The 43,000 Growth-associated Protein Functions as a Negative Growth Regulator in Glioma

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ABSTRACT

Previous molecular analyses of human astrocytomas have identified many genetic changes associated with astrocytoma formation and progression. In an effort to identify novel gene expression changes associated with astrocytoma formation, which might reveal new potential targets for glioma therapeutic drug design, we used the B8-RAS-transgenic mouse astrocytoma model. Using multiplex gene expression profiling, we found that growth-associated protein 43 (GAP43) RNA and protein expression were lost in select human and mouse glioma cell lines. In this study, we demonstrate that re-expression of GAP43 in deficient C6 glioma cells results in growth suppression in clonogenic assays, as well as in multiple independently derived C6 glioma cell lines in vitro. GAP43-expressing C6 cells also exhibit reduced tumor growth as s.c. explants in immunocompromised mice in vivo. In addition, GAP43-expressing C6 clones demonstrate impaired cell motility and increased homophilic aggregation. GAP43 re-expression is also associated with reduced mitogen-activated protein kinase and AKT activation in C6 cells, suggesting that GAP43 functions as a novel glioma growth suppressor by modulating mitogenic signaling pathways.

INTRODUCTION

Astrocytomas (or gliomas) are one of the most devastating central nervous system tumors, with a median survival of 9–12 months after diagnosis (1). Previous studies of the molecular genetic events associated with glioma formation and progression have identified a number of specific genetic alterations, including amplification or mutation of the epidermal and platelet-derived growth factor receptors and loss of cell cycle growth regulators such as p53 and p16 (2–5). In addition to these known genetic changes, it is highly likely that there exist other alterations in gene expression associated with glioma pathogenesis, which may yield novel insights into potential new targets for therapeutic drug design. In an effort to identify some of these previously unidentified glioma-associated gene expression changes, we used gene expression profiling on a recently developed transgenic mouse model of glioma.

One of the most common signaling abnormalities observed in gliomas is activation of the Ras mitogenic signaling pathway (6). This increased Ras pathway activation may occur as a result of aberrant receptor tyrosine kinase signaling. In this respect, constitutive activation of the epidermal growth factor receptor leads to increased Ras activation and increased Ras pathway mitogenic signaling relevant to astrocytoma growth (2). Inhibition of Ras pathway activation with molecules that inhibit Ras posttranslational modification and proper membrane localization required for efficient effector molecule signal propagation results in marked reduction in glioma growth in vitro and in vivo (7). In addition, individuals with the NF13 inherited tumor syndrome develop astrocytomas at an increased frequency. The NF1 tumor suppressor protein, neurofibromin, contains a central domain with Ras-GTPase activating protein activity. Loss of NF1 expression results in impaired neurofibromin-mediated Ras inactivation and leads to increased Ras pathway activation and tumorigenesis (8). Because of these observations, we and our colleagues generated a transgenic mouse in which an oncogenic activated H-Ras molecule was specifically expressed in astrocytes (9). These Ras transgenic (B8) mice develop high-grade gliomas by 3–4 months of age that are histologically identical to their human counterparts. The availability of the B8 glioma transgenic mouse provided a unique opportunity to compare the gene expression profiles of wild-type astrocytes, nonneoplastic B8 astrocytes, and B8 astrocytoma cells. Using this approach, we were able to identify a number of novel gene expression changes that were specifically associated with the neoplastic state (10). We were able to validate some of these changes on the protein level in both mouse and human glioma cell lines, confirming the results obtained on the RNA level. One of these novel gene expression changes was loss of GAP43 expression in human and mouse glioma cell lines.

