with puromycin 3 days later. However, during the course of drug selection, GFP-PLAGL2-expressing cells disappeared and we were unable to obtain cell lines. A previous study reported that PLAGL2 can promote cellular apoptosis (25); thus, exogenous expression of PLAGL2 in ES-2 and HEY cells may have induced apoptosis. We expressed
PLAGL2 depletion promotes stress fiber formation. (A) ES-2 and HEY cells were transfected with either control or PLAGL2 siRNAs and 72 h later, PLAGL2 mRNA expression levels were determined by reverse transcription–polymerase chain reaction. siPLAGL2-1 and siPLAGL2-2 indicate cells transfected with PLAGL2 siRNA1 and PLAGL2 siRNA2, respectively. siCtrl indicates cells transfected with control siRNA. (B) Cells cultured on fibronectin-coated glass coverslips were transfected with either control or PLAGL2 siRNAs; 72 h later, the cells were fixed and immunostained for F-actin and vinculin (scale bar = 20 μm). (C) ES-2 and HEY cells were treated as in B and cells with actin stress fiber formation or focal adhesion formation were counted. The graph indicates the percentage of cells with stress fiber formation or focal adhesion formation (means ± SD). Fifty cells were evaluated in each experiment, and three independent experiments were performed. Note that ES-2 cells are devoid of stress fiber and focal adhesion formation; thus, there is no value for ES-2 cells transfected with Ctrl siRNA. (D) ES-2 cells were infected with retroviruses that encoded either GFP or GFP-PLAGL2; 24 h later, the cells were transfected with control siRNA or PLAGL2 siRNA1 (siPLAGL2-1). Seventy-two hours later, the cells were fixed and stained for F-actin (scale bar = 20 μm). The graph indicates the percentage of cells with stress fiber formation (means ± SD). Fifty cells were evaluated in each experiment, and three independent experiments were performed. Note that ES-2 cells-expressing GFP or GFP-PLAGL2 are devoid of stress fiber formation; thus, there is no value for cells transfected with Ctrl siRNA.
PLAGL2 in MDA-MB-231 breast cancer cell line, which showed reduced expression of PLAGL2 compared with ES-2 and HEY cells (Figure 4A). Expression of PLAGL2 in MDA-MB-231 cells significantly induced lamellipodia formation and disruption of stress fibers (Figure 4B and C). Consistent with the formation of lamellipodia, Rac1 was activated by PLAGL2 overexpression, whereas the activity of RhoA was reduced (Figure 4D). Activity of Cdc42 was not affected by PLAGL2 overexpression (Figure 4D). Cell migration analysis showed that exogenous expression of PLAGL2 promoted the migration of MDA-MB-231 cells (Figure 4E).

To confirm that the transcriptional activity of PLAGL2 is required for the dynamic changes in cellular morphology, we created deletion constructs of PLAGL2 and expressed them in MDA-MB-231 cells (Figure 5A). A previous study showed that in addition to C2H2 domain, PLAGL2 has an essential transcriptional activation domain in the C-terminus and a repressor domain between the C2H2 domain and the activation domain (29). Recombinant retroviruses that encoded each GFP-tagged mutant PLAGL2 construct were used to infect MDA-MB-231 cells and stress fiber formation was examined. Full-length, ΔN and ARep accumulated to the nucleus, but Δ243 and Δ2CH2 were localized to both the nucleus and the cytoplasm (Figure 5B). Interestingly, ΔAct, which is deleted of activation domain, predominantly localized to the nucleolus. It appears that the activation region is required to prevent PLAGL2 accumulation to the nucleolus. ΔN and ARep induced disruption of stress fibers and production of lamellipodia similar to full-length PLAGL2, whereas Δ2CH2, Δ243 and ΔAct did not promote changes in the organization of stress fibers or lamellipodia (Figure 5B and C). These results show that transcriptional activity is required for the PLAGL2-mediated disruption of stress fibers and promotion of lamellipodia formation in MDA-MB-231 cells.

