Suppression of Rac Activity Induces Apoptosis of Human Glioma Cells but not Normal Human Astrocytes

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ABSTRACT

Tumors of glial origin such as glioblastoma multiforme (GBM) comprise the majority of human brain tumors. Patients with GBM have a very poor survival rate, with an average life expectancy of <1 year. We asked whether we could identify a survival pathway in high-grade glioma and oligodendroglioma cells that when suppressed, would induce apoptosis of these tumor cells but not of normal human adult astrocytes. To identify these pathways, we selectively suppressed the activity of a number of proteins (Ras, Rac1, Akt1, Rhoa, c-Jun, and MEK1/2) hypothesized to play roles in cell survival. We found that suppression of Rac1, a small GTP-binding protein, inhibited survival and produced apoptosis in three human glioma cell lines (U87, U343, and U373). Serum induced the activity of Rac1 and the activity or phosphorylation state of p21-activated kinase 1 and c-Jun NH2-terminal kinase (JNK), two intracellular targets of Rac1. Suppression of Rac1 also induced apoptosis in 19 of 21 short-term cultures of human primary cells from grades II and III oligodendroglioma and grade IV glioblastoma that varied in p53, epidermal growth factor receptor, epidermal growth factor receptor VIII, MDM2, and p16/p19 mutational or amplification status. In contrast, inhibition of Rac1 activity did not induce apoptosis of normal primary human adult astrocytes. In both established glioma cell lines and primary glioma cells, apoptosis induced by the inhibition of Rac was partially rescued by activated mitogen-activated protein kinase kinase kinase 1, an activator of JNK, suggesting that JNK functions downstream of Rac1 in glioma cells. These results indicate that Rac1 regulates a major survival pathway in most glioma cells, and that suppression of Rac1 activity stimulates the death of virtually all glioma cells, regardless of their mutational status. Agents that suppress Rac1 activity may therefore be useful therapeutic treatments for malignant gliomas.

INTRODUCTION

In humans, neoplasms derived from astrocytes constitute the majority of primary brain tumors. The malignant progression of astrocytomas is thought to occur as a multistep process that results in the deregulation of cellular growth control. These steps include: (a) the loss of function of the tumor suppressor p53; (b) the loss of function of cell cycle inhibitors, such as p16 and p19; (c) the amplification and mutation of genes encoding growth factor receptors, such as the EGFR; (d) activation of genes and proteins encoding growth-regulatory proteins, such as Ras and protein kinase C; and (e) the loss of function of the candidate tumor suppressor PTEN. Each of these proteins has been shown to play roles in tumor progression, cell proliferation, resistance to radiation treatment or chemotherapeutic agents, and cell migration (reviewed in Refs. 1–5). However, little is known about the signaling proteins that regulate survival of glioma cells. The purpose for this study was to identify a survival pathway in high-grade gliomas and oligodendrogliomas, that when suppressed, would induce apoptosis of virtually all tumor cells but not affect normal human adult astrocytes. Such an approach would allow us to identify novel therapies that selectively kill glioma tumor cells but leave the surrounding glia intact.

Several proteins have been suggested to play roles in the survival of glioma cells, including Ras and PI3K/Akt. Suppression of Ras activity using FTIs induces apoptosis in established glioma cell lines (6), and inhibition of PI3K or Akt activity blocks hepatocyte growth factor-induced protection of a glioma cell line from death caused by DNAdamaging agents (7). However, it is not clear whether FTIs inhibit the activity of other Ras family members (8) and whether Ras or PI3K and Akt regulate the survival of primary human glioma cells. Another potential survival protein is Rac1. Although Rac1 has not been shown previously to mediate the survival of glial cells, activated forms of Rac induce the survival of Rat1 fibroblasts and M14 melanoma cells (9, 10) and of BaF3 hematopoietic cells by stimulating Akt activity (11), and they suppress Ras-induced apoptosis in 3T3 fibroblasts (12, 13). Rac1 can also play a proapoptotic role. Rac1 activity is required for Fas ligand-mediated apoptosis in T cells and for the death of sympathetic neurons when nerve growth factor is withdrawn (14). Here, we investigate how Rac1 regulates the survival of established glioma cell lines and primary human glioma cells derived directly from surgical specimens.