GAP43 was originally identified as a neuronal protein important in cytoskeleton-associated processes relevant to nerve growth cone extension and sprouting (11–15). GAP43 belongs to a family of proteins that include CAP23 and MARCKS, which have also been implicated in neuronal outgrowth (16). GAP43 expression and function has been studied in neurons (17–21), but little is known about its role in other cell types in the brain. Several reports have described the expression of GAP43 in type 1 astrocytes (22–24), as well as type 2 astrocyte precursors and mature type 2 astrocytes (25–27). Because astrocytes are not postmitotic cells like neurons and continue to divide in the adult brain, abnormal GAP43 function in astrocytes might have different effects than previously reported for neurons. In this respect, expression of the GAP43 family member, MARCKS, is dramatically reduced in various cell lines after oncogenic or chemical transformation (28–33). In addition, re-expression of MARCKS in melanoma cell lines results in inhibition of both anchorage-dependent and anchorage-independent cell growth (34). These observations prompted us to examine the growth regulatory properties of GAP43 in gliomas.

MATERIALS AND METHODS

Constructs, Antibodies, and Cell Culture. The pcDNA3.chicken GAP43 plasmid was kindly supplied by Dr. Pico Caroni. The pcDNA3.mouse GAP43 plasmid was generated by reverse transcription-PCR using mouse brain RNA and mouse-specific primers 5’-TCTAGATCTGCTGTATGAGAAAATGAAAG/3’ and antisense 5’-GAACC-3’. Anti-GAP43 polyclonal antibody was purchased from Chemicon (Temecula, CA). The anti-phospho MAPK (T202/Y204) antibody (9106S), anti-phospho AKT (Ser473) antibody (9271S), and anti p44/42 MAPK antibody (9102) were used according to the manufacturer’s recommendations. Anti-NF1 antibodies were used according to the manufacturer’s recommendations.

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3 The abbreviations used are: NF1, neurofibromatosis 1; GAP43, growth-associated protein 43; MAPK, mitogen-activated protein kinase.
C6 rat glioma cells were passaged in DMEM containing 10% fetal bovine serum and penicillin/streptomycin.

GAP43 and vector-transfected stable C6 glioma cell lines were generated by transfecting C6 cells with pcDNA3 (vector) or pcDNA3.GAP43, followed by selection in 750 μg/ml geneticin. Individual clones were picked and analyzed for GAP43 expression. Two vector (V2, V3) and two GAP43-expressing (G13, G14) clones were selected for additional analysis. An additional two GAP43-expressing clones (G8, G10) were also generated.

Clonogenic Assay. Clonogenic assays were performed by transfecting C6 rat glioma cells with equimolar amounts of pcDNA3.chicken GAP43, pcDNA3.mouse GAP43, or pcDNA3 vector using Lipofectamine (Life Technologies, Inc., Life Sciences). Five 100-mm plates for each transfection were grown for 14 days in the presence of 750 μg/ml G418. The number of colonies > 2 mm in diameter was counted after Crystal Violet staining, and the mean and SD were determined for each transfection. Each experiment was performed at least three times with identical results.

Direct Cell Counting and Growth in Soft Agar. The different C6 clones were seeded in 60-mm dishes at a density of 1 × 10^4 cells/dish. The cells were cultured with DMEM containing 10% serum. The cells were then harvested and counted on days 3, 6, and 9 before reaching confluency. Three dishes were counted at each time point for the different clones. Each experiment was performed three times with identical results. The mean and SD were determined for each clone.

Soft agar growth assays were performed in quadruplicate using chicken GAP43-expressing and vector-transfected C6 clones as described previously (35). Briefly, 1000 C6 cells were equally divided into four 24-well plate wells GAP43-expressing and vector-transfected C6 clones as described previously. We performed three times with identical results. The mean and SD were determined for each clone.

Cell Adhesion. Cell adhesion was performed in 96-well plates precoated with 10 μg/ml fibronectin (Sigma) in sterile PBS at 4°C overnight. The wells were washed twice with PBS and incubated with 2% heat-inactivated BSA for 2 h at 37°C. The cells were digested with 0.05% trypsin and resuspended up to 1 × 10^5 cells/ml with serum-free DMEM and incubated for 2 h at 37°C. One hundred μl of cells were added to each well and allowed to adhere for 1 and 4 h. At the end of the incubation period, cells were washed three times in PBS and stained with 0.5% crystal violet for 30 min at room temperature. The wells were then washed twice in PBS, and 50 μl of 1% SDS were added to each well overnight at room temperature. The number of adherent cells was quantitated by determining the absorbance at 540 nm. Each experiment was performed at least three times with identical results.