**Induction of CHN1 is required for Rac1 inactivation and inhibition of cell migration by PLAGL2 knockdown**

To determine how PLAGL2 depletion affected the activity of small GTPases, we searched for genes whose expression was modulated by PLAGL2 knockdown. ES-2 cells were transfected with control or PLAGL2 siRNAs, and 72 h later, total RNA was extracted and subjected to DNA microarray analysis (Supplementary Tables S1 and S2, available at Carcinogenesis Online). In this analysis, we found that the levels of CHN1 mRNA were increased in cells transfected with either PLAGL2 siRNA1 or PLAGL2 siRNA2 compared with control siRNA-transfected cells. Previous studies have shown that CHN1 is a RacGAP (30,31); thus, we speculated that CHN1 expression was responsible for the inactivation of Rac1 in PLAGL2-depleted cells. We first confirmed the induction of CHN1 mRNA by reverse transcription–polymerase chain reaction analysis. Consistent with the DNA microarray analysis, the levels of CHN1 mRNA were increased ~3-fold in cells transfected with PLAGL2 siRNA (Figure 6A). To address whether CHN1 expression regulates stress fiber formation and the activity of small GTPases, we produced ES-2 cells that constitutively expressed GFP-CHN1. Expression of GFP-CHN1 did not induce any significant changes in production of stress fibers (Supplementary Figure S1, available at Carcinogenesis Online). Pull-down assays revealed that exogenous expression of GFP-CHN1 in ES-2 cells did not affect the activity of RhoA or Cdc42 but reduced Rac1 activity (Figure 6B). To address whether inhibition of Rac1 activity and cell migration by PLAGL2 knockdown was mediated by CHN1 induction, we first produced ES-2 cells that constitutively expressed GFP-CHN1. Expression of GFP-CHN1 did not induce any significant changes in production of stress fibers (Supplementary Figure S1, available at Carcinogenesis Online). Levels of CHN1 mRNA were efficiently reduced in ES-2/shCHN1 cells compared with ES-2/shLuc cells (Figure 6C). ES-2/shCtrl and ES-2/shCHN1 cells were transfected with control or PLAGL2 siRNA1 and stress fiber formation and small GTPase activity were examined. Both cell lines showed stress fiber organization upon PLAGL2 siRNA transfection (Figure 6D). Consistently, activity of RhoA was increased by PLAGL2 knockdown (Figure 6E). However, PLAGL2 depletion did not induce inactivation of Rac1 in ES-2/shCHN1 cells (Figure 6F). We next examined cell migration of ES-2/shCHN1 cells after PLAGL2 siRNA transfection. ES-2/shLuc and ES-2/shCHN1 cells were transfected with either control or PLAGL2 siRNA1, and 72 h later, cell migration was determined using a Boyden chamber. Depletion of CHN1 restored cell migration delay induced by PLAGL2 knockdown (Figure 6F). These results suggest that the CHN1 expression is associated with inactivation of Rac1 and inhibition of cell migration by PLAGL2 suppression. We also examined whether CHN1 expression was induced by exogenous
expression of PLAGL2 in MDA-MB-231 cells; CHN1 expression was not affected by PLAGL2 overexpression (Figure 6G).

Discussion

In this report, we showed that PLAGL2 is associated with the organization of the actin cytoskeleton and the activity of small GTPases such as RhoA and Rac1. Depletion of PLAGL2 in ES-2 and HEY cells promoted robust organization of actin stress fibers and focal adhesions. Rescue experiments demonstrated that stress fiber formation induced by PLAGL2 siRNA transfection was not due to the off-target effect of siRNA. Small GTPases are crucial factors for the regulation of actin cytoskeletal organization. Pull-down assays revealed that RhoA was activated by PLAGL2 depletion, whereas Rac1 was suppressed. Suppression of RhoA activity by chemical inhibitors or dominant-negative RhoA inhibited stress fiber formation induced by PLAGL2 depletion. These results show that PLAGL2 depletion promotes stress fiber formation by activating RhoA. A number of transcriptional factors have been reported to regulate dynamic remodeling of actin cytoskeleton (32–35). For example, Myc expression modulates RhoA signaling pathway and induces reorganization of actin stress fibers and focal adhesions (36). Our results demonstrate novel regulatory mechanisms of actin cytoskeleton organization mediated by transcriptional factors. To determine how the activities of RhoA or Rac1 were modulated by PLAGL2 knockdown, we performed DNA microarray analysis. Although we were unable to identify genes that regulate RhoA activity, we found that CHN1, a RacGAP, was induced in the absence of PLAGL2 in ES-2 cells. CHN1 expression in ES-2 cells induced inactivation of Rac1, but did not affect the activities of RhoA or Cdc42. In addition, silencing of CHN1 by shRNA expression suppressed Rac1 inactivation induced by PLAGL2 depletion. These results indicate that PLAGL2 depletion induces Rac1 inactivation by promoting the expression of CHN1. Rac1 is known to inhibit RhoA activity through the activation of p190RhoGAP or inactivation of NET1, which is a RhoGEF (37,38). However, we think it is unlikely that Rac1 inactivation caused by
PLAGL2 knockdown induced the activation of RhoA, because exogenous expression of CHN1 reduced Rac1 activity but did not affect RhoA activity.