Rac1 is a member of the Rho family of small GTP-binding proteins, which includes RhoA andcdc42. Rho family members are active when bound to GTP and inactive when bound to GDP (15). Rac1 is best known for its role in regulating actin polymerization and the assembly of associated integrin complexes (16–18), gene transcription, and G1 cell cycle progression (19–21). The multifunctionality of Rac is achieved through direct or indirect interactions with multiple effector proteins. These include several protein kinases with roles in cell proliferation or cytoskeletal organization, including Pak (22, 23), Mlk2 (24, 25), JNK, and Mekk1 (26–28). A kinase-inactive form of Mekk1 blocks Rac-mediated activation of JNK, suggesting that Rac, Mekk1, and JNK may act sequentially in a signaling pathway (29).

In this study, we used a recombinant adenovirus-based approach to identify survival proteins in established human glioma cell lines and primary glioma cells isolated from surgical specimens. We found...
using recombinant adenoviruses expressing DN forms of signaling proteins, that suppression of Rac1 activity via DN Ral1 induced the death of glioma cell lines and 19 of 21 primary glioblastoma and oligodendroglioma cells but not normal human adult astrocytes. These results suggest that Rac1 is a key contributor to glioma cell survival.

**MATERIALS AND METHODS**

**Preparation of Recombinant Adenoviruses.** Replication-defective recombinant adenovirus vectors encoding DN forms of Akt (DN Akt (30), c-Jun (DN Jun; Ref. 31), N17Raas (DN Raas; Ref. 32), or activated-MEKK (MEKK); Ref. 33) were prepared and purified as described previously by our group. c-myc-tagged DN N17Rac1, DN N17RhoA, and DN N17cdc42 (a kind gift of C. Bazenet, Esei, Inc., London, United Kingdom) were cloned into the pAd-CMV-FI-ires-EGFP bicistronic expression vector (32). Expression of the constructs and GFP proteins were confirmed in transiently transfected 293 cells by Western blot analysis. Replication-defective recombinant adenoviruses were prepared and purified and titered as described (32, 34). As controls, GFP or Escherichia coli β-galactosidase-expressing recombinant adenoviruses (Aegera Therapeutics, Montreal, Quebec, Canada and Dr. F. Graham, McMaster University, Hamilton, Ontario, Canada, respectively), generated using the same viral backbone as above, were used.

**Cell Lines.** Established human glioma cell lines, U87, U343, and U373 (obtained from A. Guha, Toronto, Ontario, Canada) derived from grade III astrocytoma or grade IV GBM, were grown in 10% FCS in DMEM supplemented with glutamine (2 mM), penicillin (50 units/ml), and streptomycin (50 µg/ml) at 37°C in 5% CO2.

**Primary Astrocyte Cultures.** Tissues were obtained from patients undergoing temporal lobe resection for the surgical treatment of intractable epilepsy. Tissues were treated with trypsin (0.25%) and DNase (50 µg/ml), filtered through a 70 µm mesh, followed by Percoll gradient centrifugation (Pharmacia LKB Biotechnology, Uppsala, Sweden) at 15,000 rpm for 30 min. The dissociated cells at the interface were washed several times with PBS and then suspended in DMEM with 10% FCS and plated (2 × 10^5 cells/ml) onto Falcon tissue culture flasks. The following day, oligodendrocytes remained floating and were removed. Remaining adherent cells, consisting of astrocytes and microglia, were further separated by mechanically dissociating the astrocytes from the tissue culture flask by shaking cultures for 3 h on a Belco orbital shaker. The dissociated cells were resuspended in 10% FCS in DMEM. Astrocytes were identified using mouse monoclonal anti-GFAP antibody (Boehringer-Mannheim, Mannheim, Germany), followed by goat CY3-conjugated antishive IgG. The purity of the nontransformed adult human astrocyte cultures varied between 25 and 75% (as determined by GFAP expression), with the contaminating cells predominantly microglia and a small percentage of oligodendrocytes.

**Primary Human Glioma Cultures.** Biopsy samples of human gliomas were obtained during brain tumor surgery, and short-term cultures were established from samples with high tumor cell content as assessed by morphometric analysis. Biopsy samples of human gliomas were obtained during brain tumor surgery, and short-term cultures were established from samples with high tumor cell content as assessed by morphometric analysis. Biopsy samples of human gliomas were obtained during brain tumor surgery, and short-term cultures were established from samples with high tumor cell content as assessed by morphometric analysis. Biopsy samples of human gliomas were obtained during brain tumor surgery, and short-term cultures were established from samples with high tumor cell content as assessed by morphometric analysis.

**Viral Infections.** The human glioma cell lines U87, U343, and U373 were plated at appropriate cell numbers for each type of assay. Cells were infected 1 day after plating with recombinant adenovirus at various MOIs, typically that transduced 100% of the cultured cells in the presence of 10% FCS. The next day, the medium was changed with fresh media in DMEM. For pharmacological studies, 50 µM U0126 or 20 µM LY294002 was present at all times. Assays were performed at the indicated times.