Motility Assay. GAP43-expressing C6 cells and vector-transfected clones were suspended in serum-free medium at 5 × 10^5 cells/ml. Two hundred μl of cell suspension (1 × 10^5 cells/ml from each clone were added in each of triplicate transwells with an 8.0-μm pore size (Corning Costar Incorporated, Cambridge, MA). Six hundred μl of complete medium DMEM containing 10% serum were added into each well of 24-well plate. Transwells were placed over the wells of 24-well plate. The plates were incubated at 37°C for 4 h or overnight (~16 h). The membranes of the transwells were fixed, stained, and removed. The numbers of cells on the lower surface of the membranes and the bottom of lower chamber (well of the 24-well plate) were photographed at ×200 magnification. The assay was at least repeated three times with identical results.

Fluorescence Immunocytochemistry. C6 cells were grown in 6-well plates to 50% confluency. Cells were then washed twice with warm PBS and fixed for 30 min at room temperature with 3.7% formaldehyde followed by a permeabilization wash with Triton X-100 for 10 min. Cells were next washed and incubated with 1% BSA for 30 min. Primary anti-GAP43 antibody was used at a 1:200 dilution overnight at 4°C and incubated with rabbit-Alexa 488-conjugated fluorescent antibody (Molecular Probe, Eugene, OR) at a 1:500 dilution at 37°C for 30 min. The cells were photographed using a Nikon Optiphot fluorescence microscope.

Western Blotting. Western blotting was performed as previously described (36) using enhanced chemiluminescence detection (Amersham, Arlington Heights, IL). Cells were lysed in MAPK lysis buffer (20 mM Tris (pH 7.5), 10 mM EGTA, 40 mM β-glycerophosphate, 1% NP40, 2.5 mM MgCl₂, and 2 mM sodium orthovanadate). A total of 100 μg of total protein was separated by 12% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). The anti-GAP43 antibody was used at a 1:4000 dilution at 4°C overnight. The other antibodies (tubulin, actin, MAPK, MAPK-P, and AKT-P) were used at a 1:1000 dilution at 4°C overnight.

Growth in Athymic Immunocompromised Mice. GAP43-expressing (clones G13 and G14) and vector-transfected (clones V2 and V3) cells were trypsinized and washed with PBS without Ca²⁺ and Mg²⁺ and resuspended to 1 × 10^6 cells/ml. A total of 1 × 10^6 cells in 100 μl of total volume was s.c. injected into the flank of 6-8-week old male athymic nude mice. Four to five mice were injected for each clone. All procedures followed the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education, issued by the New York Academy of Sciences’ Ad Hoc Committee on Animal Research. The tumor volumes were measured with calipers every week after injection. On day 19 or 21, the mice were euthanized, and the tumors were measured and removed. Tumor volume was calculated according to the formula: tumor volume (mm³) = (L × W²) / 2, where L represents the longest dimension and W the shortest dimension of the tumor (37–39). Half of each tumor was homogenized and lysed in MAPK lysis buffer. A total of 100 μg of total protein from each tumor was analyzed by Western blot to confirm GAP43 expression.

Statistical Methods. Data are presented as the mean ± SD and were analyzed with ANOVA followed by the t test with significance set at P < 0.01.

RESULTS

GAP43 Expression Inhibits C6 Glioma Growth In Vitro. The observation that GAP43 expression was lost in select human and mouse glioma cell lines suggests that GAP43 loss might be related to dysregulated growth control in gliomas. As an initial test of the hypothesis that GAP43 functions as a growth regulator in gliomas, we used a clonogenic assay to determine the effect of GAP43 re-expression on glioma cell growth in vitro. We chose to use C6 glioma cells for these studies, rather than the B8 RAS transgenic astrocytoma cells, because B8 glioma cells are not a clonal cell line and represent a heterogeneous population of transformed astrocytes. In these experiments, equimolar amounts of pcDNA3.chicken GAP43, pcDNA.mouse GAP43, and pcDNA3 vector were transfected into C6 rat glioma cells, and the number of colonies ≥ 2 mm in diameter was determined after G418 (geneticin) selection for 14 days. Western blotting demonstrated GAP43 expression in cells pooled from parallel dishes transfected with chicken and mouse GAP43, compared with absent GAP43 expression in the vector-transfected cells (Fig. 1A). An 88.6–91.7% decrease in colony number was observed in both chicken and mouse GAP43-transfected dishes compared with vector-transfected dishes (Fig. 1, B and C), suggesting that both chicken and mouse GAP43 impair glioma cell growth.