Although we failed to establish ES-2 and HEY cells that constitutively expressed PLAGL2, exogenous expression of PLAGL2 in MDA-MB-231 cells disrupted stress fiber formation and promoted lamellipodia production. Consistently, the activation of Rac1 and the inactivation of RhoA were observed in PLAGL2-expressing MDA-MB-231 cells. However, CHN1 expression was not affected by PLAGL2 expression in this cell line. Experiments with deletion mutants of PLAGL2 demonstrated that transcriptional activity was required for lamellipodia production and stress fiber disruption. Thus, expression of small GTPase regulatory proteins other than CHN1 is required for the remodeling of the actin cytoskeleton induced by
PLAGL2 expression in this cell line. Although it still remains largely unknown how PLAGL2 regulates small GTPases, our results clearly indicate that the expression level of PLAGL2 is associated with Rac1 and RhoA activity and with actin cytoskeletal organization.

There are two critical regions in PLAGL2 for the transcription of target genes. C2H2 region is essential for the binding of PLAGL2 to the specific DNA sequences, and the C-terminal activation region is required for the transcriptional activation (29). Although it is still unknown how the activation region of PLAGL2 regulates transactivation, it is considered that the activation region recruits other factors, such as transcription initiation complex, to process transcription. Interestingly, we found that the deletion of the activation region induced accumulation of PLAGL2 in the nucleolus. Therefore, the activation region may be required for the transcriptional activation as well as proper localization of PLAGL2.

Rac1 activation is known to promote both cellular transformation and apoptosis in a cell type-dependent manner (39,40). In a colorectal model, Rac1 overexpression promoted, and Rac1 depletion inhibited, tumor progression (41). A recent study reported that Rac1 mutation was associated with cancer progression (42). In contrast, Rac1 can induce
Fig. 6. Induction of CHN1 by PLAGL2 knockdown suppresses Rac1 activity. (A) ES-2 cells were transfected with control or PLAGL2 siRNAs; 72 h later, total RNA was extracted and expression of CHN1 was determined by quantitative reverse transcription–polymerase chain reaction (RT–PCR). The graph indicates the relative levels of CHN1 mRNA. (B) ES-2 cells that constitutively expressed GFP-CHN1 were established by retrovirus infection, and the activity of the indicated small GTPases was examined. (C) ES-2 cells that constitutively expressed shRNA-targeting luciferase (shCtrl) or CHN1 (shCHN1) were established by retrovirus infection. CHN1 mRNA levels were determined by quantitative RT–PCR analysis. The graph indicates the relative levels of CHN1 mRNA. (D) ES-2/shCtrl and ES-2/shCHN1 cells were transfected with control or PLAGL2 siRNA1, and 72 h later, the cells were fixed and immunostained for F-actin (scale bar = 20 μm). (E) ES-2/shCtrl and ES-2/shCHN1 cells were transfected with control or PLAGL2 siRNA1, and 72 h later, RhoA and Rac1 activities were examined. (F) ES-2/shCtrl and ES-2/shCHN1 cells were transfected with control or PLAGL2 siRNA, and 72 h later, the cells were subjected to migration assay. Cells were counted in five randomly selected fields from three independent experiments. The graph indicates the average number of cells per field (means ± SD; *P < 0.01; n.s., P > 0.05). (G) Total RNA was extracted from MDA-MB-231 cells that expressed GFP or GFP-PLAGL2 and subjected to quantitative RT–PCR analysis. The graph indicates the relative levels of CHN1 mRNA.
apoptosis under specific conditions. Activation of Rac1 in NIH3T3 cells under serum-deprived conditions results in increased levels of ceramide and Fas ligand, which are involved in apoptosis (43). In addition, suppression of Rac1 activity can inhibit tumor necrosis factor-induced apoptosis in U937 cells (44). Similar to Rac1, PLAGL2 expression is associated with both transformation and apoptosis. Our results suggest that the opposing actions of PLAGL2 may be mediated, in part, by the regulation of Rac1 activity. Future studies of the PLAGL2-mediated regulation of small GTPases may provide further insight into the role of PLAG family genes in transformation and apoptosis.

Supplementary material

Supplementary Tables S1 and S2 and Figure S1 can be found at http://carcin.oxfordjournals.org/

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