**Survival Assays, Cell Death ELISA, and Pharmacological Treatments.** Survival assays were performed 72–96 h after viral infection. Cultures were assayed for survival using a nonradioactive cell proliferation assay, Alamar blue (36, 37). Briefly, Alamar blue reagent (BioSource International), a vital dye that is an indicator of mitochondrial function, was added to the cultures for 3–4 h and read via a fluorometer (FL600; BioTek).

For survival assays using selective pharmacological inhibitors, cultures were exposed to 50 µM MEK1 inhibitor U0126 (38, 39) or 20 µM PI3K inhibitor LY294002 (40) in 10% FCS-containing medium. We established that these concentrations attained maximal effects in glioma cultures. Cells treated with the appropriate volume of filtered DMSO, used to solubilize the drug stocks, served as controls. The volume of DMSO never surpassed 0.5%.

Apoptosis was also assessed by a quantitative sandwich-enzyme immunoassay that measures apoptotic cell death using monoclonal antibodies directed against DNA and histone, and which measures the enrichment of nucleosomes, a hallmark of cells undergoing apoptosis (Roche Molecular Biochemicals, Laval, Quebec, Canada). Glioma cell lines were plated at 10,000 cells/well and primary human gliomas or normal human glial cells plated at 30,000–50,000 cells/well were assayed for apoptosis 72–96 h after viral infection.

**Western Blot Analysis and Kinase Assays.** Forty-eight to 72 h after infection, cells were washed twice in PBS and lysed as described (41). Equal amounts of protein (50–100 µg) were separated on 7.5% (for Akt, MAPK, and PK) and 10% or 7–15% gradient (for Rac1, JNK, and c-myc) polyacrylamide gels (PAGE). Western blots were performed using the following primary antibodies: anti-phospho Akt (Ser-172/211) and anti-Akt (New England Biolabs, Beverly, MA); anti-phospho-JNK (New England Biolabs); anti-JNK1/2 (New England Biolabs); anti-phospho-MAP (Promega Corp., Madison, WI); anti-MAPK (Santa Cruz Biotechnology, Santa-Cruz, CA); anti-c-myc (9E10; Pharmingen); anti-PI3K (Santa Cruz Biotechnology); and anti-Rac1 (Santa Cruz Biotechnology), and anti-Rac1 (New England Biolabs), and visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL) and XAR X-ray film.

For JNK assays, cells lysates were immunoprecipitated with 4 µl of anti-JNK1/2 (Santa Cruz Biotechnology) for 3 h, and then 40 µl of protein A-Sepharose beads was added to each tube for an additional hour. The immune complex was washed twice in lysis buffer and twice in JNK kinase buffer (25 mM HEPES (pH 7.4), 25 mM MgCl2, and 1 mM DTT) and resuspended in 30 µl of JNK kinase buffer. The kinase reaction was assayed for 20 min at 30°C in the presence of 2 µg of GST-c-Jun (Santa Cruz Biotechnology) as a substrate and 5 µCi of [γ-32P]ATP. The reaction was stopped by adding electrophoresis sample buffer, and proteins were separated on 8% SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography.

PAK kinase assays were performed after immunoprecipitation with anti-PAK-agarose conjugate (Santa Cruz Biotechnology) for 4 h at 4°C. PAK agarose beads were washed twice with lysis buffer and twice with PAK kinase buffer (20 mM Tris-HCl (pH 7.5), 20 mM MgCl2, 10 mM MnCl2, 1 mM EDTA, 1 mM EGTA, and 40 µM ATP). PAK kinase activity was assayed for 15 min at 30°C in PAK kinase buffer containing 1 µg of MBP and 5 µCi of [γ-32P]ATP. The reaction was stopped by adding electrophoresis sample buffer, and proteins were separated on 8% SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography.

Rac1 activation assays were performed using a nonradioactive Rac Activity Assay kit (Upstate Biotechnology, Lake Placid, NY). Briefly, cell lysates were
immunoprecipitated with a GST fusion-protein corresponding to the p21-binding domain (residues 67–150) of human PAK1 bound to glutathione-agarose, run on 7–15% SDS-PAGE, and Western blotted using monoclonal anti-Rac (Upstate Biotechnology). In vitro GTPyS and GDP protein loading were used for positive and negative controls, respectively. For all kinase assays, cells were lysed 24–48 h after viral infection.