To validate these initial findings, we generated constitutive GAP43-expressing stable C6 cell lines. Chicken GAP43 was used in these studies because this cDNA has been extensively characterized in studies involving neurons and transgenic mice (14–17). A total of four independent GAP43-expressing clones (G8, G10, G13, and G14) were generated and exhibited similar effects on cell growth (data not shown). Two vector (V2 and V3) and two GAP43-expressing (G13 and G14) clones were chosen for additional analyses. Western blot confirmed that GAP43 clones G13 and G14 expressed fairly equivalent levels of the M, 43,000 GAP43 protein, whereas the vector clones V2 and V3 did not express GAP43 (Fig. 2A). Using these clones, we determined the effect of GAP43 re-expression on anchorage-dependent and anchorage-independent cell growth. During exponential phase growth in serum, we observed a significant reduction in cell growth in the GAP43-expressing clones (Fig. 2B). In these experiments, we observed a 61.2–70% decrease in cell number compared with those of vector clones on day 9 (Fig. 2B). Anchorage-independent cell growth was assessed by growth in soft agar. Similarly, we noted an average decrease of 33.7% in colony number in the GAP43-
expressing C6 clones compared with vector (Fig. 2C). These data suggest that GAP43 inhibits C6 cell growth in vitro. No changes in cell viability were observed in GAP43-expressing clones (data not shown).

**GAP43 Inhibits C6 Tumor Growth in Vivo.** To determine whether GAP43 expression inhibits C6 glioma growth in vivo, 10 6–8-week old male athymic *nu/nu* mice were divided into two groups, and 5 mice each received 1 × 10⁶ of GAP43-expressing (clone G14) or vector-transfected (clone V2) C6 cells by s.c. injection into the right flank. Tumor volumes were measured with calipers every week. Compared with vector-transfected C6 cells, GAP43-expressing C6 gliomas grew significantly slower than vector controls at all time points examined (Fig. 3A). We observed a reduction in tumor sizes at 7, 14, and 19 days (27, 36, and 50% reduction in tumor size, respectively). In a duplicate experiment terminated after 19 days (Fig. 3B), we also observed reduced tumor growth in the GAP43-expressing G14 cells compared with V2 vector cells (31, 44, and 31% reductions in tumor size at 7, 14, and 19 days, respectively). Similar results were obtained with additional, independently isolated GAP43-expressing (G13) and vector (V3) C6 glioma cell lines (Fig. 3B). We observed a reduction in tumor sizes at 7, 14, and 19 days (19, 12, and 32% reductions in tumor size at 7, 14, and 19 days, respectively). By the third week after injection, the majority of the tumors derived from vector-transfected C6 cells developed visible necrosis and hemorrhage (Fig. 3C, arrows), whereas none of the GAP43-expressing C6 tumors exhibited those changes. Lastly, we verified persistent GAP43 expression in tumors harvested at the end of the experiment by Western blotting (Fig. 3D). Collectively, these experiments demonstrate that GAP43 inhibits glioma cell growth both in vitro and in vivo.