RESULTS

Rac1 Activity Is Important for the Survival of Several Glioma Cell Lines. To determine the intracellular signaling proteins that regulate glioma cell growth and survival, we used the human glioma cell lines U87, U373, and U343. These cell lines have been used extensively in the glioma field are well characterized for mutations in p53, p16, platelet-derived growth factor receptor, and EGFR and reflect the heterogeneity of glioma molecular characteristics (3, 4). To examine the role of several signaling proteins in glioma cell survival, DN forms of signaling proteins known to play important roles in controlling the proliferation and survival in other cell types were introduced into the glioma cell lines using a recombinant adenovirus-based approach. Recombinant adenoviruses have been previously used to assess the function of cell cycle genes in glioma cell lines (42, 43). Two important advantages of recombinant adenoviruses are the ability to obtain high virus titers (>10¹⁰ viral particles/ml) that result in infections of entire populations of cells in a culture dish for several weeks and the ability of the virus to infect nondividing or slowly proliferating cells at low toxicity. By using DN proteins to perturb signaling pathways in human glioma, we were able to assess their necessity for glioma growth and survival.

Glioma cells were infected with recombinant adenoviruses encoding DN Rac1, DN Ras, DN Akt1, DN MEK1, DN c-jun, and DN RhoA, at a MOI (or plaque-forming units of virus added per cell) of 50 (Fig. 1A). The viruses were used at MOIs that inhibited the activities of MAPK (in the case of DN Ras and DN MEK) or Akt (for DN Akt; data not shown) and that inhibited PAK1 (for DN Rac1, see below). Two days after infection, cells were assayed for survival by adding Alamar blue, a vital dye that quantifies mitochondrial enzyme function and is an assay for cell survival and growth (36, 37). As shown below, the results of this assay correlated well with TUNEL staining, a widely used assay for apoptosis that measures fragmentation of DNA. Suppression of the activities of Ras, Akt1, MEK, c-jun, or RhoA inhibited the survival of the cell lines up to 30%. Suppression of Akt at MOIs of 100 also did not inhibit cell survival by >30%. However, the effects were cell-line dependent, and in some cases (DN MEK in U87), cell survival was slightly stimulated. In contrast, inhibition of Rac1 activity using DN Rac1 significantly inhibited survival in all of the cell lines by 38–60%. DN Rac1 also suppressed survival in U118 and U138 human glioma cells (data not shown). The suppression of survival was dose dependent, with maximal suppression seen at 200 MOI (Fig. 1, B and C). Infection with control adenoviruses expressing either GFP or β-galactosidase had no effect on survival or growth (Fig. 2 and data not shown). In contrast, infection with a recombinant adenovirus expressing the Rho family member cdc42 had either stimulatory effects on U87 cells or mildly inhibitory effects on the survival of U373 cells, as compared with infection with DN Rac1 (Fig. 1D). These results suggest that Rac1 mediates the survival of glioma cell lines by a pathway independent of either cdc42 or RhoA.

DN Rac1 Induces Apoptosis of Glioma Cell Lines. To determine whether suppression of Rac1 induced apoptosis, we assessed apoptosis by two different methods, TUNEL assay and cell death ELISA. U87, U373, and U343 glioma cells were mock infected (no virus; Fig. 2A) or infected with adenovirus expressing DN Rac1 (Fig. 2B), and TUNEL assay was performed after 2 days. Cells were immunostained with α-tubulin to visualize the cell morphology. Cells expressing DN Rac1 are indicated by the appearance of GFP (Fig. 2B) that is coexpressed in the DN Rac1 recombinant adenovirus. No apoptosis was seen in uninfected cells (Fig. 2A), whereas DN Rac1 induced apoptosis in the three cell lines, as indicated by the appearance of TUNEL labeling (Fig. 2B). As a second measure of apoptosis, a cell death ELISA was performed on the cell lines infected with increasing concentrations (25–200 MOI) of DN Rac1-expressing adenovirus or with a control adenovirus expressing only GFP (Fig. 2C). This ELISA measures the enrichment of nucleosomes in the cytoplasm of cells undergoing apoptosis. DN Rac1 expression increased apoptosis by up to 15-fold in U373, 6.5-fold in U87, and 5.5-fold in U373, whereas the
DN Rac1 Does Not Induce Apoptosis in Primary Human Adult Astrocytes. To assess whether signaling proteins that inhibit glioma cell survival do not similarly affect normal astrocytes, biopsy samples of normal adult brain tissue were obtained, and primary cultures of human glia were established. The cultures were enriched for astrocytes but also contained microglia and a small percentage of endothelial cells and oligodendrocytes. The astrocytes were readily infected by recombinant adenovirus, as shown by expression of GFP control virus (Fig. 5A, left panel) or GFP encoded by the DN Rac1 virus (Fig. 5A, right panel). Parallel cultures of normal human astrocytes and primary human GBMs were infected with 50 MOI of adenovirus expressing either DN Rac1 or control KD-TrkA (Fig. 5B). Cultures were characterized by immunofluorescence for the intermediate filament GFAP (green) and TUNEL (red) 48 h after infection. DN Rac1 failed to induce any apoptosis in the human astrocytes while inducing extensive apoptosis in the GBM. The Rac1 signaling pathways, we asked whether DN Rac1 inhibited the serum-induced activation of PAK1 and JNK. DN Rac1 suppressed the activity of PAK1 (Fig. 3B, U373), the phosphorylation of JNK (Fig. 3C, and the activity of JNK as measured by the ability of immunoprecipitated JNK to phosphorylate a c-jun exonogenous substrate in vitro (Fig. 3D, U373).

DN Rac1 Induces Apoptosis in 19 of 21 Primary Human GBM and Oligodendroglioma Cultures. To assess whether DN Rac1 could induce apoptosis of primary human glioma cultures in addition to established glioma cell lines, we cultured cells from patients with GBM or oligodendroglioma. Twenty-one human GBMs and oligodendroglomas were assayed for their susceptibility to DN Rac1. Frozen sections and intraoperative smear preparations established the diagnoses and grades of all gliomas tested, which ranged from grade II oligodendroglioma to grade IV GBM as assessed by the WHO classification. Fresh glioma tissue collected at the time of surgery was dissociated and grown in 10% FCS (see “Materials and Methods”). Characterization of tumor cells at 24–48 h (Table 1) by molecular and immunohistochemical assays for p53 amplification, MDM2 overexpression, EGFR amplification, and the EGFR variant type III (EGFR-vIII; a constitutively active, naturally occurring mutation found in glial tumors) revealed the heterogeneity of the tumors. These data were compared with a similar analysis of frozen tissue obtained from the tumor at the time of resection. The percentage of expression of tumor markers was similar between the tissue sections and cultured cells from the tumor. To determine whether DN Rac1 would induce apoptosis of primary human gliomas, cells were infected with DN Rac1 (50 MOI) at 24 h after plating, and apoptosis was assessed by TUNEL staining or cell death ELISA 2 days after infection. In Fig. 4, we show data from one representative GBM (A) and three oligodendrogliomas (B). DN Rac1 induced virtually complete apoptosis of a primary GBM with amplified p53 (Fig. 4A), whereas a control adenovirus expressing a kinase-inactive form of TrkA (KD-TrkA; Ref. 30) showed no apoptosis. Similarly, DN Rac1 induced significant apoptosis of cells from three patients with oligodendroglioma (Fig. 4B), as assessed by TUNEL (red in a–f) or cell death ELISA (g–i), whereas infection with two control adenoviruses (KD-TrkA or GFP) showed only small increases in apoptosis (g–i).

Table 1 shows the data for DN Rac1 killing of 12 GBMs and 9 oligodendrogliomas. Of the 21 gliomas tested, 19 were found to be susceptible to DN Rac1-induced apoptosis. In all cases, apoptosis was evident in most or all of the cells in each culture. The two tumors that were not found to be susceptible to DN Rac1-induced apoptosis (99–101) were low-grade oligodendrogliomas with proliferation indexes of <3 and <10% as assessed by MIB-1 staining. These results suggest that suppression of a Rac1 survival pathway induces the apoptosis of glioma cells independent of their genetic background.
mRNA of the cells resembled control virus-infected cells (Fig. 5B). A, endogenous Rac1 activity is stimulated by serum treatment. U373 cells grown in serum-free medium overnight were treated with 10% serum (+S) for 20 min. Extracts were precipitated with a GST fusion protein containing the Rac-binding domain of PAK1 that binds only activated (GTP-bound) Rac1, and the activated Rac1 is detected in Western blots using anti-Rac1. The negative control represents cell lysate containing inactive GDP-bound Rac1, and the positive control represents cell lysate containing activated GTP-bound Rac1. B, serum stimulates the activity of the Rac1 substrate PAK1, and DN Rac1 inhibits this activity. U373 cells were infected with DN Rac1 (100 MOI), and cell lysates were immunoprecipitated with anti-PAK1. PAK1 activity was measured by innumocomplex kinase assays using MBP as an exogenous substrate. Shown is the phosphorylation of MBP by PAK1, quantified by measuring the amount of phosphorylated MBP compared with total immunoprecipitated PAK1. C, serum stimulates the activity of the Rac1 substrate JNK, and DN Rac1 inhibits this activity. U87, U373, and U343 cells grown in serum-free medium overnight were treated with 10% serum for 20 min. Cells were lysed, and equivalent amounts of protein from cells were assessed for activation of JNK using anti-phospho-JNK in Western blots. As controls, the blots were probed with anti-JNK to detect total JNK or with anti-c-myc to detect the c-myc epitope tag on DN Rac1. The arrows point to JNK1, 2, and an unidentified JNK (JNKX) stimulated by serum treatment. The graphs show quantification of phosphorylated JNK as compared with total JNK. D, DN Rac1 inhibits the kinase activity of JNK. U373 cells infected with DN Rac1 or uninfected cells were grown in serum-free medium overnight and treated with 10% serum for 20 min. JNK activity was measured by assessing the ability of immunoprecipitated JNK to phosphorylate its substrate, c-jun, in vitro. Cells were also infected with constitutively activated MEKK (MEKK*), an activator of JNK, as a control.