**GAP43 Expression Reduces C6 Cell Motility.** One of the established properties of GAP43 is its ability to modulate cytoskeleton-associated processes. To examine this property of GAP43 in gliomas, we studied the effect of GAP43 overexpression on motility, attachment, and adhesion in C6 cells. Using a modified Boyden chamber motility/migration assay, we demonstrated that GAP43 expression reduced C6 cell motility by 48–57% at 4 h (Fig. 4A) and even more significantly after an overnight incubation (Fig. 4B). In contrast, we observed no effect on GAP43 expression on C6 cell attachment to fibronectin-coated surfaces (Fig. 4C). Similarly, we observed no differences in actin cytoskeleton organization during the initial phases of...
C6 cell spreading onto laminin-coated surfaces in GAP43-expressing cells compared with vector controls (data not shown). However, GAP43 overexpression resulted in an increase in homophilic C6 cell aggregation (Fig. 4D). These results demonstrate that GAP43 impairs cytoskeleton-mediated processes in gliomas as have been reported in other cell types.

**GAP43 Impairs MAPK and AKT Pathway Activation.** To determine the mechanism by which GAP43 expression inhibits C6 cell growth, we analyzed the effect of GAP43 overexpression on known intracellular signaling pathways. We observed no obvious changes in tyrosine phosphorylation or Rap1 activation in GAP43-expressing clones compared with vector controls (data not shown). We next examined the effect of GAP43 expression on MAPK and AKT kinase activation using phospho-specific antibodies in GAP43-expressing clones. In response to stimulation with 10% fetal bovine serum, we observed a significant reduction in MAPK and AKT activation. This reduction was observed at 5, 10, 30, and 60 min after serum stimulation compared with vector controls at the same time points (Fig. 5). No activity of either AKT or MAPK was seen in unstimulated cells (data not shown). Total MAPK p44/42 was used as a control for equal protein loading. These results suggest that GAP43 modulates MAPK and AKT activity in C6 glioma cells.

**DISCUSSION**

The regulation of cell growth in gliomas is a complex and intricately orchestrated process involving multiple interrelated signaling pathways. Studies from a number of laboratories have demonstrated that signaling pathways downstream of Ras are critical for facilitating tumor cell proliferation, inhibiting cell death, and maintaining the tumor phenotype (40, 41). Activation of the RAF/MAPK pathway has been shown to provide a mitogenic growth advantage for astrocytoma cells in vitro and in vivo (2, 6, 42). Inhibition of MAPK activation in these cells results in reduced proliferation and tumor growth (7, 43). Similarly, activation of the AKT pathway as a result of PTEN loss or activation of receptor tyrosine kinases provides a survival advantage by inhibiting programmed cell death or apoptosis (44, 45). In addition, activation of this pathway is also important for the maintenance of the tumor phenotype by promoting cell motility and cell shape changes important for tumorigenesis (46, 47). Lastly, activation of the Rac/Rho and S6 kinase signaling pathways in gliomas impacts not only on cell proliferation but also cell motility, cell size, and the transcriptional program (48–50).

Previous studies from our laboratory using gene expression profiling have identified GAP43 as a protein lost in select human and...
mouse glioma cell lines compared with normal human or mouse astrocytes (10). These results raised the question as to whether GAP43 functions as a growth suppressor in gliomas. In this study, we demonstrate that the neuronal growth-associated protein GAP43 functions as a negative growth regulator when reintroduced into GAP43-deficient glioma cells. We additionally demonstrate that GAP43 inhibits cell growth by impairing activation of the AKT and MAPK signaling pathways. The finding that GAP43 performs a growth regulatory function in glioma cells expands our understanding of the proteins involved in gliomagenesis.

Much is known about the role of GAP43 in neuronal cells. GAP43 is primarily found in axons and presynaptic terminals where it is predominantly localized to the cytoplasmic side of the plasma mem-