DN Rac1 May Suppress Survival in Part by Inhibiting MEK/MAPK Activity. The data above indicate that JNK may be one of the proteins regulating Rac1-induced survival. In other cell types, Rac1 has been suggested to regulate survival by stimulating the activity of the MEK/MAPK or PI3K/Akt signaling pathways (11, 12, 45). We asked whether Rac1 used similar mechanisms in glioma cells. We first determined whether DN Rac1 expression would suppress the activities of MAPK or Akt. U87 or U373 cells expressing DN Rac1 were assessed for MAPK, MEK, or Akt activities using activation and phosphorylation state-specific antibodies for Akt, MAPK1/2, or MEK. DN Rac1 suppressed the activation of MAPK1/2 (Fig. 6A) and MEK (data not shown) but had no effect on the phosphorylation of Akt (Fig. 6A). Similar results were observed in U343 cells, which unlike U87 and U373, express PTEN (data not shown). The inhibition of phosphorylation of MEK and MAPK suggested that DN Rac1 induces apoptosis, in part, by inhibiting the MEK/MAPK signaling pathway. If this were true, then DN Rac1 should only induce the same amount of cell death as inhibition of survival using a pharmacological inhibitor of the MEK/MAPK signaling pathway, U0126 (28, 39). Furthermore, DN Rac1 should not induce any more cell death than that induced by U0126. U373, U87, and U343 cell lines were assessed for survival after either infection with DN Rac1, treatment with U0126 (50 μM), or treatment with U0126 (50 μM) combined with infection with DN Rac1 (50 MOI). The dose of U0126 used in these experiments was sufficient to completely inhibit all serum-induced MAPK phosphorylation (Fig. 4C). Survival assays were performed using Alamar blue, because survival measured using this technique correlated well with techniques directly measuring apoptosis.

Activation of JNK Rescues Apoptosis Induced by DN Rac1. Serum treatment of glioma cells stimulates the activity of JNK (Fig. 3), which has been shown to mediate several of the biological responses of cells to Rac1 activation (19, 20). To determine whether JNK may function in a survival pathway regulated by Rac1, we asked whether cell death induced by DN Rac1 could be rescued by activation of JNK. Cells expressing DN Rac1 were coinfected with a recombinant adenovirus expressing a constitutively activated form of MEKK1, a protein kinase that activates JNK1, JNK2, and an unidentified JNK (JNKX) stimulated by serum treatment of U87 cells (Fig. 3C). U87 cells were infected with 25–100 MOI of DN Rac1 or coinfected with DN Rac1 (25–100 MOI) and 10 MOI of activated-MEKK (MEKK*). Expression of activated MEKK1 partially rescued the apoptosis induced by DN Rac1 at all MOIs tested (Fig. 5C). We also found that activated MEKK1 could partially rescue the apoptosis induced by DN Rac1 in primary GBM cultures from patients. Apoptosis in these cultures was significantly reduced by coinfection of the DN Rac1 virus with activated MEKK1 (Fig. 5, D and E). These data suggest that JNK activity may function in a signaling pathway downstream of Rac1 and may in part mediate Rac1-induced survival.
Although DN Rac1 suppressed survival by 75–95% (at 200 MOI), U0126 was only able to inhibit survival by 25–39%, indicating that DN Rac1 inhibits survival more effectively than U0126. Treatment of cells with both U0126 and DN Rac1 (50 MOI) inhibited survival by 45–93%. These results suggest that although DN Rac1 might mediate a portion of its apoptotic effects by inhibiting MEK/MAPK, the majority of Rac1-induced survival occurs via a MEK/MAPK-independent pathway.

Table 1 Summary of apoptosis of gliomas induced by DN Rac1

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<tr>
<th>Tumor Type</th>
<th>p53</th>
<th>MDM2</th>
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<td>00-1472</td>
<td>OLIGO II</td>
<td>NEG</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>5%</td>
</tr>
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</table>

a DN Rac1 induced the apoptosis of >80% of the cultured tumor cells, as assessed by TUNEL.

b nd, test not completed.
c Recurrent tumors, 99-467 (98-589) and 00-69 (98-715).

(Fig. 2). Although DN Rac1 suppressed survival by 75–95% (at 200 MOI), U0126 was only able to inhibit survival by 25–39%, indicating that DN Rac1 inhibits survival more effectively than U0126. Treatment of cells with both U0126 and DN Rac1 (50 MOI) inhibited survival by 45–93%. These results suggest that although DN Rac1 might mediate a portion of its apoptotic effects by inhibiting MEK/MAPK, the majority of Rac1-induced survival occurs via a MEK/MAPK-independent pathway. To determine
whether DN Rac1 induced apoptosis by suppressing either PI3K or Ras activities, DN Rac1-infected cells were treated with the PI3K inhibitor LY294002 or coinfected with DN Ras. DN Rac1 stimulated apoptosis regardless of whether PI3K (Fig. 6B) or Ras (data not shown) was active in the cells.

**DISCUSSION**

The signals that regulate the survival of cultured human glioma cells are not well defined. Here, we show that: (a) Rac1 mediates the survival of glial tumor cells and not of normal glia; (b) Rac1, and not other Rho family members, appears to regulate a dominant survival pathway in glial tumor cells; and (c) DN Rac1 induces cell death of glial tumor cells regardless of p53, MDM2, or EGFR mutational or amplification status. Suppression of Rac1 activity in 5 of 5 (100%) glioma cell lines and 19 of 21 (90%) primary cultures from patients with GBM or oligodendroglioma induced apoptosis within 48 h. In contrast, inhibition of Rac1 activity did not markedly alter the cytoskeleton. In addition, cell cycle analysis by flow cytometry did not indicate a block of DN Rac1-expressing glial tumor cells in any stage of the cell cycle.8 Rather, apoptosis was induced within 24–48 h of DN Rac1 expression. Rac1 activity, as well as the activity of Rac1 and that of PAK1 and JNK (two substrates of Rac1) were stimulated by serum treatment of glioma cells, and DN Rac1 expression blocked Rac1, PAK1, JNK, and MAPK activities. These data, together with the rescue of DN Rac1-induced cell death by MEKK-induced activation of JNK activity, suggest that at least two signaling pathways, JNK and MAPK, may contribute to Rac1-mediated survival (Fig. 7).

Cell death induced by specific suppression of Rac1 activity has not been shown previously. Rac1 regulates many cellular processes, including actin cytoskeleton organization, transcriptional activation, and cell proliferation (17, 20, 46–49). In contrast with other cell types, suppression of Rac1 activity in glial tumor cells did not markedly alter the cytoskeleton. In addition, cell cycle analysis by flow cytometry did not indicate a block of DN Rac1-expressing glial tumor cells in any stage of the cell cycle.8 Rather, apoptosis was induced within 24–48 h of DN Rac1 expression. Rac1 activity, as well as the activity of PAK1 and JNK, two intracellular targets of Rac1 in other cell types, was induced by serum treatment of glioma cells, which is

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8 D. L. Senger and D. R. Kaplan, unpublished data.
required for their optimal survival. Our results suggest that Rac1 is one of the signals regulating the survival of glioma cells. These data contrast with those obtained in normal neural cells expressing DN Rac1, by our group and others (14).9 In normal adult glia cultures, which contain primarily astrocytes but also microglia and oligodendrocytes, we failed to observe any apoptotic cells after suppression of Rac1 activity (Fig. 5). Surprisingly, in cultured sympathetic neurons, DN Rac1 is a prosurvival rather than proapoptotic protein (14). Thus, although DN Rac1 induces the death of glial tumor cells, it has no observable affects on normal glia and stimulates the survival of neurons. Rac1 may therefore be an ideal target for therapeutic approaches for the treatment of brain tumors, because at least in vitro, DN Rac1 selectively induces apoptosis of tumor cells and not normal glia and neurons.