brane in axonal growth cones (12, 51). GAP43 is widely expressed in developing neurons throughout the central nervous system, and its synthesis and accumulation declines as animals mature and neurons differentiate (52). GAP43 and its related proteins, CAP23 and MARCKS, promote nerve sprouting in transgenic mice and process outgrowth in cultured cells (17), suggesting a role in cytoskeleton-mediated processes. In C6 glioma cells, we demonstrate that GAP43 re-expression results in impaired cell motility and increased homophilic cell aggregation. In contrast, we observed no effect of GAP43 expression on glioma cell attachment or actin cytoskeleton organization during the initial phases of cell spreading. Our findings parallel observations made in neurons deficient in GAP43 or CAP23. In CAP23-deficient neurons, the cytoskeleton abnormalities could be mimicked by inhibiting microtubule assembly (17), suggesting that GAP43 family members impair cytoskeleton-associated processes. The effects of GAP43 on cytoskeleton-associated processes may account for the impaired motility observed upon GAP43 re-expression in glioma cells because the motile phenotype of many cell types can be modulated by interference with microtubule or actin cytoskeleton assembly (53, 54). Similarly, the ability to extend filopodia is highly regulated and impacts on cell motility. Previous studies in neurons have shown that GAP43 is directly involved in the regulation of filopodial extension and nerve sprouting (18). Lastly, GAP43 is responsible for neural cellular adhesion molecule (CAM) stimulation of cell growth (20). CAM proteins are important mediators of cell adhesion (55) such that their altered function in GAP43 re-expressing glioma cells may promote homophilic cell adhesion. Our observations are supported by the findings of Mariani et al. (56) who describe up-regulation of GAP43 expression in the G112 glioma cell line when exposed to extracellular matrix. Interestingly, we observed no effect of GAP43 re-expression on cell attachment to fibronectin or actin cytoskeleton organization during the initial phases of cell spreading on laminin-coated surfaces. Collectively, these results suggest that

Fig. 4. GAP43 re-expression suppresses C6 glioma cell motility. A, GAP43-expressing C6 cells demonstrate reduced cell motility compared with the vector-transfected C6 cells at both 4 h and overnight (~16 h; B). The left panel shows that more vector-transfected C6 cells traversed the transwell membrane and attached to the lower surface as well as to the bottom of the wells (lower chamber) compared with the GAP43-expressing C6 cells (right panel). The single asterisk denotes statistically significant differences between GAP43-expressing C6 cells and vector-transfected C6 cells (P < 0.01; magnification ×100). C, GAP43-expressing C6 cells did not exhibit significant differences in cell adhesion at 1 and 4 h onto fibronectin-coated plates compared with the vector-transfected cells. D, GAP43-expressing clones exhibit increased homophilic aggregation under standard growth conditions compared with vector-transfected C6 cells. Fluorescence staining demonstrates GAP43 expression in GAP43-expressing C6 cells (magnification, ×200).

Fig. 5. GAP43 inhibits MAPK and AKT kinase activation. GAP43-expressing C6 cells and vector-transfected C6 cells were serum starved for 48 h and then stimulated with 10% serum for 5, 10, 30, and 60 min. Phospho-MAPK p44/42 and phospho-AKT Ser473 expression at different time points after serum stimulation were detected by Western blotting. No activity was observed in serum-starved cells (data not shown). GAP43-expressing C6 cells exhibited a significant reduction in phospho-MAPK p44/42 and phospho-AKT Ser473 expression at 5, 10, 30, and 60 min after serum stimulation. MAPK expression is included as a loading control.
GAP43 confers specific properties on glioma cytoskeleton-mediated processes. There are conflicting reports regarding the expression of GAP43 in type 1 astrocytes, with some groups clearly identifying GAP43 in differentiated fibrillary astrocytes (22–24) and others describing GAP43 expression only in oligodendrocyte-type 2 astrocyte progenitor lineage cells (25–27). We consistently observe GAP43 expression in pure neocortical cultures of mouse type 1 astrocytes, as well as normal human fetal astrocyte cultures (10). During cell growth arrest in response to serum starvation or contact inhibition, we did not observe any change in astrocyte GAP43 expression (N. Hedrick and D. H. Gutmann, unpublished observations). It is possible that GAP43 expression is developmentally regulated during astrocyte differentiation, rather than strictly modulated by growth arrest. Support for this observation derived from experiments demonstrating that GAP43 expression is down-regulated during oligodendrocyte differentiation (26). Similarly, GAP43 expression is up-regulated in astrocytes in response to cerebral ischemia (24). These results suggest that GAP43 may play a critical role in promoting cell differentiation such that its loss in tumors favors a relatively undifferentiated and proliferative state. Additional studies to directly address this issue will be required.