Why is DN Rac1 activity so effective as an apoptotic agent? The DN Rac1 adenovirus did not induce cell death of normal glia, and recombinant adenoviruses expressing DN RhoA, DN cdc42, DN MEK1, kinase inactive TrkA, GFP, and LacZ did not induce apoptosis of glioma cells. These results rule out any potential toxicity of the adenovirus vectors. We therefore conclude from our results that Rac1 mediates an important survival pathway in glial tumor cells. Other survival pathways stimulated by serum treatment or by survival factors, such as PI3K/Akt (50, 51) and Ras (52), are also likely to play roles in the survival of glioma cells. However, because suppression of Rac1 activity is much more effective than suppression of Akt or of Ras at inducing apoptosis, Rac1 activity may be a more necessary component of the cell survival machinery than Akt and Ras. The only other agents that we have found to be as effective as DN Rac1 in inhibiting glioma cell survival are FTIs (6),10 which may very well suppress the activity of Rac1 in addition to Ras and Rho (8). In this regard, we have observed that FTIs will inhibit the activity of Rac1 in glioma cells lines, although further experimentation will be required to confirm this result.8 Suppression of Rac1 activity also induced apoptosis irrespective of the mutational status of proto-oncogenes or tumor suppressor genes. Rac1 thus appears to be a dominant survival-inducing protein for glial tumor cells and not for other normal cell types.

Fig. 6. DN Rac1 may suppress survival in part by suppressing MEK/MAPK activity. A, DN Rac1 suppresses MAPK but not Akt activity. U87 and U373 cells infected with DN Rac1 (25–100 MOI), activated MEKK (10 MOI; MEKK*), or GFP were lysed, and activated Akt or MAPK was detected using activation-specific antibodies for Akt (P-Akt) and p44 and p42 MAPK (P-MAPK) in Western blots. Blots were also probed with anti-MAPK to visualize total MAPK and anti-c-myc to detect DN Rac1 expression. B, glioma cell lines U87, U373, and U343 were infected with recombinant adenovirus expressing DN Rac1 (25–200 MOI) or DN Rac1 (50 MOI) in the absence or presence of the MEK inhibitor U0126 (50 μM) or the PI3K inhibitor LY294002 (20 μM). After 3 days, cells were assayed for survival by Alamar blue. Results are expressed as the percentage of cell survival of infected cells as compared with uninfected cells (Control). Bars, SD.

Fig. 7. Diagram of signaling pathways used in glioma cells to regulate cell survival. Rac1, which stimulates PAK and JNKX activities, appears to be the dominant pathway regulating glioma cell survival. Rac1 can also stimulate MAPK activity. Ras regulates cell survival independently of Rac1 by stimulating PI3K and Akt activities and MEK and MAPK activities.

9 I. E. Mazzoni and D. R. Kaplan, unpublished data.

How does Rac1 mediate glioma cell survival? DN Rac1 inhibited the activities of two known downstream targets of Rac1 activity, PAK1 and JNK. It also inhibited the activities of MEK and MAPK but not of Akt, one of the most effective antiapoptotic proteins for a variety of cell types (53). Suppression of MEK activity by treatment of cells with the selective MEK inhibitor U0126 also inhibited glioma cell survival, although only mildly compared with expression of DN Rac1 (Fig. 6), and expression of activated MEK did not rescue DN Rac1 killing of glioma cells. We suggest that inhibition of MEK may mediate a small portion of the apoptosis-inducing effects of DN Rac1. A second protein that may be involved in mediating Rac1-induced survival effects is JNK. Serum treatment stimulated JNK activity in glioma cells, assessed by the phosphorylation of c-jun by immunoprecipitated JNK, and DN Rac1 inhibited this activity. The JNK isoform that was activated by serum treatment, however, was not identified, because the phosphorylated JNK isoform in serum-treated glioma cells migrated at a different molecular weight than JNK 1, 2, or 3. Expression of constitutively activated MEKK, a potent activator of the JNKs, enhanced the phosphorylation of the unidentified JNK isoform in U87 cells and partially rescued these cells from DN Rac1-mediated cell death, suggesting that Rac1-induced activation of JNK plays a role in glioma cell survival. Rac1 likely regulates cell survival by stimulating multiple signaling pathways, including MEK/MAPK and JNK. These results are consistent with the observation that overexpression in fibroblasts of EGFRvIII, a constitutively active, naturally occurring mutation of the EGFR found in many human tumors including glioma (54–56), results in an increase in basal JNK activity (57). JNK isoforms may therefore mediate the prosurvival and proliferative responses of glioma cells to Rac1 and EGFRvIII activation.

ACKNOWLEDGMENTS

We thank C. Bazenet for the DN Rac1 cDNA, F. D. Miller for the activated MEKK adenovirus, and M. Boudreau and L. LeSauteur for advice and assistance.

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