Although GAP43 has not been directly implicated in tumor growth regulation, it has been implicated in processes in neurons that typically require Ras pathway activation, such as process extension. Previous studies have demonstrated a relationship between growth arrest, the differentiated state, and GAP43 expression. For example, treatment of human SK-N-SH neuroblastoma cells with ciliary neutrotrophic factor increased GAP43 expression and resulted in cellular differentiation (57). Similarly, 12-O-tetradecanoylphorbol-13-acetate stimulation of human SH-SY5Y neuroblastoma cells is associated with increased GAP43 mRNA expression and neuronal differentiation (58), and transforming growth factor β treatment of neuroblastoma LAN-5 cells reduces cell growth and dramatically induces increased GAP43 expression (59). Lastly, overexpression of the chicken ovalbumin upstream promoter-transcription factor in PCC7 teratocarcinoma cells delays the induction of GAP43 expression and leads to blockade of morphological differentiation (60).

In addition, there is indirect evidence for an association between the transformed tumor phenotype and GAP43 expression. Expression of the GAP43 family member, MARCKS, is reduced in various cell lines after oncogenic or chemical transformation (28–33) and re-expression of MARCKS in deficient melanoma cell lines results in reduced cell growth (34). Our findings provide the first direct analysis of the function of GAP43 in glioma cell growth regulation. In this article, we demonstrate that GAP43 re-expression in deficient glioma cells reduces both anchorage-dependent and anchorage-independent cell growth in vitro and glioma tumor growth in vivo.

In an effort to define the mechanism by which GAP43 re-expression reduces glioma cell growth, we analyzed the mitogenic signaling pathways relevant to astrocytoma growth control and demonstrated that GAP43 re-expression reduced both AKT and MAPK activity. Preliminary studies in our laboratory have shown that oncogenic Ras can reduce GAP43-expressing C6 glioma cell growth suppression, but GAP43 overexpression was not associated with measurable differences in Ras activity, measured using the RAF-GBD pulldown technique (Z.-y. Huang and D. H. Gutmann, unpublished observations). Collectively, these results suggest that GAP43 impairs activation of Ras effector proteins such as MAPK and AKT. However, these data do not preclude the possibility that GAP43 growth suppression leads to reduced AKT and MAPK signaling through other intracellular signaling pathways, independent of Ras. Moreover, it is also conceivable that the ability of oncogenic Ras to reduce GAP43 growth suppression is not directly attributable to reversion of GAP43-mediated signaling but involves nonoverlapping growth regulatory pathways important for glioma proliferation.

In this article, we used multiple constitutively GAP43-expressing C6 glioma cell clones to define the mechanism of GAP43 growth suppression. Some of the potential problems with using a small number of clonal isolates relate to biological effects that reflect the site of transgene integration or the selection of cells with specific physiological properties that do not directly result from transgene expression. To reduce the possibility that our results on GAP43 growth regulation reflect cellular properties unrelated to GAP43 transgene expression, we used multiple independently derived clonal isolates. Regulatable systems using tetracycline- or ec dysone-inducible promoters to control transgene expression afford an excellent alternative to generating multiple independent constitutively expressing clones and allow one to directly analyze the consequences of transgene expression. Future studies using inducible GAP43-expressing glioma cell lines are planned to more precisely define how GAP43 impairs AKT and MAPK signaling and glioma cell growth. Insights into this novel role of GAP43 in glioma cell regulation may lead to the development of additional strategies to modulate Ras pathway signaling and astrocytoma cell growth.

REFERENCES

15. Caroni, P., Aigner, L., and Schneider, C. Intrinsic neuronal determinants locally intrinsic to generating multiple independent constitutively expressing clones and allow one to directly analyze the consequences of transgene expression. Future studies using inducible GAP43-expressing glioma cell lines are planned to more precisely define how GAP43 impairs AKT and MAPK signaling and glioma cell growth. Insights into this novel role of GAP43 in glioma cell regulation may lead to the development of additional strategies to modulate Ras pathway signaling and astrocytoma cell growth.

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